

**Anti-cancer and anti-inflammatory activities of apigenin
nanoencapsulated in dairy proteins**

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Shan Hong

May 2020

Copyright © 2020 by Shan Hong

All rights reserved.

ACKNOWLEDGEMENTS

I would like to first express my heartfelt gratitude to my major supervisor Dr. Qixin Zhong for giving me the precious opportunity to pursue my doctoral study. His guidance, patience, encouragement, and feedback on my experiments made me move forward and complete my dissertation. I would like to thank the financial support of the American National Institutes of Health grant (5R21EB018937-02), and express my deep appreciation to Dr. Seung Joon Baek for designing animal experiments. I am also very grateful to my doctoral committee members, Dr. Vermont P. Dia for training with my cell work and conducting my animal studies. His door is always open for me to ask questions. Dr. Tao Wu and Dr. Ling Zhao, for their intellectual input, feedback and suggestions throughout my doctoral program.

I want to express a big thank you to Svjetlana Matic, for her time and help in the entire duration of the animal study. I also would like to express my special thank you to Andrea Nieto, for always be open for me to discuss any questions on my work. I wish to thank all the labmates, Anyi Wang, Nan Li, Jennifer Vuia- Riser, Inseob Choi, Melody Fagan, and Mary Lamsen for encouragement and company. Also I want to express my gratitude to Dr. Philipus Pangloli, for his help with my experiments. I am also grateful for the faculty, staff and graduate students in the department.

I would like to express my deep thanks to my parents. Their love, understanding, encouragement, continued support and guidance have helped me become the person I am today. Also heartfelt thanks for those whom I have missed to acknowledge.

ABSTRACT

Dietary polyphenols such as apigenin are the secondary plant metabolites reported to reduce incidence of many chronic diseases including cancers and chronic inflammation. However, the high hydrophobicity of lipophilic polyphenols results in low water solubility and bioavailability, which limits the therapeutic efficacy and impedes further applications in aqueous food systems. Nanoencapsulation is a potential approach to address the above problems. Thus, the objective of this dissertation was to develop nanodelivery systems of apigenin utilizing dairy proteins as carriers, followed by evaluating the *in vitro* and *in vivo* anticancer efficacy and anti-inflammatory effects. The first delivery system was fabricated by increasing mixtures with 0.6-2.0 mg/mL apigenin and 1% w/v whey protein isolate (WPI) to pH 13, followed by neutralization (the pH-cycle). The encapsulation efficiency was 93.44-98.38% and the loading capacity was 57.19-195.78 mg-apigenin/g-WPI. Nanodispersions maintained stable hydrodynamic diameters of 180-240 nm during 30-day ambient storage. Nanoencapsulation enhanced the cellular uptake of apigenin in human HCT-116 and HT-29 colorectal cancer cells, and the oral bioavailability in C57BL/6J mice. *In vivo* anti-colorectal cancer efficacy was evaluated in Apc^{Min+} mice. Results showed that neither free apigenin nor encapsulated apigenin positively suppressed colon carcinogenesis, by insignificantly affecting polyps numbers, tissue apoptosis, tumor marker expressions (COX-2, β -catenin, cyclin D1, and c-myc), and levels of inflammatory cytokines (TNF- α , IL-6, and IL-1 β). The second nanodelivery system was fabricated by co-encapsulating apigenin and curcumin at various mass ratios in sodium caseinate using the pH cycle method. Curcumin enhanced the

encapsulation efficiency and dispersion stability. When evaluated for anti-inflammatory activities in LPS-induced RAW 264.7 macrophages under non-toxic concentrations, unencapsulated polyphenol mixtures with an increased proportion of curcumin showed synergistic anti-inflammatory activities, evidenced by a combination index of smaller than 1, in inhibiting nitric oxide production, suppressing iNOS enzyme expression, decreasing pro-inflammatory cytokines (TNF- α and IL-6), and reducing ROS production. These synergistic anti-inflammatory effects of apigenin and curcumin were enhanced after coencapsulation. Findings from the present dissertation may provide insights to develop functional foods incorporated with lipophilic phytochemicals as potential strategies for disease prevention.

Keywords: apigenin, dairy proteins, nanoencapsulation, pH-cycle, bioavailability, anticancer, anti-inflammatory, synergistic interactions

TABLE OF CONTENTS

Chapter 1. Introduction	1
1.1 Colon cancer	2
1.1.1 Incidence and mortality.....	2
1.1.2 Development of colon cancer	2
1.1.3 Risk factors of colon cancer.....	4
1.1.4 Current therapeutic approaches and challenges	6
1.2 Chronic inflammation	7
1.2.1 Inflammatory response.....	7
1.2.2 Chronic inflammation and cancer development	9
1.2.3 Oxidative stress and chronic inflammation.....	10
1.2.4 Inflammatory mediators.....	11
1.2.5 Current treatments for inflammation	14
1.3 Dietary polyphenols	15
1.3.1 Classification and chemical structures of polyphenols	15
1.3.2 Pharmacological properties of polyphenols.....	16
1.3.3 Challenges of applying polyphenols for disease intervention	18
1.3.4 Apigenin.....	20
1.4 Synergistic interactions between bioactive compounds	21
1.4.1 Synergistic anticancer effects of dietary compounds.....	22
1.4.2 Synergistic anti-inflammatory activities of dietary compounds	23
1.5 Nanoencapsulation systems and underlying problems	24
1.5.1 Emulsions.....	24
1.5.2 Solid lipid nanoparticles and nanostructured lipid carriers.....	26
1.5.3 Liposomes	27
1.5.4 Micelles.....	29
1.5.5 Polymeric nanoparticles.....	29
1.5.6 Inclusion complexes.....	30

1.5.7 Nanocrystals.....	31
1.6 Bovine milk proteins as effective encapsulation carriers.....	32
1.6.1 Caseins	32
1.6.2 Whey proteins	33
1.6.3 Unique physicochemical properties and structures of bovine milk proteins ..	35
1.6.4 Bovine milk protein-based particles as delivery systems	39
1.7 Hypothesis and objectives.....	39
References.....	42
Appendix.....	77
Chapter 2. Nanoencapsulation of apigenin with whey protein isolate: physicochemical properties and <i>in vitro</i> activities against colorectal cancer cells.....	79
2.1 Abstract	80
2.2 Introduction.....	82
2.3 Materials and methods	84
2.3.1 Materials	84
2.3.2 Nanoencapsulation of apigenin.....	84
2.3.3 Physicochemical characterizations of nanoencapsulated apigenin.....	85
2.3.4 In vitro gastrointestinal digestion.....	86
2.3.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ...	87
2.3.6 Cell culture and cell proliferation assay.....	87
2.3.7 Cellular uptake studied with high-performance liquid chromatography (HPLC)	88
2.3.8 Cell cycle analysis by flow cytometry	89
2.3.9 Statistical analysis.....	90
2.4 Results and discussion	90
2.4.1 Physicochemical characteristics of nanoencapsulated apigenin	90
2.4.2 <i>In vitro</i> anti-proliferative activity.....	93
2.4.3 Cellular uptake of apigenin.....	95
2.4.4 Effects of apigenin on cell cycle distribution and apoptotic ratio.....	96

2.5 Conclusions.....	96
References.....	98
Appendix.....	102
Chapter 3. Oral bioavailability and <i>in vivo</i> anti-colorectal cancer effects of apigenin nanoencapsulated with whey protein isolate.....	112
3.1 Abstract	113
3.2 Introduction.....	115
3.3 Materials and methods	117
3.3.1 Materials	117
3.3.2 Preparation of nanoencapsulated apigenin.....	117
3.3.3 <i>In vivo</i> bioavailability of nanodelivered apigenin.....	118
3.3.4 <i>In vivo</i> anticancer activities.....	119
3.3.5 Statistical analysis.....	122
3.4 Results and discussion	123
3.4.1 <i>In vivo</i> bioavailability of apigenin influenced by nanoencapsulation.....	123
3.4.2 <i>In vivo</i> anticancer studies	124
3.5 Conclusions.....	128
References.....	129
Appendix.....	134
Chapter 4. Synergistic anti-inflammatory activity of free and co-encapsulated apigenin and curcumin in lipopolysaccharide-stimulated RAW 264.7 macrophages	139
4.1 Abstract	140
4.2 Introduction.....	142
4.3 Materials and methods	145
4.3.1 Materials	145
4.3.2 Co-encapsulation of apigenin and curcumin.....	145
4.3.3 Physicochemical characterizations of dispersions	146
4.3.4 Cytotoxicity assay	147
4.3.5 Determination of NO production	147

4.3.6 Preparation of whole cell lysates and western blot analysis	148
4.3.7 Measurement of pro-inflammatory cytokines.....	149
4.3.8 Determination of intracellular reactive oxygen species (ROS)	149
4.3.9 Determination of synergism.....	150
4.3.10 Statistical analysis.....	151
4.4 Results and discussion	151
4.4.1 Physicochemical characteristics of dispersions	151
4.4.2 Cell viability and toxicity of apigenin and curcumin.....	152
4.4.3 Synergistic inhibition of NO production.....	153
4.4.4 Decreased expression of iNOS and COX-2.....	154
4.4.5 Synergistic suppression of IL-6 and TNF- α	156
4.4.6 Synergistic inhibition of ROS production.....	157
4.5 Conclusions.....	158
References.....	160
Appendix.....	164
Chapter 5. Concluding remarks and future work.....	181
5.1 Conclusions.....	182
5.2 Future work.....	184
Reference	186
Vita	187

LIST OF TABLES

Table 1-1. Composition of proteins in bovine milk ^{1,2}	77
Table 2-1. Zeta-potential of pH 7.0 nanodispersions with 1% w/v and different amount of apigenin after the pH-cycle treatment.....	102
Table 4-1. Encapsulation efficiency and zeta-potential of dispersions after the pH-cycle treatment of 2% w/v sodium caseinate (NaCas) and 0.2 mg/mL polyphenols or with different mass ratios of apigenin (AP) : curcumin (CUR) at a constant AP concentration of 0.2 mg/mL.	164
Table 4-2. Concentrations of free or encapsulated (En) apigenin (AP) and curcumin (CUR) to inhibit 50% of NO production (IC ₅₀) and combination index (CI) calculated for combinations with various mass ratios of free or co-encapsulated (CoEn) AP and CUR on NO production in LPS-stimulated RAW 264.7 cells.	165
Table 4-3. Combination index (CI) calculated for combination effects of coencapsulated (CoEn) apigenin (AP) and curcumin (CUR) at different mass ratios on expression of iNOS triggered by LPS activation.....	166
Table 4-4. Concentrations of free or encapsulated (En) apigenin (AP) and curcumin (CUR) to inhibit 50% of IL-6 level (IC ₅₀) and combination index (CI) calculated for combinations with various mass ratios of free or co-encapsulated (CoEn) AP and CUR on IL-6 production induced by LPS stimulation.....	167
Table 4-5. Concentrations of free apigenin (AP) and curcumin (CUR) to inhibit 50% of TNF- α level (IC ₅₀) and combination index (CI) calculated for combinations with various mass ratios	

of free AP and CUR on TNF- α level induced by LPS stimulation. 168

Table 4-6. Concentrations of free or encapsulated (En) apigenin (AP) and curcumin (CUR) to inhibit 50% of ROS production (IC₅₀) and combination index (CI) calculated for combinations with various mass ratios of free or co-encapsulated (CoEn) AP and CUR on ROS production in LPS-induced RAW 264.7 cells. 169

LIST OF FIGURES

Figure 2-1. (A) Encapsulation efficiency and loading capacity as affected by the amount of apigenin (AP); (B) Particle size distributions of nanodispersions prepared with (1) 0.6, (2) 1.0, (3) 1.5, or (4) 2.0 mg/mL AP during 30-day storage at 21 °C; (C) AFM image of nanodispersion prepared with 2.0 mg/mL AP in height channel (top) and 3-dimensional view (bottom);	103
Figure 2-2. (A) DSC thermograms of pristine AP, pH-treated WPI and powder lyophilized from nanodispersion sample with 2.0 mg/mL AP; (B) FTIR spectra of (a) pristine AP, (b) unprocessed WPI, (c) pH-treated WPI and (d) powder freeze-dried from nanodispersion with 2.0 mg/mL AP.	104
Figure 2-3. Electrophoretic profile of (1) Native WPI, (2) pH-treated WPI, (3) nanodispersion with 2 mg/mL AP; (4) native WPI, (5) pH-treated WPI, and (6) nanodispersion after simulated gastric digestion; (7) native WPI, (8) pH-treated WPI, and (9) nanodispersion after simulated gastric and intestinal digestions. Lane Mw represents the molecular weight marker.....	105
Figure 2-4. Proposed structural changes leading to nanoencapsulation of apigenin in WPI during the pH-cycle.....	106
Figure 2-5. Anti-proliferative activity of 0, 5, 10, and 20 µM of free (pre-dissolved in DMSO) apigenin (AP) and nanoencapsulated AP (En-AP) against (A) HCT-116 and (B) HT-29 colon cancer cells after 72-h incubation at 37 °C. Error bars are standard deviations ($n \geq 3$). Treatments labeled with $p < 0.05$ represent a significant difference between each other.....	107
Figure 2-6. Anti-proliferative activity of 0, 10, and 20 µM of (1) free (pre-dissolved in DMSO) apigenin (AP) and (2) nanoencapsulated AP (En-AP) against (A) HCT-116 and (B) HT-29	

colon cancer cells before and after *in vitro* digestions after 72-h incubation at 37 °C. Error bars are standard deviations ($n \geq 3$). Treatments labeled with $p < 0.05$ represent a significant difference between each other..... 108

Figure 2-7. Cellular uptake of 5, 10, and 20 μM of free (pre-dissolved in DMSO) apigenin (AP) and nanoencapsulated AP (En-AP) in (A) HCT-116 and (B) HT-29 colon cancer cells after 24-h incubation at 37 °C. Error bars are standard deviations ($n = 3$). Different letters above columns indicate significant differences ($p < 0.05$). 109

Figure 2-8. Flow cytometry analysis of relative apoptotic ratio (A) and the percentage of (B, C) cell cycle distribution of HCT-116 cell line treated by 0, 5, 10, and 20 μM of free (pre-dissolved in DMSO) apigenin (AP) and nanoencapsulated AP (En-AP) for 24 h at 37 °C. Relative apoptotic ratio was calculated with reference to control cells without AP treatment. Error bars are standard deviations ($n = 3$). Different letters above columns indicate significant differences ($p < 0.05$). 110

Figure 2-9. Flow cytometry analysis of relative apoptotic ratio (A) and the percentage of (B, C) cell cycle distribution of HT-29 cell line treated by 0, 5, 10, and 20 μM of free (pre-dissolved in DMSO) apigenin (AP) and nanoencapsulated AP (En-AP) for 24 h at 37 °C. Relative apoptotic ratio was calculated with reference to control cells without AP treatment. Error bars are standard deviations ($n = 3$). Different letters above columns indicate significant differences ($p < 0.05$). 111

Figure 3-1. Apigenin concentration of mice after oral gavage with 50 mg/kg-body weight of free apigenin (AP) and encapsulated AP (En-AP) in the serum (A) and colon mucosa (B). Error

bars are standard deviations ($n \geq 3$). 134

Figure 3-2. Tumorigenesis evaluation of mice treated with vehicle (0.5% w/v methylcellulose), pH-treated WPI, 50 mg/kg-mice-body-mass free apigenin (AP) or nanoencapsulated AP (En-AP): Location (A), size distribution (B) and total number (C) of polyps in the small intestine of mice. 135

Figure 3-3. TUNEL assay of apoptotic cells in colon mucosa within different treatment groups: (A) Green staining represented apoptotic cells (10 $\times$ magnifications); (B) Green intensity calculated from 10 randomly selected fields with each section. Error bars represent standard deviations. 136

Figure 3-4. Western blot of tumor markers (COX-2, β -catenin, c-myc, cyclin D1). “T” and “C” represent tumor and normal colonic tissue, respectively, in (A). Relative expression is calculated based on standard marker of β -actin (B). Error bars are standard deviations ($n \geq 3$). 137

Figure 3-5. Effects of apigenin over inflammatory response *in vivo* as analyzed for cytokines (TNF- α , IL-1 β , IL-6) in mice serum (A) and colon mucosa (B). Error bars are standard deviations ($n \geq 3$). 138

Figure 4-1. Molecular structure of curcumin, (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione. 170

Figure 4-2. (A) Hydrodynamic diameter distributions of pH 7.0 dispersions of sodium caseinate (NaCas), pH-cycle treated NaCas (pH-NaCas) and coencapsulated apigenin (AP) and curcumin (CUR) at different mass ratios; (B) TEM images of (a) NaCas; (b) pH-NaCas, and

(c) dispersion with AP and CUR at 1:1 mass ratio. 171

Figure 4-3. (A) Visual appearance of nanodispersions prepared in 2% w/v NaCas and constant 0.2 mg/mL apigenin (AP) at mass ratio of AP:curcumin (CUR) (1) 1:1, (2) 1:2, (3) 1:3, (4) 1:4, and (5) 1:0; (B) Particle size distributions of the dispersions prepared with AP and CUR at mass ratios of (a) 1:1, (b) 1:2, (c) 1:3, and (d) 1:4 during ambient storage (21 °C) for up to 30 days. 172

Figure 4-4. Effects of 0-30 µg/mL apigenin (A) and 0-15 µg/mL curcumin (B) on viability of lipopolysaccharide (LPS+) activated macrophages. Error bars are standard deviations ($n \geq 3$). Different letters above columns indicate significant differences ($p < 0.05$)..... 173

Figure 4-5. Percentage of inhibition on NO production in LPS-induced RAW 264.7 cells by free (A) or encapsulated (B) apigenin (AP) or curcumin (CUR); free AP and CUR at different mass ratios (C); and AP and CUR co-encapsulated at different mass ratios (D). Error bars are standard deviations ($n \geq 3$). Different letters above columns in A and B indicate significant differences ($p < 0.05$). 174

Figure 4-6. Western blot analysis of iNOS expression in LPS-induced RAW 264.7 cells as inhibited by 0-6 µg/mL free or encapsulated (En) apigenin (AP) and curcumin (CUR) at mass ratios from 1:0 to 0:1: images (A) and relative expression (B, free; C, En). GAPDH represents the housekeeping protein. The first “0 µg/mL” represents the negative control without LPS and polyphenols. The second “0 µg/mL” represents the positive control with LPS but without polyphenols. Relative expression of iNOS protein was calculated compared to the positive control cells. Error bars are standard deviations ($n = 3$). 175

Figure 4-7. Western blot analysis of COX-2 expression in LPS-induced RAW 264.7 cells as inhibited by 0-6 µg/mL free or encapsulated (En) apigenin (AP) and curcumin (CUR) at mass ratios from 1:0 to 0:1: images (A) and relative expression (B, free; C, En). GAPDH represents the housekeeping protein. The first “0 µg/mL” represents the negative control without LPS and polyphenols. The second “0 µg/mL” represents the positive control with LPS but without polyphenols. Relative expression of COX-2 protein was calculated compared to the positive control cells. Error bars are standard deviations (n = 3). 176

Figure 4-8. IL-6 production in RAW 264.7 cells stimulated with 1µg/mL LPS for 24 h as inhibited by 1.5-6.0 µg/mL free (A) or encapsulated (B) apigenin (AP) and curcumin (CUR) at different mass ratios. The inhibition percentage was calculated with respect to the LPS-treated positive control. Error bars are standard deviations (n = 3). 177

Figure 4-9. TNF-α production in 1µg/mL LPS-induced RAW 264.7 cells for 24 h as inhibited by 1.5-6.0 µg/mL free (A) or 6.0 µg/mL encapsulated (B) apigenin (AP) and curcumin (CUR) at different mass ratios. The inhibition percentage was calculated with respect to the LPS-treated positive control. Error bars are standard deviations (n = 3). Different letters above columns in B indicate significant difference ($p < 0.05$). 178

Figure 4-10. Fluorescence microscopy analysis of intracellular reactive oxygen species (ROS) production as inhibited by 6 µg/mL free (A) and encapsulated (B) apigenin and curcumin at mass ratios of (3)1:0, (4) 0:1, (5) 4:1, (6) 3:1, (7) 2:1, (8) 1:1, (9) 1:2, (10) 1:3, and (11) 1:4. Cells were treated for 24 h and were activated by 1 µg/mL LPS in the last 18 h. Figure 1 shows the negative control without LPS and polyphenols. Figure 2 shows the positive control with

LPS but without polyphenols. Pictures were taken at 20× magnification after incubation with 10 µM of 2',7'-dichlorofluorescein diacetate. 179

Figure 4-11. Production of reactive oxygen species (ROS) as inhibited by 1.5-6.0 µg/mL free (A) or encapsulated (B) apigenin (AP) and curcumin (CUR) at different mass ratios investigated by fluorescence spectrophotometry at 485 nm/528 nm. Cells were treated for 24 h and were activated by 1 µg/mL LPS in the last 18 h. Inhibition percentage was calculated based on fluorescence intensity at 485 nm/528 nm with respect to the positive control. Error bars are standard deviations (n = 3).....180

Chapter 1. Introduction

1.1 Colon cancer

1.1.1 Incidence and mortality

Cancer, a malignant neoplasm caused by the abnormal proliferation and differentiation of cells, continues to threat human life as the second leading cause of death globally, only exceeded by heart disease.¹⁻⁴ There were 18.1 million new cases and 9.6 million deaths from cancer worldwide estimated in 2018.⁵ The global burden of cancer is predicted to reach 21.7 million newly diagnosed cases and 13 million mortalities by 2030.⁶

According to the Global cancer statistics, colorectal cancer (CRC) has been ranked the third most commonly diagnosed cancer, accounting for 10.2% of all incident cancers.⁵ In 2018, over 881,000 deaths were estimated among the total 1.8 million new CRC cases, i.e., about 1 death in 2 CRC cases, which made it the second most prevailing cause of cancer-related death in the overall population.⁵ In the past two decades, there has been a decline in the incidence and mortality rates in the adults aged 50 years and over, which is largely attributable to the screening to prevention and early detection.⁷ However, the number of new cases is rising among young adults aged 20-49 years.^{5, 8, 9} It is projected that the global population has to bare a 22% increase of the CRC worldwide burden to more than 2.2 million new cases and 1.1 million cancer deaths by 2030.¹⁰

1.1.2 Development of colon cancer

Colon carcinogenesis is a complex multistage process conventionally defined as three major phases: initiation, promotion and progression.^{4, 11, 12} Along these steps, CRC develops as a consequence of the progressive accumulation of genetic mutations and epigenetic alterations

that cause conversion and progression of normal functioning colonic epithelium mucosa to adenoma and, eventually, carcinoma.¹³⁻¹⁵

This “adenoma-carcinoma sequence” is resulted from series activation of oncogenes and inactivation of tumor suppressor genes.¹⁴ One of the critical events in colorectal tumorigenesis is the aberrant activation of β -catenin, which is an important cancer target that vitally involved in both cell adhesion and intracellular signaling.¹⁴⁻¹⁶ β -catenin cooperates with adenomatous polyposis coli (APC), a tumor suppressor gene, forming a complex, which is degraded by the ubiquitination process under normal circumstances.¹⁷ However, mutations in APC genes cause the incapability to bind with β -catenin, resulting in a larger amount of β -catenin in the cytosol, which subsequently translocates into the nucleus and finally promotes cells proliferation.¹⁸⁻²⁰ Thus, APC inactivation along with the aberrant activation of β -catenin are key initial events in the tumorigenic process, resulting in the transition of preneoplastic cells to aberrant crypt foci (ACF) and toward development of adenoma or polyps, which always start out as benign.²¹⁻²³ Further progression of such early-stage adenomas to carcinoma in situ and invasive metastatic carcinoma requires several additional pathways, involving mutational activation of oncogenes such as Kirsten rat sarcoma viral oncogene homolog (K-ras) and proto-oncogene B-Raf, as well as inactivation of tumor suppressors including Transforming growth factor beta receptor II (TGF β R2), p53 and the proapoptotic protein Bax.^{2, 24-29} Cyclooxygenase 2 (COX-2) expression is also increased in the transition of benign adenoma to malignant adenocarcinoma.³⁰⁻³²

1.1.3 Risk factors of colon cancer

A number of risk factors are associated with the development of colon cancer, including inherited genetic factors, lifestyle and environmental factors.³³ Hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome), which is induced by mutations in the DNA mismatch repair system, and familial adenomatous polyposis (FAP) that results from the germline mutation in the tumor suppressor APC gene, make up the two main forms of inherited colon cancers.³⁴⁻³⁶ However, only approximately 5% of CRC patients are diagnosed to have such inherited genetic predisposition. Sporadic CRC, on the other hand, accounts for the majority of cases.^{33, 37}

Incidence of CRC varies greatly over the world, with a higher rate found in parts of Europe, New Zealand, Australia and North America, while the occurrence in most regions of Africa and South Asia remains relatively low.^{5, 33} In addition, rates are elevated due in part to economic development and the adoption of a western lifestyle. Japan provides a striking example of an Asian country where CRC is traditionally uncommon.³⁸ The booming prosperity and the dramatically shifted dietary pattern with sharply increased intake of meat and fat have been met with the remarkable concomitant growth in both prevalence and mortality of colon cancer since the middle of the twentieth century.^{39, 40} Such changes clearly suggest that occurrence of colon cancer is highly positively associated with the poor environmental and lifestyle factors such as unhealthy diet, smoking, alcohol abuse, as well as lack of physical exercise.^{1, 41, 42}

Epidemiological studies indicate that dietary factors are significantly involved in the occurrence of CRC, which are estimated to be responsible for 30% to 50% of CRC

worldwide.³² A high intake of red and processed meat, refined cereals and starches, saturated and trans-fatty acids, and sugars have been correlated to the augment rate of CRC incidence, while diets rich in fruits, vegetables, fibers, fish, unsaturated fats and whole grains are likely to lower CRC risk.⁴³⁻⁴⁵ Nutritional recommendations from the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) suggest the importance of increased consumption of vegetables and fruits, to help reduce the potential of cancer development and progression.⁴⁶ A meta-analysis concluded that high intake of fruits and vegetables were found to decrease 13% and 40% lower CRC risk, respectively.⁴⁴ The protective effects of fruits and vegetables may be largely attributable to the abundant bioactive ingredients consist in them, which have beneficial health activities. In addition, consumption of alcohol has implications in CRC development due to the mutagenic and pro-carcinogenic activities of acetaldehyde metabolized from alcohol.⁴⁷ Besides, tobacco makes contributions to approximately 20% of CRCs, as more than 7000 dangerous components containing various carcinogens exist in tobacco smoke.^{48, 49}

Apart from these, age, sex and race are also the factors that affect CRC risk. While the probability of developing colon cancer augments significantly over 50 years of age,⁵⁰ men have two times higher risk than women.⁵¹ Besides, African American populations are more prone than Caucasian populations to develop colon cancer, while incidence of CRC is lower in Asian Americans.³⁵ Individuals with some diseases including obesity, type 2 diabetes and inflammatory bowel disease (IBD) like ulcerative colitis or Crohn's disease are also at considerably greater risks for developing colon cancer.^{52, 53}

1.1.4 Current therapeutic approaches and challenges

CRC is a highly complex cancer resulted from a various genetic mutations and epigenetic alterations, which leaves great challenges to combat this calamity. Current therapeutic treatments of CRC involve a combination of surgery, radiation, and chemotherapy based on the different diagnosed stages.⁵⁴ Surgical resection remains curative when it is feasible. However, only 70% of colorectal tumors are resectable and CRC is in high probability of relapse, which drives the importance of the adjuvant chemotherapy.⁵⁵ Drug discovery endeavors have yielded a wide array of chemotherapeutic agents such as oxaliplatin (OX), irinotecan (IT), 5-Fluorouracil (5-FU) and so forth.⁵⁶⁻⁵⁹ Main mechanisms for chemoprevention are to suppress the uncontrolled cell proliferation, induce cell apoptosis or autophagy, regulate cell cycle, inhibit tumor cell migration and invasion, and simultaneously stimulate immune response of patients.^{54, 60}

Despite significant advancements in adjuvant therapies of CRC treatment, approximately one half of patients will suffer local recurrence or distant metastasis during the process of the illness.⁶¹ The 5-year survival success rate for patients with advanced CRC, additionally, remains less than 10% as ever.⁶² The gradually developing resistance of colon tumor cells to the majority chemotherapy drugs largely accounts for these depressing facts. For instance, the treatment response rates of 5-FU in the advanced CRC yielded only 10-15%, which limited its usage.⁵⁸ Resistance to oxaliplatin is evidenced to associate with the reduced ability to induce mitochondrial-mediated apoptosis.⁶³ Combinational chemotherapy is used to eliminate the ineffectiveness due to resistance, which is targeting multiple molecular pathways to induce cell

death.^{56, 64} Nevertheless, cancer cells still develop resistance in later rounds of therapy, especially when it tends to relapse and metastasize.⁵⁴ In addition, existing chemotherapeutic drugs are not selective enough, and are mostly cytotoxic and not only work on cancer cells, but also affect normal but fast-dividing cells such as hair follicles and epithelial cells in the digestive system,⁶⁵ which lead to severe adverse side effects, including gastrointestinal complications, alopecia, bone marrow suppression, acute and chronic neuropathy, and hypersensitivity reactions.^{66, 67} As such, alternative chemopreventive agents that are able to target multiple hallmarks of cancer meanwhile exhibit limited toxicity are urgently needed.

1.2 Chronic inflammation

1.2.1 Inflammatory response

Inflammation is characterized as a defensive response of the immune system against various biological, chemical, or physical stimuli, such as pathogens (e.g. bacteria and viruses), trauma (wounds or burns), detrimental chemicals, as well as allergic or auto-immune reactions, which causes a disruption of tissue homeostasis.⁶⁸⁻⁷⁰

Depending on the type of stimulus and the efficiency to remove such stimulus, inflammation can be typically acute or chronic.^{71, 72} Acute inflammation is an innate and initial response that occurs within minutes following infections or tissue injuries. It is a short-term process, lasting few hours or several days, in which fluid and plasma proteins exudate and leukocytes (mostly neutrophils) emigrate from blood to the extravascular damaged tissues to eliminate the agents that could potentially cause tissue injury and restore homeostasis to the affected tissue.^{71, 73-75} These processes are always along with increased temperature in local

tissues, redness, swelling, pain, and loss of tissue function.^{74, 76} However, acute inflammation is a self-limited beneficial response and disappears when immune system successfully removes damaging stimulus and the homeostatic state recovers.⁷⁷ In contrast, failure to clear such detrimental sources will cause a chronic phase of inflammation.

Chronic inflammation is an extended inflammation period, persisting for weeks, months or even years. Upon an immunogenic stimulus, inflammatory responses are initiated through the identification of exogenous pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by pathogen-recognition receptors (PRRs).⁷⁸⁻⁸² Some of the extensively studied PRRs belong to the toll-like receptors (TLRs) family which are membrane receptors on macrophages, dendritic cells and so on, playing a major role in the immune system.⁸³ During inflammation, immune cells such as macrophages and lymphocytes are firstly activated by recognizing PAMPs and DAMPs with TLRs. Activated TLRs recruit several adapter proteins within the cytosol such as MyD88, TRIF, TRAF, and IRAK. Binding of TLRs with adapter proteins stimulates inflammatory responses and sequentially induces signaling cascades involving with intracellular inflammatory signaling molecules, and ultimately activates inflammatory signaling pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), interferon regulatory factor (IRF), and activator protein-1 (AP-1) pathways.⁸⁴⁻⁸⁶ Various pro-inflammatory mediators are activated in inflammatory signaling pathways, such as nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-alpha (TNF- α), interleukins (IL-1 β and IL-6), and pro-inflammatory enzymes, including inducible nitric oxide synthase (iNOS) and cyclooxygenases (COX-2) are

expressed.^{71, 87, 88} Excessive amount of inflammatory mediators may be resulted from the chronic inflammation, which may promote the occurrence of various chronic diseases.^{89, 90} Increasing studies indicate that inflammation in its chronic phase has been widely associated with the development of numerous chronic diseases such as asthma, rheumatoid arthritis, inflammatory bowel diseases, atherosclerosis, obesity, diabetes and various cancers.^{89, 91-96}

1.2.2 Chronic inflammation and cancer development

It is well documented that chronic inflammation acts as key risk factors on the initiation and progression of several diseases especially cancers.⁹⁷⁻⁹⁹ Over 15% of cancer malignancies are initiated by inflammation.¹⁰⁰ For instance, almost 15-20% of smokers with bronchitis are susceptible to develop lung cancer during their lifetime.¹⁰¹ Chronic form of gastritis, an inflammation of stomach lining that is induced by the infection of *Helicobacter pylori* has been shown to lead to gastric cancer.^{102, 103} Besides, one well established link between chronic inflammation and human cancer is that inflammatory bowel disease (such as ulcerative colitis) serves as a precursor to colon cancer.^{104, 105}

Mechanisms contributing to the oncogenic roles of chronic inflammation have been proposed by a large body of epidemiological studies, including induction of genomic instability, alteration of the genomic epigenetic status, enhancement of cell growth, inhibition of cell apoptosis, provocation of angiogenesis, and promotion of tumor cells invasion and metastasis.^{102, 106} Tumor progression are correlated to the increased production of various markers, including transcription factors such as NF- κ B, AP-1, and signal transducer and activator of transcription 3 (STAT3), pro-inflammatory mediators including cytokines,

chemokines, 5-lipoxygenase (5-LOX), COX-2, matrix metalloproteinases (MMPs), hypoxia-inducible factor 1 α (HIF- α), and reactive oxygen species.^{90, 107} Therefore, suppressing inflammatory molecules and thus controlling chronic inflammation has been regarded as a potential strategy for the prevention and treatment of cancers.

1.2.3 Oxidative stress and chronic inflammation

Reactive oxygen species (ROS) are free oxygen or nitrogen radicals that are formed as natural byproducts of normal metabolism of oxygen through cellular mitochondrial respiratory system,⁷¹ including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\bullet$), and nitric oxide ($NO\bullet$).¹⁰⁸ In normal metabolic conditions, such oxidants functions in the germicidal effect of phagocytic cells and are eliminated by the biological protective antioxidant system involving several antioxidant enzymes and biomolecules.¹⁰⁹ However, persistent and long term immune responses, or over exposure to various stimuli such as xenobiotics, pollutants, smoke, drugs, toxic chemicals, and heavy metal ions, result in excessive ROS production.¹¹⁰ The abnormal and elevated level of ROS yields an imbalance between oxidants and antioxidant defense system, leading to detrimental effects, which is termed as oxidative stress.¹¹¹ Overproduction of ROS stimulates immune cells to release a variety of mediators, which provokes recruitment of additional macrophages into the inflammatory site.¹¹² In addition, owing to the presence of an unpaired electron in the outermost shell of electrons, ROS are highly reactive, attacking and damaging vital biomolecules, such as intracellular proteins or enzymes, membrane lipids, carbohydrates, and nuclear DNA in cells and tissues, thereby causing undesirable protein modification, lipid peroxidation, DNA fragmentation and then cell

death.^{113, 114} Therefore, the persistent recruitment of inflammatory cells, repeated stimulation of pro-inflammatory mediators and ROS generation, and continued proliferation of genomically unstable cells are believed to participate in the etiology of several chronic diseases, such as inflammation and cancers.^{115, 116}

1.2.4 Inflammatory mediators

Inflammation involves a set of complex chemical signals responding to traumatic, infectious, toxic and allergic agents and is modulated by immune cells.⁷⁰ A number of mediators, including cytokines, chemokines, pro-inflammatory transcription factors, and pro-inflammatory enzymes molecularly participate in this process¹¹⁷

1.2.4.1 Cytokines

Cytokines, proteins that mediate cell-to-cell communications, are believed to at least partially control the systemic immune response.⁷³ They are mainly produced by activated lymphocytes and macrophages as well as endothelial and epithelial cells and are capable of regulating diverse physiological processes, including cell growth, differentiation, migration, and immune response.^{73, 118} Cytokines have pleiotropic functions that not only can modulate an antitumoral response, but also can induce cell transformation and malignancy during chronic inflammation.⁷⁰ Thus, they can be broadly classified as pro-inflammatory cytokines such as IL-1, IL-2, IL-6, IL-1 β , and IFN- γ , and anti-inflammatory ones like IL-4, IL-10, and TGF- β .^{118, 119} Among them, TNF- α and IL-6 are two widely studied cytokines, playing predominant roles in the inflammatory process and the downstream chronic inflammation-related diseases.^{75, 120}

Specifically, tumor necrosis factor-alpha (TNF- α) is a potent pro-inflammatory cytokine

that exhibits various biological activities by binding to general receptors on the surface of target cells.¹²¹ TNF- α promotes various chemokines and cytokines to commence acute and chronic phases of inflammation and is implicated in the development of carcinogenesis.^{122, 123} It can induce DNA damage, facilitate angiogenesis, and provoke COX-2 expression to initiate and promote tumor growth.¹⁰⁵ Various human cancer cells, such as those of breast, prostate, colorectal, and bladder have been detected in an overexpression of TNF- α .¹²⁴⁻¹²⁷ Interestingly, studies have shown a dual role of TNF- α in carcinogenesis. A high level of TNF- α was found to be able to both causes necrosis and promote tumor cells growth.¹¹⁸ Pleiotropic functions of TNF- α , with both pro-inflammatory and immune-regulatory activities, were also demonstrated during chronic inflammation.¹²⁸

Interleukin (IL)-6 is another vital pro-inflammatory cytokine playing a key role in the acute response associated with the stimulation of acute phase proteins synthesis by liver.^{75, 121} IL-6 instructs the transition from acute to chronic inflammation in combination with its soluble receptor sIL-6R α , by alternating the structure of leucocyte infiltration from polymorphonuclear neutrophils to monocyte/macrophages.⁷⁵ As such, IL-6 is known to favor chronic inflammation and related diseases. For instance, elevated serum level of IL-6 has been implicated in rheumatoid arthritis, psoriasis, systemic lupus erythematosus, and some systemic cancers.¹²⁹⁻¹³² Besides, it has been demonstrated that IL-6 modulates genes expressions that involved in cell cycle progression and suppression of apoptosis, mainly through the JAK (Janus kinase)-STAT signaling pathway.¹¹⁸ Thus, IL-6 has been turned into a potential drug target in the treatment of chronic inflammatory diseases.

1.2.4.2 Nitric oxide (NO) and inducible nitric oxide synthase (iNOS)

Nitric oxide (NO), a gaseous free radical, is another important inflammatory mediator linking chronic inflammation and a variety of chronic diseases.¹¹⁸ The production of NO results from converting L-arginine into NO and L-citrulline by nitric oxide synthase (NOS), involving endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).^{133, 134} NO produced by constitutively expressed eNOS and nNOS at low physiological levels is essential to maintain immune homeostasis,¹³⁵ whereas prolonged larger amounts of NO are synthesized by iNOS, which is a dimeric and Ca^{2+} independent enzyme, highly expressed by inflammatory stimuli.¹³⁶ As a reactive radical, excessive production of NO causes DNA damage by impairing the functionality of cellular machinery that is responsible for DNA repair.¹³⁷ Overproduction of NO also induces p53 gene mutations,¹³⁸ thereby inducing genomic instability.¹³⁹

On the other hand, iNOS is not detectable in healthy tissues but is highly expressed in response to continuous stimulation, including lipopolysaccharide (LPS) and pro-inflammatory cytokines, which in turn can increase the production of NO.^{136, 140} It has been reported that improper up-regulation of iNOS and NO is involved in the pathophysiology of diverse malignant tumors, such as breast, colorectal, lung, prostate cancers, and melanoma.^{138, 141, 142}

1.2.4.3 Cyclooxygenase-2 (COX-2)

In addition to iNOS, cyclooxygenase-2 (COX-2) is another pro-inflammatory enzyme involved in the inflammatory process, which converts arachidonic acid (AA) into prostaglandins.^{76, 117, 143} There are two prostaglandin H synthase isoforms, COX-1 and COX-2. While the expression of COX-1 exists in many tissues, mitogens, tumor promoters, and growth

factors regulate the expression of COX-2 that can also be stimulated by pro-inflammatory cytokines and oncogenes.^{76, 139} Aberrant induction of COX-2 has been implicated in several types of cancers, by up-regulating generation of prostaglandins, which promote cell survival, angiogenesis, and metastasis.^{16, 144-146} It is frequent to find elevated levels of COX-2 in numerous human malignancies, such as breast, gastric and colon tumors, suggesting a role of COX-2 as a regulator between inflammation and cancers.¹⁴⁷ For instance, overexpression of COX-2 has been detected in up to 90% of colon carcinomas and 40% of colon adenomas.¹³⁹ Thus, COX-2 is a vital target for anti-inflammation therapy as well.

1.2.5 Current treatments for inflammation

Based on the World Health Organization (WHO) report, chronic inflammation has been the greatest threat to public health, as three of every five individuals die from chronic inflammation induced diseases like diabetes, heart disorders, and cancers.¹⁴⁸ It is anticipated that the prevalence of such diseases will rise steadily in the next 30 years.¹⁴⁸ In this respect, effectively treating chronic inflammation is of primary importance.

Currently, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) are commonly applied to combat inflammation.⁷⁶ However, these NSAIDs are non-selectively and irreversibly inhibit the activity of both COX-1 and COX-2, whereas constitutively expressed COX-1 is essential for homeostatic functions.^{149, 150} Due to the unexpected COX-1 inhibition, long-term administration of NSAIDs results in severe adverse effects, especially gastric ulceration, due to the protective effects of COX-1 for the stomach lining.^{71, 76} In addition to gastrointestinal disturbances, NSAIDs also can cause salt and water retention, renal

dysfunction, bronchospasm, and hypersensitivity reactions.⁷¹ Compare to non-selective NSAIDs, COX-2 selective inhibitors (known as Coxibs) such as celecoxib and rofecoxib, have the established efficacy in the treatment of inflammation with better gastrointestinal safety.⁷¹ Nevertheless, Coxibs can promote renal sodium reabsorption, thus predisposing to hypertension and edema, which is related to cardiovascular complications.^{151, 152} In consequence, there is still a pressing need to develop safer, less toxic and more cost-effective therapeutic alternatives.

1.3 Dietary polyphenols

1.3.1 Classification and chemical structures of polyphenols

As noted, poor diet habits have been evidenced to further increase the cancer burden, and researchers therefore have turned to dietary factors to intervene and prevent cancers. A great deal of studies have associated the increased consumption of fruits and vegetables with the reduced cancer incidence. Possible mechanisms accounting for the protective effects of such natural foods are largely ascribed to the abundant bioactive compounds existing in them, such as polyphenols, which possess health beneficial effects.

Dietary polyphenols are naturally-occurring secondary plant metabolites that are formed to protect plants against environmental stressors and stimulators such as reactive oxygen species and photosynthetic stress.^{153, 154} They are characterized by the presence of at least two phenolic groups in the structure and are ubiquitous in diverse fruits, vegetables, nuts, seeds, legumes, and plants.^{155, 156} Over thousands of polyphenols have been identified, ranging from

low molecular weight phenolic acids to the large and highly polymerized tannins.^{156, 157} Polyphenols are differentiated from different numbers of phenolic rings and the way they bond, and are broadly divided into four common classes, including phenolic acids (such as caffeic acid and ferulic acid), flavonoids, lignans and less common stilbenes (e.g. resveratrol).^{158, 159}

Among these, flavonoids are the most widely studied group, which can be further grouped into several sub-classes. Flavones include luteolin and apigenin; flavonols include mostly quercetin, myricetin, and kaempferol; flavan-3-ols include the class of catechins; anthocyanins mainly include malvidin, cyanidin, delphinidin, and pelargonidin; and isoflavones include genistein and daidzein.¹⁶⁰⁻¹⁶² Flavonoids share the same structural skeleton, a 15-carbon phenylchromone structure (C6-C3-C6) composed of two benzene rings (A and B) linked via a heterocyclic pyrane C ring.¹⁶³ Modification to the structure skeleton, via different levels of oxidation and substitutions to the C ring, makes contribution to diverse types of flavonoids, and their versatile biological functions.

1.3.2 Pharmacological properties of polyphenols

Proverbially, natural phenolic compounds are believed to confer a broad spectrum of pharmacological activities, such as antioxidant, anti-inflammatory, anti-carcinogenic, anti-viral, anti-bacterial and anti-atherogenic activities.^{164, 165} Numerous studies have contributed to this postulation.

1.3.2.1 Polyphenols and colon cancer

There is a strong epidemiological evidence suggesting that consumption of diets high in fruits, vegetables and plant foods is associated with the decreased risks of diverse cancers,

especially reduced the prevalence of colorectal cancer, which appears to be the most relevant to dietary factors.^{44, 166} Therapeutic effects of numerous polyphenols such as curcumin, resveratrol, quercetin, and green tea catechins on combating versatile cancers have been well documented.^{154, 167, 168} Curcumin has been recognized as a promising antitumor drug via inhibiting tumor initiation, promotion, invasion, and metastasis through a variety of mechanisms.¹⁶⁹⁻¹⁷³ Resveratrol has been reported to significantly reduced β -catenin, a key component of the Wnt signalling pathway in colon cancer regulation.¹⁷⁴ In vitro experiments showed that proanthocyanidins effectively inhibited the PI3k/PKB pathway and induced apoptosis in colon cancer cells.¹⁷⁵ Lycopene was also found to inhibit the proliferation of human colon cancer cells HT-29 via inhibition of the Akt signaling pathway.¹⁷⁶

1.3.2.2 Polyphenols and chronic inflammation

As chronic inflammation is intimately associated with the progression of various cancers, mounting epidemiological studies have reported the role of polyphenols in the remedy of inflammation. Oxidative damage to biomolecules such as proteins, lipids and DNA is supposed to be closely linked to human health concerns, including chronic inflammation and the downstream related chronic diseases such as carcinogenesis, neurodegenerative disorders and cardiovascular diseases.^{71, 177} In this regard, neutralizing the over generated free radicals such as ROS by supplemental antioxidants is a promising approach to attenuate inflammation. Polyphenols are the most frequent antioxidants in human diets.^{157, 178} The acceptance an electron by the phenolic groups of polyphenols to form relatively stable phenoxyl radicals results in the disruption of oxidation chain reactions in cellular components.¹⁷⁹

Apart from anti-oxidative activity, polyphenols perturb the inflammatory response by mediating various inflammatory factors, including cytokines, pro-inflammatory enzymes and also transcription factors.^{180, 181} Green tea catechins was reported to down-regulate COX-2 expression in LPS-induced macrophages and inhibit IL-1 β dependent pro-inflammatory signal transduction in cultured respiratory epithelial cells^{182, 183}. Using LPS activated macrophages, quercetin was found to inhibit the iNOS expression, which subsequently resulted in suppression of NO generation.¹⁸⁴ Besides, inhibition of NF- κ B may account for the anti-inflammatory effects of many polyphenols including resveratrol, 6-gingerol, and caffeic acid phenethyl ester (CAPE).⁷⁶

1.3.3 Challenges of applying polyphenols for disease intervention

In spite of the wide spectrum of pharmacological effects, the further use of dietary polyphenols in the pharmaceutical field is impeded due to some physiological limitations, such as the poor aqueous solubility, low stability, and the resultant poor bioavailability.

1.3.3.1 Solubility and stability

Chemically, dietary polyphenols have phenyl rings and phenolic groups in the structure. Due to these structural characteristics, many dietary polyphenols are lipophilic, thus resulting in a low solubility in aqueous environments. For instance, curcumin is only sparingly soluble in water, with the solubility of only about 11 ng/mL,¹⁸⁵ while it has a high solubility in organic solvents such as dimethyl sulfoxide, acetone and ethanol.¹⁸⁶ Similarly, resveratrol has a water solubility of less than 0.05 mg/mL, which consequentially leads to an undesired oral bioavailability.¹⁸⁷

In addition, the structural characteristics of polyphenols are responsible for the susceptibility to degradation by various environmental factors, such as pH, ionic strength, temperature, oxygen and light,¹⁸⁸ which results in considerable changes in their physicochemical and functional properties.¹⁸⁹ Typical reactions leading to chemical degradation of bioactives include oxidation, reduction, hydrolysis, and isomerization.¹⁹⁰ In regard to curcumin, the lipophilic molecule with phenolic groups and conjugated double bonds, is degraded rapidly by light and basic pH, while only less than 20% of total curcumin decomposed at 1h under acidic conditions.^{191, 192}

1.3.3.2 Bioavailability

The therapeutic efficacy of phenolic compounds depends primarily upon their bioavailability. Bioavailability is defined as the rate and extent to which the bioactive ingredient is absorbed into systemic circulation and becomes available at the targeted site.¹⁹³ It relies on the forms of the bioactive compounds that present *in vivo*, the relative absorption from the intestine, and also the rate of metabolism and clearance from the body.¹⁹⁴ Many lipophilic phenolic components have low bioavailability within the human gastrointestinal tract, which may result from the low bioaccessibility, poor absorption, high rate of metabolism or rapid elimination, as well as their poor solubility, high melting point, and chemical instability.^{195, 196} As an example, most of the ingested EGCG is not absorbed and is there metabolized by the gut microbiota; the bioavailability and thus potential bioactivity can be low.¹⁹⁷ Therefore, enhancing the bioavailability of dietary polyphenols is critical to enable the health effects *in vivo*.

1.3.4 Apigenin

1.3.4.1 Role of apigenin in colon cancer

Apigenin (4',5,7-trihydroxyflavone) (Figure 1-1), one of the most promising polyphenols, is predominantly distributed in several fruits, vegetables, and medicinal plants, including parsley, paper mulberry and chamomile.¹⁹⁴ Owing to the superiority of non-mutagenesis and low intrinsic toxicity,¹⁹⁸ apigenin has received great attention as a potential pharmaceutical agent for benefiting human health. The potential mechanisms of apigenin acting as a cancer suppressor are recognized for targeting diverse molecules and modulating versatile kinase pathways, including promoting cell cycle arrest,¹⁹⁹ inducing apoptosis,²⁰⁰⁻²⁰⁴ inhibiting mutagenesis,²⁰⁵ and suppressing signal transduction^{189,196,197}. It was reported that apigenin induced ERK and p38MAPK pathway, but ineffective on the Jun kinase activity in HCT-116 cells.²⁰⁶ Apigenin was able to suppress the Wnt/ β -catenin signaling pathway in colon cancer cell, thereby inhibiting cell growth, migration, and invasion.^{207, 208} Apigenin reduced the proliferation of colorectal cancer cells via arresting cell cycle at the G2/M phase along with a decreased expression of cyclin B1.²⁰⁹ Besides, an *in vivo* anti-tumorigenic study using a carcinogen-induced mice model showed that apigenin was capable of reducing ACF formation, the early preneoplastic lesions in colonic mucosa.²¹⁰

1.3.4.2 Role of apigenin in chronic inflammation

The protective effects of apigenin on chronic inflammation have also been well established. Studies showed that apigenin inhibited the production of cytokines,²¹¹ suppressed NO production in LPS-induced macrophages,²¹² and blocked the collagenase activity that is

involved in rheumatoid arthritis.²¹³ Apigenin exhibited anti-inflammatory activity against ulcerative colitis (UC) in C57BL/6J mice in mechanisms involving the inhibition of inflammatory cytokines, COX-2 expression, and the reduction in immune cell infiltration in colonic tissues.²¹⁴ Apigenin also served as a potent therapeutic agent in a murine dextran sulphate sodium (DSS) colitis model through the reduction of matrix metalloproteinase (MMP-3) level.²¹⁵

1.3.4.3 Major limitations of apigenin

Despite the profound therapeutic benefits given above, apigenin has a very low solubility in water (1.35 µg/mL).²¹⁶ The highest aqueous solubility reported is only 2.16 µg/mL at pH = 7.5,²¹⁷ leading to poor intestinal absorption. Apigenin is extensively metabolized after oral administration and is mainly eliminated by the first-pass metabolism via glucuronidation and sulfation in human bodies.^{161, 218} The rapid metabolism causes a considerable accumulation of conjugates in the systemic circulation and thereby causes low bioavailability.²¹⁹ Taken together, the improvement of solubility and bioavailability is urgently required for developing applications of apigenin.

1.4 Synergistic interactions between bioactive compounds

Additive and synergistic interactions of multiple phytochemicals present in fruits and vegetables are suggested for their health benefits, resulting the recommendation of consuming whole foods.²²⁰ This interesting “whole foods” theory has boosted numerous studies focusing on the achievement of synergistic health beneficial effects by combinations of bioactive compounds. Combinations of two or more purified bioactive agents or phytochemical-enriched

plant extracts can modulate the antioxidant, anti-inflammatory and anti-cancer activities, as well as the bioavailability and in vivo activity of each component. Mixtures of components can produce additive, synergistic or antagonistic effects. When the effectiveness of combinations is equal to the sum of individual components in the mixture, such interactions are additive; whereas the greater or less effects of combinations than the additive effects result in synergistic or antagonistic interactions.²²¹ These interactions are always analyzed by the combination index (CI), as $CI > 1$, $= 1$, < 1 represents antagonism, synergism, and addition effects, respectively.²²²

1.4.1 Synergistic anticancer effects of dietary compounds

Current cancer chemotherapy has resulted in severe side effects and drug resistance. Therefore, combination of different naturally occurring compounds has been employed to treat different cancers, to potentially reduce drug dosages and alleviate the side effects of one drug, to overcome drug resistance, as well as to potentiate the therapeutic outcome by targeting multiple signal transduction pathways.^{223, 224} Studies have reported that EGCG synergistically inhibited cancer cell viability and migration, induced apoptosis and suppressed angiogenesis when combined with other natural molecules, such as ascorbic acid, curcumin, quercetin and proanthocyanidins.²²⁵⁻²²⁷ Besides, a study in patients with familial adenomatous polyposis reported that the therapy combining curcumin and quercetin reduced the number and size of polyps from baseline and the adverse reactions were negligible.²²⁸ Mixtures of resveratrol, chrysin and curcumin exerted synergistic anti-proliferative activity against Caco-2 colon cancer cells.²²⁹ Likewise, blends of grape seed extracts and resveratrol enhanced the

suppression of HCT-116 colon carcinogenic cells.²³⁰ The above studies suggest that mixtures of bioactives with similar or complementary anti-cancer mechanisms can be studied for potential synergy in the anti-cancer efficacy.

1.4.2 Synergistic anti-inflammatory activities of dietary compounds

Due to the complexity of inflammatory process, several combinations of dietary compounds attempted to enhance the efficiency of inflammation treatments. For instance, synergistic protective effects occurred in LPS-induced RAW 264.7 cells when combined luteolin with tangeretin.²³¹ Combinations of polyunsaturated fatty acids (DHA or EPA) with a low dose of curcumin have been found to synergistically inhibit NO production, and suppress expressions of prostaglandin E2 (PGE2), iNOS, COX-2, 5-lipoxygenase (5-LOX) and cPLA2 in LPS-activated RAW 264.7 macrophages.²⁰² Different combinations of polyphenolic compounds, including nobiletin and sulforaphane, lycopene, lutein, β -carotene, and carnolic acid have also shown synergistic effects by modulating various inflammatory mediators in LPS-induced RAW264.7 cells^{117, 232} The synergistic anti-inflammation activity of such polyphenol combinations can result from multi-target effects of the individual compounds that are otherwise impossible for a single component.

In addition to anticancer and anti-inflammatory effects, combination of phytochemicals may also result in other biological changes, such as the antioxidant activity and the bioavailability of individual components. Hyperoside acted in synergy with EGC on ABTS $\cdot$ and DPPH $\cdot$ radical scavenging activities.²³³ Besides, β -carotene was found to increase the bioavailability of lycopene in human plasma.²³⁴ Some natural molecules such as caffeine,

genestein, piperine, and quercetin, were also found to enhance the bioavailability of EGCG in mice.²³⁵⁻²³⁸ Overall, combinations of bioactive ingredients could induce changes in biological activities and bioavailability of the component. Constructing such combination systems with synergistic biological benefits may serve as effective strategy to deal with cancers and chronic inflammation.

1.5 Nanoencapsulation systems and underlying problems

Given the low water solubility and bioavailability of many hydrophobic dietary polyphenols limiting their therapeutic efficacy and further applications, nanoencapsulation has appeared as an innovative and effective strategy to address these challenges. A number of nanoencapsulation systems have been developed, including emulsions, liposomes, micelles, polymeric nanoparticles, inclusion complexes, and nanocrystals. Their developments, effectiveness, and underlying problems are specifically discussed.

1.5.1 Emulsions

Emulsions are thermodynamically unstable dispersions typically formulated by mixing two immiscible liquids in the presence of surfactants with droplet dimensions of 0.1 to 100 μm .^{188, 239, 240} According to the droplet size, nanoemulsions are further defined as dimensions less than 100 nm.²⁴¹ Emulsion structures are differentiated from the spatial distribution of the oil and water phases. Oil-in-water (O/W) emulsion refers to the system consists of oil droplets dispersed in the water phase, whereas water-in-oil (W/O) emulsion is formed through the aqueous solution dispersed in oil phase.²⁴² However, when exposed to environment stresses,

such as extreme temperatures, pH values, and salt concentrations, simple emulsions are often physical instable due to coalescence or Ostwald ripening.²⁴³ Thus, more sophisticated multiple systems have been developed, such as water-in-oil-in-water (W/O/W) emulsions, oil-in-water-in-oil (O/W/O) emulsions.^{242, 243} Distinguished from conventional emulsions, microemulsions are isotropic and thermodynamically stable systems with dispersed droplet diameters varied from 10 to 50 nm.²⁴⁰ Usually, nanoemulsions are typically resulted from high-energy methods, such as homogenization, microfluidization or sonication,^{239, 244} while microemulsions are formed spontaneously by self-assembly with a relatively higher amount of surfactants.^{188, 245}

Emulsion-based delivery systems are widely applied to encapsulate lipophilic or hydrophilic ingredients, protecting them against harsh environmental factors, and improving biological activities.^{243, 246} For instance, degradation of curcumin at pH 5.9 was prevented by nanoencapsulation into nanoemulsions containing Tween 80, lecithin, ethyl oleate, and water during two month storage.²⁴⁷ In comparison to the free state curcumin, the resulted nanoemulsions with droplet size of 114 nm resulted in a 1400-fold increment of the solubility. Quercetin formulated in nanoemulsions increased the bioaccessibility by two times than that solubilized in bulk oils.¹⁸⁸ Olive leaf phenolic extracts were incorporated in W/O/W multiple emulsions stabilized by WPC and pectin.^{248, 249} The antioxidant activity of the resulted emulsion in soybean oil was enhanced compared with the non-encapsulated phenolic extracts. Besides, food-grade microemulsions made of lecithin, caprylic/capric triglycerides, isopropyl myristate, alcohols, and water were produced to nanoencapsulate natural antioxidants, such as gallic acid, p-hydroxybenzoic acid, protocatechuic acid, and tyrosol, with droplet size of

smaller than 10 nm.²⁵⁰ The increased bioavailability of diacylglycerol oils dissolved in microemulsions have also been reported.²⁵¹ Nevertheless, the high cost of energy input and the large amount of surfactants or co-surfactants which may lead to mucosal toxicity are the major disadvantages existing in these systems.

1.5.2 Solid lipid nanoparticles and nanostructured lipid carriers

Similar to conventional O/W emulsions, solid lipid nanoparticles (SLNs) also consist of a hydrophobic core and a hydrophilic shell, however, the liquid oils are replaced by the solid state lipids, including fatty acids (e.g. decanoic acid and stearic acid), triglycerides (e.g., tristearine), waxes (e.g., cetyl palmitate), and steroids (e.g. cholesterol).^{252, 253} Surfactants or co-surfactants are applied to stabilize the lipid phase.²³⁹ SLNs are usually fabricated by initially preparing an O/W emulsion using different strategies at elevated temperatures above the melting point of the oil phase, then cooling down to promote lipid crystallization.^{244, 254} High-energy methods including high shear homogenization, high-pressure homogenization (hot and cold), and ultrasonication are commonly used to yield SLNs.^{188, 253, 255} SLNs can also be prepared by laboratory-scale approaches such as phase inversion and solvent emulsification/evaporation.^{253, 256} Encapsulating lipophilic compounds into the solid lipid core have been reported to increase the stability, reduce chemical degradation, and prolong the release of components since the solidified lipid phase is able to inhibit molecular diffusion.^{239, 246, 257} For example, enhanced refrigerated and ambient storage stability as well as bioavailability of curcumin were reported after incorporating into SLNs which were prepared by high shear homogenization and ultrasonication followed by coating with chitosan.²⁵⁸

Resveratrol was loaded into SLNs formulated with surfactants of Tween 80 and polyvinyl alcohol (PVA).²⁵⁹ The resulted particles showed a sustained release of resveratrol and promising anti-neoplastic activity in brain tissues.

Despite the widely use of SLNs as delivery systems, SLNs suffer a number of limitations, such as the low ingredient loading capacity, possibility of compound leakage during storage, and susceptibility of particle aggregation.^{244, 260} As for this, nanostructure lipid carriers (NLCs) with a mixture of solid–liquid lipids that crystallize into more irregular structures were developed.^{260, 261} Compared with the highly ordered solid structure formed in SLNs, the irregular solid lipid phase in NLCs resulted in more effective encapsulation of bioactive compounds, with a higher loading capacity, higher encapsulation efficiency, and higher bioavailability.²⁴⁴ In a study, resveratrol were incorporated in both SLNs and NLCs by homogenization. SLNs were developed using glyceryl behenate while NLCs were prepared by using liquid migliol oil as a replacement of a part of the solid lipid. The resulted NLCs were found to have 18% higher encapsulation efficiency of resveratrol and smaller particle sizes, which promote the superiority of NLCs for further dermal applications.²⁶² Curcumin loaded NLCs showed high encapsulation efficiency of 91.76% with a nanometric size of 263.9 nm.²⁶³ Enhanced skin permeation, anti-oxidation, and anti-inflammation of quercetin were also reported in a NLC system prepared by evaporation-solidification at low temperature.²⁶⁴

1.5.3 Liposomes

Liposomes are spherical vesicles self-assembled from phospholipid bilayers surrounding an aqueous core.^{244, 245} The interactions between the amphiphilic phospholipids and the water

molecules driving the hydrophilic heads exposed to the interior aqueous phase and exterior environment while the hydrophobic fatty acid tails to the bilayer are accounting for the formation of liposomes.²⁵³ Due to the biphasic character, liposomes are able to deliver both hydrophilic and hydrophobic bioactive agents.²⁶⁵ Besides, the nanometric size of liposomes which refers to as nanoliposomes have higher potential to improve the bioavailability of entrapped compounds due to the larger interfacial area contacting with biological tissues produced by the smaller size.²⁶⁶ Consequently, higher energy input is required to reduce the size of liposomes. Nanoliposomes are commonly produced by high pressure homogenization, microfluidization, extrusion, and sonication.^{239, 253} Liposomes have been evidenced to enhance the bioactivity and bioavailability of phenolic compounds by a number of studies. In a study, quercetin loaded in liposomes and administered by intranasal administration showed anxiolytic and cognitive beneficial effects when compared to pristine quercetin administered orally.²⁶⁷ Apigenin-loaded liposomes, fabricated by a lipid film hydration method in the presence of methanol, showed enhanced chemotherapeutic efficacy against colorectal cancer based on flavone-membrane interactions.²⁶⁸ Nanoliposomes were also carried out for encapsulation of essential fatty acids to protect them against oxidation and increase their bioavailability.^{269, 270} Due to the susceptibility to oxidation and physical instability, liposomes are always coated by polymers to achieve steric stabilization.^{239, 271} As an example, curcumin loaded nanoliposomes coated with chitosan were demonstrated to protect curcumin against digestion in mouth and gastric tract *in vitro*, and promote absorption in the small intestine and thereby enhancing the bioavailability.²⁷² Despite these reports, the residual organic solvent, the high cost of the

phospholipids and surfactants are potential problems restricting the wide applications of liposomes in food systems.

1.5.4 Micelles

Micelles are thermodynamically stable systems formed spontaneously when the concentration of surfactants exceeds their critical micelle concentration (CMC) in water.^{188, 239} Recently, polymeric micelles that composed of amphiphilic block-copolymers are gaining popularity as delivery systems.²³⁹ The hydrophobic and hydrophilic monomer units existed in the molecule formulate a core-shell micelle structure which can accommodate lipophilic drugs to the hydrophobic core, thereby increasing the solubility, enhancing the permeability and protecting them from the biological environments such as the gastrointestinal tract.^{239, 271, 273} For instance, apigenin-loaded polymeric micelles made of Pluronic P123 and Solutol HS 15 were reported to improve cytotoxicity against HepG2 and MCF-7 cancer cells.²⁷⁴ It has been found that the solubility of curcumin was greatly increased after incorporation in polymeric micelles composed of methoxy poly (ethylene glycol)-block-polycaprolactone diblock copolymers (MePEG-b-PCL).²⁷⁵ Quercetin was delivered by a nanomicelle prepared by the diblock copolymer of polyethylene glycol (PEG)-derivatized phosphatidylethanolamine (PE) and the micellar formulation presented an enhanced anti-cancer activity both in *in vitro* A 549 lung cancer cells and *in vivo* murine xenograft model.²⁷⁶

1.5.5 Polymeric nanoparticles

Polymeric nanoparticles are colloidal delivery systems fabricated from food proteins and polysaccharides, as well as synthetic polymers such as polylactide-co-glycolide (PLGA),

polyglycolic acid (PGA), and polylactic acid (PLA).^{253, 277} Due to the high biodegradability, biocompatibility, low toxicity, slow and sustained drug release, polymer-based nanoparticles have been regarded as popular delivery systems for the encapsulation of various therapeutic ingredients.^{253, 278} Incorporation of curcumin into zein nanoparticles with 100-150 nm increased the stability of curcumin against different pH values and UV irradiation.²⁷⁹ Casein nanoparticles were developed to protect vitamin D from thermal treatment as well as increase the long-term cold storage stability and bioavailability.²⁸⁰ Quercetin loaded PLA nanoparticles were prepared by a simple evaporation method.²⁸¹ Besides, apigenin encapsulated in PLGA nanoparticles with a dimension of about 100 nm, prepared by solvent displacement, were investigated for anti-proliferative potentials in A375 cells *in vitro*.²⁸² Chitosan nanoparticles are of great interest being used as an absorption enhancer due to its positive charge and mucous adhesiveness.^{246, 283, 284} In an attempt to encapsulate EGCG into peptide/chitosan nanoparticles, more than two-fold enhancement permeation rate across CaCO-2 cells was observed.²⁸⁵ Thus, it is clear that the versatile polymeric nanoparticles have great potential to deliver functional dietary components, however, the residual organic solvent produced during the formation process such as solvent evaporation,²⁵³ nanoprecipitation,²⁷⁷ and desolvation²⁴⁴ may possess potential toxicity.

1.5.6 Inclusion complexes

In molecular inclusion, encapsulated molecules are entrapped by the shell materials, generally cyclodextrins (CDs), through hydrogen bonding, van der Waals forces or hydrophobic interactions.^{188, 242, 286} CDs are naturally occurring cyclic oligosaccharides derived

from starch by bacterial enzymatic conversion, consisting of six, seven or eight glucopyranose units known as α -, β -, and γ -CDs, respectively.^{188, 253, 287} β -CD is the most commonly applied CD for encapsulation.²⁴² The molecular structure of CDs is characterized with an internal hydrophobic cavity and an external hydrophilic rim.²⁸⁸ which ensure their ability to encapsulate poorly water soluble phytochemicals into the apolar interior part through hydrophobic interactions, thereby increasing their aqueous solubility, stability and bioavailability.^{242, 244} Kim et al. investigated the inclusion of apigenin in (2-hydroxypropyl)- β -cyclodextrin (HP- β -CD), and increased the solubility by 11.5 times.²⁸⁹ Significant improvement of the solubility and anti-proliferative activity against human erythroleukaemia and cervix cancer cells of quercetin occurred after inclusion into the sulfobutyl ether-7 β -cyclodextrin.²⁹⁰ Curcumin-loaded β -CD was prepared using a solvent evaporation technique. Compared with the free curcumin, the resulted complex showed increased stability at pH 7.4, 37°C and anti-inflammatory effects in prostate cancer cell lines.²⁹¹ Apart from phytochemicals, volatile organic molecules have been encapsulated in inclusion complexes to mask odors and flavors, or preserve aromas.²⁸⁶

1.5.7 Nanocrystals

Nanocrystals are another approach widely used to overcome the solubility issue of bioactive compounds. Main advantages of nanocrystals are not only the increased drug surface area, the increased dissolution rate, but also the increased penetration and cellular uptake by epithelium cells due to the small particle dimensions, which significantly improve the bioavailability of phytochemicals.²⁹²⁻²⁹⁴ For example, loading quercetin in nanosuspension yielded a 70-fold increment of the solubility, and the clearance rate in plasma was significantly

reduced with an increased bioavailability of quercetin.²⁹⁵ Nanocrystals are commonly produced by the “top-down” technology, for example, bead milling or high pressure homogenization.^{288,}
²⁹⁶ These methods require no harsh solvents, however, the high energy input, high cost, and low power efficiency are underlying problems remain to be resolved.

In conclusion, in spite of extensive studies, many of these approaches remain problematic for food applications. In this respect, a constant demand for new types of non-toxic nanocarriers and low-cost, low-energy technologies are still significantly needed.

1.6 Bovine milk proteins as effective encapsulation carriers

Food proteins provide nutrition and functions.²⁹⁷ Proteins isolated from bovine milk are chemically and structurally versatile, and are promising natural nano-vehicles for delivering bioactives.^{297, 298} Bovine milk proteins consist of two major categories: caseins and whey proteins (Table 1-1).²⁹⁸

1.6.1 Caseins

Caseins account for almost 80% of the total bovine milk proteins and are inexpensive, widely available,²⁹⁸ biocompatible, and biodegradable.²⁹⁷ Caseins are composed of approximate 94% protein and 6% minerals known as colloidal calcium phosphate.²⁹⁹ Mainly four types of caseins, α_{s1} -, α_{s2} -, β - and κ -casein exist in bovine milk, differing in amino acid, number of phosphorus atoms, content of carbohydrate, functional properties, and genetic variations.³⁰⁰⁻³⁰² α_{s1} -, α_{s2} -, and β -casein are phosphoproteins with serine-phosphate residues for calcium binding.³⁰³ In particular, α_{s1} -casein has the highest negative charge and phosphate

content; α_{s2} -casein is more sensitive to Ca^{2+} than others, whereas β -casein has the highest portion of prolines (17% proline residues).²⁹⁹ κ -casein, on the other hand, is a glycoprotein with two cysteines and is able to form disulfide bridges.³⁰⁴ Due to the high levels of hydrophobic and hydrophilic amino acid residues, caseins are amphiphilic proteins and are naturally self-assembled into micelles in aqueous solutions with an average size of 150-200 nm.^{304, 305} Casein micelles are proposed to be held together by calcium phosphate nanoclusters binding with phosphopeptides in the core and stabilized by the charged surface coverage with the hydrophilic segment known as glycomacropeptide of κ -casein, providing electrostatic and steric repulsions.³⁰⁶⁻³⁰⁸ Caseins are in open structure due to the high content of proline.³⁰³ The highly random and flexible structure enables their thermal stability, however, results in a susceptibility to changes in temperature, pH, ionic strength, and high hydrostatic pressure which will alter their size distribution.³⁰² Lowering temperature favors the dissociation of casein micelles.³⁰⁹ Casein micelles can also be disrupted under alkaline conditions, but aggregate and precipitate at isoelectric point of 4.6–4.8, due to the alteration of calcium phosphate solubility and electrostatic interactions.^{300, 310} Besides, high pressure homogenization,³¹¹ hot ethanol (> 35%) aqueous solution with above 60 °C are also able to disrupt the micellar structure.^{312, 313}

1.6.2 Whey proteins

In contrast, whey proteins constitute the rest 20% of bovine milk proteins and are generally produced as a co-product of the cheese industry.³¹⁴ They are typically globular proteins of variable compositions and diverse biological functions.²⁴⁰ The functional properties

of commercial whey protein products are mainly controlled by the predominant whey protein, β -lactoglobulin (β -lg).³¹⁵ β -lg is a small globular protein made up of 162 amino acids.²⁹⁹ The structure of β -lg is characterized as a β -barrel calyx folded up by eight antiparallel β -strands (strands A-H), an important part on the dimer interface formed by the ninth strand (strand I), and an α -helix on the outer surface.^{299, 316} Five cysteines exist in the β -lg sequence, resulting in two disulfide bonds and a free thiol group which is responsible for the pH dependent activity and plays an important role in protein aggregation and denaturation.^{297, 316} It is mainly found as dimers at neutral pH, whereas at acidic pH below 3.5 or above 7.5, β -lg always dissociates into monomers due to the electrostatic repulsions between the subunits.^{299, 316} Due to the stability of globular conformation in acidic conditions, β -lg is resistant to peptic degradation,²⁴³ which makes it capable to protect bioactives in the gastric tract.³¹⁷ As the second most abundant whey protein, α -lactalbumin (α -la) is a smaller globular metalloprotein stabilized by four disulfide bridges.³¹⁸ α -la is partially responsible for the synthesis of lactose in the mammary gland.³¹⁹ In addition, minor whey protein, bovine serum albumin (BSA), is a larger protein with 582 amino acids and structurally contains a main α -helix, 17 intramolecular disulfide bonds, and a free thiol.^{304, 320, 321} It is found both in blood serum and milk, with the capability to bind and deliver various small ligands.³⁰⁴ Bovine lactoferrin is a monomeric globular glycoprotein with around 700 amino acid residues, belonging to the transferrin family.^{299, 304} Lactoferrin consists of two lobes which have iron binding ability.²⁹⁹ It is in a broad spectrum of biological activities, such as antimicrobial, anti-inflammatory, anti-cancer, immunomodulatory, and enzymatic activities.^{299, 322}

1.6.3 Unique physicochemical properties and structures of bovine milk proteins

In addition to the non-toxicity and high nutritious value, dairy proteins are unique in many structural and physicochemical properties which confer them as natural nanovehicles to deliver drugs and nutrients. These attributes include binding of hydrophobic compounds, excellent self-assembly properties, superior gelation properties, as well as surface activities.

1.6.3.1 Binding hydrophobic molecules

Bovine milk proteins have gained special interest for transporting bioactive molecules due to their binding properties.^{304, 323} Mechanisms involved in the binding reactions include covalent and noncovalent interactions.³⁰³ Covalent bonds commonly lead to irreversible bindings of hydrophobic agents to free reactive nucleophilic groups such as amino ($-\text{NH}_2$) and thiol ($-\text{SH}$) groups on the surface or inside cavities of proteins.^{324, 325} Noncovalent interactions including hydrophobic interactions, van der Waals forces, and hydrogen bonds are more common compared to covalent bonds and generally result in the reversible binding reactions.³⁰⁴ Proline residues of proteins are well-known primary binding sites for various polyphenols.³²⁶ Caseins, proline-rich proteins with open and no fixed tertiary structures, highly tend to associate with phenolic compounds as well as other ligands, predominantly via hydrophobic interactions and hydrogen-bonding.^{246, 326, 327} For instance, vitamin D has been successfully nanoencapsulated into β -casein micelles possibly due to adhesion of hydrophobic vitamin D to the hydrophobic residues of caseins.³²⁸ Sodium caseinate has been reported as a carrier for ω -3 polyunsaturated fatty acids. Each caseinate molecule can bind 3 to 4 DHA molecules; the DHA-loaded casein nanoparticles showed good colloidal stability with a size of approximately

300 nm.³²⁹ Apart from caseins, β -lg also acts as a good carrier for various hydrophobic molecules.²⁹⁹ As a member of lipocalins superfamily, β -lg has three possible binding sites: a central β -barrel calyx with hydrophobic interior as the main site, the secondary site on the external surface of the β -barrel near the α -helix, and the ternary site at the dimer interface.^{299, 316, 330} As for this, numerous studies have confirmed the binding and transporting role of β -lg for many hydrophobic compounds. One β -lg molecule was found to be able to bind three folic acid molecules through hydrophobic interactions.³³¹ Besides the native β -LG, thermally modified β -LG can also be used for the development of delivery systems. β -lg binds with phenolic phytochemicals, including resveratrol and epigallocatechin gallate (EGCG), and can be strengthened after prior heat denaturation of the protein to expose more hydrophobic amino acid residues.^{332, 333} Compared with β -lg, α -la gained less research as vehicles, however, was still demonstrated to bind polyphenols such as genistein and kaempferol mainly via hydrogen bonds.³³⁴ In addition, BSA is always a natural carrier for hydrophobic vitamins and hormones.³²⁴

1.6.3.2 Self-assembly properties

Molecular self-assembly plays quite an important role in the nanotechnology. As for caseins, self-assembly is well documented as micelle formation. Structurally, casein micelles have a hydrophobic core and a hydrophilic-charged κ -CN layer, which are able to encapsulate lipophilic compounds via hydrophobic interactions.^{299, 335} β -casein micelles have been suggested to deliver curcumin, for enhanced solubility as well as bioavailability and antioxidant activity.³³⁶ The cytotoxic effects of curcumin on MCF7 cells was demonstrated to be enhanced

in the presence of β -casein micelles.³³⁷ Reconstituted casein micelles were achieved by applying high pressure homogenization followed by depressurization, and were used to encapsulate vitamin D₂ to protect against degradation under UV-light.³³⁸ β -carotene was also incorporated in reassembled casein micelles to elude the degradation induced by industrial process such as pasteurization and high hydrostatic pressure.³³⁹ Besides, self-assembly of β -lg was reported recently in the presence of lysozyme (Lyso) using electrostatic interactions between the two oppositely charged proteins.³⁴⁰ The developed β -lg/Lyso microspheres were able to efficiently entrap vitamin D₃ with an encapsulation efficiency of $90.8 \pm 4.8\%$. Additionally, novel nanotubes were formed by self-assembling of enzymatically hydrolyzed α -la.³⁴¹

1.6.3.3 Gelling properties

Gelation of food proteins enables the development of colloidal particles with the embedded bioactive cores, and is extensively studied.^{323, 342} The desired gelled structures of milk proteins can be induced by acid, enzymes, or thermal treatment.³²⁴ Acid gelation is based on the isoelectric point (pI) precipitation of caseins.³⁰⁴ Due to the lack of rigid tertiary structures, caseins are promising candidates for enzymatic cross-linking by transglutaminase (TGase), which covalently link the glutamine residues in the peptides with the lysine residue.³⁰² These heat-free gelation processes are advantageous for heat labile bioactives, such as probiotics.^{343, 344} Genipin, a naturally occurring cross-linking agent, was also reported to induce heat-free gelation of caseins.³⁴⁵

Heat-induced gelation usually results from denaturation and unfolding of proteins, via

hydrophobic interactions.³⁰⁴ In contrast to caseins, whey proteins are prone to thermal treatment and undergo partially unfold and associate with each other or lipophilic molecules through hydrophobic attractions when temperatures are above 70 °C.³⁴⁶ Hydrogels induced by heating whey protein concentrate have been used as carriers for caffeine.³¹⁸ It is noteworthy that whey proteins are exceptional due to the ability to form cold-set gelation by the addition of cations, preferably Ca^{2+} ions to the preheated whey protein solutions which is at pH far from the pI and in low ionic strength, or lowering pH to the pI.^{302, 304, 347} The cold-induced gelation network has been successfully used to encapsulate some heat sensitive microorganisms, for example yeasts, to maintain their viability and growth capability.³⁴⁸

1.6.3.4 Surface activity

Bovine milk proteins possess excellent surface activities and emulsifying properties due to their amphiphilic structures, thereby playing important roles in emulsion-based delivery systems.^{324, 349} They are able to adsorb at the oil-water interface and stabilize emulsions.³⁰⁴ The surface properties are influenced by various factors, such as the structure and flexibility of proteins, state of aggregation, as well as the ionic strength, the temperature and the pH of the environment.³⁰⁴ With a high flexibility, caseins are suitable emulsifiers and emulsion stabilizers.³⁵⁰ Sodium caseinate was found to have a better stabilization ability than whey protein in an O/W emulsion composed of palm kernel oil and sunflower oil.³⁵¹ The entrapped β -carotene in this system achieved an increased stability with higher protein concentrations. In another study, β -lg and lactoferrin stabilized emulsion based carrier system for β -carotene.³⁵²

1.6.4 Bovine milk protein-based particles as delivery systems

The unique physicochemical characteristics and special nanostructures as detailed above have facilitated the functionality of bovine milk proteins in delivery tasks. A wide variety of hydrophobic bioactives have been entrapped into milk protein-based colloidal particles, such as polyphenols, vitamins, and polyunsaturated fatty acids.^{298, 353} Spherical particles formed by hydrophobic interactions between β -carotene and casein-grafted dextran were stable against dilution, pH change, oxidation, and long-term storage.³⁰⁴ Vitamin B12 as a model bioactive has also been incorporated into the enzyme-assisted novel casein hydrogels under mild conditions and showed a prolonged release behavior.³⁵⁴ Besides, resveratrol was investigated to bind with β -lg to significantly increase the solubility, and bioavailability.³⁵⁵ Caffeine was encapsulated using a desolvation method by preheating the β -lg solution to 60°C, with an average particle size about 60 nm.³⁵⁵ It was also reported that β -lg in various conditions served as a promising matrix for the encapsulation of anthocyanins from sour cherry extract.³⁵⁶ Whey protein isolate based nanoparticles was successfully applied for encapsulation of vitamin D3, with the protection from oxygen.³⁵⁷

1.7 Hypothesis and objectives

As given above, apigenin has gained special attention due to its relatively low intrinsic toxicity, non-mutagenic property, and promising health beneficial effects, especially in the reduction of cancer incidence and attenuation of chronic inflammation. However, the poor water solubility and low bioavailability have hindered further applications of apigenin.

Therefore, the long-term goal of the current research is to develop food biopolymer-based nanodelivery systems to improve the solubility, bioavailability, and thereby enhance the therapeutic activity of apigenin.

The first objective of this dissertation is to nanoencapsulate apigenin in bovine milk proteins. The working hypothesis is that nanoencapsulation can improve the bioavailability and anti-colorectal cancer activity of apigenin both *in vitro* and *in vivo*. Three tasks were conducted to address this hypothesis.

Task 1 is to fabricate organic-solvent free and whey protein isolate (WPI)-based nanodelivery system, and characterize physicochemical properties of nanoparticles (Chapter 2).

Task 2 is to investigate the anti-colorectal cancer activities of nanodelivered apigenin against human colon cancer cells (HCT-116 and HT-29) *in vitro* (Chapter 2).

Task 3 is to evaluate the oral bioavailability and *in vivo* anti-colorectal cancer efficacy of nanodelivered apigenin in *Apc^{min+}* mouse model (Chapter 3).

The second objective is to test the hypothesis that co-encapsulating apigenin together with curcumin can enhance the synergistic anti-inflammatory effects in LPS-stimulated RAW 264.7 macrophages. The hypothesis was tested in two tasks presented in chapter 4.

Task 1 is to coencapsulate apigenin and curcumin with sodium caseinate (NaCas), followed by characterizing physicochemical properties of dispersions.

Task 2 is to investigate the potential synergistic anti-inflammatory activity of apigenin and curcumin in both simple mixtures and coencapsulated forms.

The present study may provide a better understanding of the biological fate of nanodelivered apigenin that may be used to combat colorectal carcinogenesis and macrophages-based inflammation.

References

1. Martin, S. L.; Royston, K. J.; Tollefsbol, T. O. The Role of Non-Coding RNAs and Isothiocyanates in Cancer. *Molecular Nutrition & Food Research* **2018**, 62 (18), 1700913 DOI: 10.1002/mnfr.201700913.
2. Subramanian, A. P.; John, A. A.; Vellayappan, M. V.; Balaji, A.; Jaganathan, S. K.; Mandal, M.; Supriyanto, E. Honey and its Phytochemicals: Plausible Agents in Combating Colon Cancer through its Diversified Actions. *Journal of Food Biochemistry* **2016**, 40 (4), 613--629 DOI: 10.1111/jfbc.12239.
3. Siegel, R. L.; Miller, K. D.; Jemal, A. Cancer statistics, 2019. *CA: A Cancer Journal for Clinicians* **2019**, 69 (1), 7--34 DOI: 10.3322/caac.21551.
4. Yan, X.; Qi, M.; Li, P.; Zhan, Y.; Shao, H. Apigenin in Cancer Therapy: Anti-cancer Effects and Mechanisms of Action. *Cell & bioscience* **2017**, 7 (1), 50.
5. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R. L.; Torre, L. A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* **2018**, 68 (6), 394--424 DOI: 10.3322/caac.21492.
6. Cancer Facts & Figures 2017. 2017.
7. Aran, V.; Victorino, A. P.; Thuler, L. C.; Ferreira, C. G. J. C. c. c. Colorectal cancer: epidemiology, disease mechanisms and interventions to reduce onset and mortality. **2016**, 15 (3), 195-203.
8. Siegel, R.; DeSantis, C.; Jemal, A. Colorectal cancer statistics, 2014. *CA: A Cancer Journal for Clinicians* **2014**, 64 (2), 104--117 DOI: 10.3322/caac.21220.
9. Levine, J. S.; Ahnen, D. J. Adenomatous Polyps of the Colon. *New England Journal of Medicine* **2006**, 355 (24), 2551--2557 DOI: 10.1056/NEJMcp063038.
10. Ferlay, J.; Soerjomataram, I.; Ervik, M.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D. M.; Forman, D.; Bray, F. GLOBOCAN 2012 v1. 0, cancer incidence and mortality worldwide: IARC CancerBase No. 11. *Lyon, France: International agency for research on cancer* **2013**, 2016.

11. Rajagopal, C.; Lankadasari, M. B.; Aranjani, J. M.; Harikumar, K. B. Targeting oncogenic transcription factors by polyphenols: A novel approach for cancer therapy. *Pharmacological Research* **2018**, 130, 273--291 DOI: 10.1016/j.phrs.2017.12.034.
12. Kinzler, K. W.; Vogelstein, B. Gatekeepers and caretakers. *Nature* **1997**, 386 (6627), 761-763 DOI: 10.1038/386761a0.
13. Hale, L.; Greer, P.; Trinh, C.; Gottfried, M. Treatment with oral bromelain decreases colonic inflammation in the IL-10-deficient murine model of inflammatory bowel disease. *Clinical Immunology* **2005**, 116 (2), 135--142 DOI: 10.1016/j.clim.2005.04.011.
14. Terzić, J.; Grivennikov, S.; Karin, E.; Karin, M. Inflammation and colon cancer. *Gastroenterology* **2010**, 138 (6), 2101-2114. e5.
15. Karin, M.; Greten, F. R. NF- κ B: linking inflammation and immunity to cancer development and progression. *Nature Reviews Immunology* **2005**, 5 (10), 749--759 DOI: 10.1038/nri1703.
16. Villegas, I.; Snchez-Fidalgo, S. a. Chemopreventive effect of dietary curcumin on inflammation-induced colorectal carcinogenesis in mice. *Molecular Nutrition and Food Research* **2011**, 55 (2), 259--267 DOI: 10.1002/mnfr.201000225.
17. Yang, D.; Zhang, X.; Zhang, W.; Thamaraiselvan, R. Vicenin-2 inhibits Wnt/ β -catenin signaling and induces apoptosis in HT-29 human colon cancer cell line. *Drug Design, Development and Therapy* **2018**, Volume 12, 1303--1310 DOI: 10.2147/DDDT.S149307.
18. Pandurangan, A. K. Potential Targets for Prevention of Colorectal Cancer: a Focus on PI3K/Akt/mTOR and Wnt Pathways. *Asian Pacific Journal of Cancer Prevention* **2013**, 14 (4), 2201--2205 DOI: 10.7314/APJCP.2013.14.4.2201.
19. Bourroul, G. M.; Fragoso, H. J.; Gomes, J. W. F.; Bourroul, V. S. O.; Oshima, C. T. F.; Gomes, T. S.; Saba, G. T.; Palma, R. T.; Waisberg, J. The destruction complex of beta-catenin in colorectal carcinoma and colonic adenoma. *Einstein (S ~ a o Paulo)* **2016**, 14 (2), 135--142 DOI: 10.1590/S1679-45082016AO3678.
20. Fodde, R.; Smits, R.; Clevers, H. APC, Signal transduction and genetic instability in colorectal cancer. *Nature Reviews Cancer* **2001**, 1 (1), 55--67 DOI: 10.1038/35094067.
21. Rajagopalan, H.; Nowak, M. A.; Vogelstein, B.; Lengauer, C. The significance of unstable

- chromosomes in colorectal cancer. *Nature Reviews Cancer* **2003**, 3 (9), 695--701 DOI: 10.1038/nrc1165.
22. Fearon, E. R.; Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* **1990**, 61 (5), 759--767 DOI: 10.1016/0092-8674(90)90186-I.
23. Morin, P. J. Activation of beta -Catenin-Tcf Signaling in Colon Cancer by Mutations in beta -Catenin or APC. *Science* **1997**, 275 (5307), 1787--1790 DOI: 10.1126/science.275.5307.1787.
24. Takaku, K.; Oshima, M.; Miyoshi, H.; Matsui, M.; Seldin, M. F.; Taketo, M. M. Intestinal Tumorigenesis in Compound Mutant Mice of both Dpc4(Smad4) and Apc Genes. *Cell* **1998**, 92 (5), 645--656 DOI: 10.1016/S0092-8674(00)81132-0.
25. Biswas, S.; Chytil, A.; Washington, K.; Romero-Gallo, J.; Gorska, A. E.; Wirth, P. S.; Gautam, S.; Moses, H. L.; Grady, W. M. Transforming Growth Factor β Receptor Type II Inactivation Promotes the Establishment and Progression of Colon Cancer. *Cancer Res.* **2004**, 64 (14), 4687--4692 DOI: 10.1158/0008-5472.CAN-03-3255.
26. Grady, W. M.; Carethers, J. M. Genomic and Epigenetic Instability in Colorectal Cancer Pathogenesis. *Gastroenterology* **2008**, 135 (4), 1079--1099 DOI: 10.1053/j.gastro.2008.07.076.
27. Bedeir, A.; Krasinskas, A. M. Molecular diagnostics of colorectal cancer. *Archives of pathology & laboratory medicine* **2011**, 135 (5), 578--587.
28. Subramaniam, R.; Mizoguchi, A.; Mizoguchi, E. Mechanistic roles of epithelial and immune cell signaling during the development of colitis-associated cancer. *Cancer research frontiers* **2016**, 2 (1), 1.
29. Kinzler, K. W.; Vogelstein, B. Lessons from Hereditary Colorectal Cancer. *Cell* **1996**, 87 (2), 159--170 DOI: 10.1016/S0092-8674(00)81333-1.
30. Oshima, M.; Dinchuk, J. E.; Kargman, S. L.; Oshima, H.; Hancock, B.; Kwong, E.; Trzaskos, J. M.; Evans, J. F.; Taketo, M. M. Suppression of intestinal polyposis in Apc δ 716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* **1996**, 87 (5), 803--809.
31. Gupta, R. A.; DuBois, R. N. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nature Reviews Cancer* **2001**, 1 (1), 11.
32. Koehne, C.-H.; Dubois, R. N. COX-2 inhibition and colorectal cancer. **2004**, 31, 12--21.

33. Lucas, C.; Barnich, N.; Nguyen, H. Microbiota, inflammation and colorectal cancer. *International journal of molecular sciences* **2017**, 18 (6), 1310.
34. Kraus, S.; Arber, N. Inflammation and colorectal cancer. *Current Opinion in Pharmacology* **2009**, 9 (4), 405--410 DOI: 10.1016/j.coph.2009.06.006.
35. Winawer, S. J.; Zauber, A. G.; Gerdes, H.; O'Brien, M. J.; Gottlieb, L. S.; Sternberg, S. S.; Bond, J. H.; Waye, J. D.; Schapiro, M.; Panish, J. F.; Kurtz, R. C.; Shike, M.; Ackroyd, F. W.; Stewart, E. T.; Skolnick, M.; Bishop, D. T. Risk of Colorectal Cancer in the Families of Patients with Adenomatous Polyps. *New England Journal of Medicine* **1996**, 334 (2), 82--87 DOI: 10.1056/NEJM199601113340204.
36. Jasperson, K. W.; Tuohy, T. M.; Neklason, D. W.; Burt, R. W. Hereditary and Familial Colon Cancer. *Gastroenterology* **2010**, 138 (6), 2044--2058 DOI: 10.1053/j.gastro.2010.01.054.
37. Burt, R.; Neklason, D. W. Genetic Testing for Inherited Colon Cancer. *Gastroenterology* **2005**, 128 (6), 1696--1716 DOI: 10.1053/j.gastro.2005.03.036.
38. Arnold, M.; Sierra, M. S.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global patterns and trends in colorectal cancer incidence and mortality. *Gut* **2017**, 66 (4), 683-691.
39. Johnson, I. T. Cruciferous Vegetables and Risk of Cancers of the Gastrointestinal Tract. *Molecular Nutrition and Food Research* **2018**, 62 (18), DOI: 10.1002/mnfr.201701000.
40. Center, M. M.; Jemal, A.; Ward, E. International Trends in Colorectal Cancer Incidence Rates. *Cancer Epidemiology Biomarkers & Prevention* **2009**, 18 (6), 1688--1694 DOI: 10.1158/1055-9965.EPI-09-0090.
41. Torre, L. A.; Bray, F.; Siegel, R. L.; Ferlay, J.; Lortet-Tieulent, J.; Jemal, A. Global cancer statistics, 2012. *CA: A Cancer Journal for Clinicians* **2015**, 65 (2), 87--108 DOI: 10.3322/caac.21262.
42. Doubeni, C. A.; Laiyemo, A. O.; Major, J. M.; Schootman, M.; Lian, M.; Park, Y.; Graubard, B. I.; Hollenbeck, A. R.; Sinha, R. Socioeconomic status and the risk of colorectal cancer. *Cancer* **2012**, 118 (14), 3636--3644 DOI: 10.1002/cncr.26677.
43. Pipertzis, A.; Muhlinghaus, M.; Mezger, M.; Scherf, U.; Floudas, G. Polymerized Ionic Liquids with Polythiophene Backbones: Self-Assembly, Thermal Properties, and Ion Conduction. *Macromolecules* **2018**, 51 (16), 6440--6450.

44. Marshall, J. R. Prevention of colorectal cancer: diet, chemoprevention, and lifestyle. *Gastroenterology clinics of North America* **2008**, 37 (1), 73--82.
45. Bruce, W. R.; Wolever, T. M. S.; Giacca, A. Mechanisms linking diet and colorectal cancer: the possible role of insulin resistance. *Nutrition and cancer* **2000**, 37 (1), 19--26.
46. Key, T. J.; Schatzkin, A.; Willett, W. C.; Allen, N. E.; Spencer, E. A.; Travis, R. C. Diet, nutrition and the prevention of cancer. *Public Health Nutrition* **2004**, 7 (1a), 187--200 DOI: 10.1079/PHN2003588.
47. Boffetta, P.; Hashibe, M. Alcohol and cancer. *The Lancet Oncology* **2006**, 7 (2), 149--156 DOI: 10.1016/S1470-2045(06)70577-0.
48. The health consequences of smoking-50 years of progress: a report of the Surgeon General; U. S. Department of Health and Human Services
Atlanta GA, 2014.
49. Shiels, M. S.; Katki, H. A.; Freedman, N. D.; Purdue, M. P.; Wentzensen, N.; Trabert, B.; Kitahara, C. M.; Furr, M.; Li, Y.; Kemp, T. J.; et al. Cigarette smoking and variations in systemic immune and inflammation markers. *Journal of the national cancer institute* **2014**, 106 (11), dju294.
50. Lopez, P. J. T.; Albero, J. S.; Rodriguez-Montes, J. A. Primary and Secondary Prevention of Colorectal Cancer. *Clinical Medicine Insights: Gastroenterology* **2014**, 7, CGast.S14039 DOI: 10.4137/CGast.S14039.
51. Freedman, A. N.; Slattery, M. L.; Ballard-Barbash, R.; Willis, G.; Cann, B. J.; Pee, D.; Gail, M. H.; Pfeiffer, R. M. Colorectal Cancer Risk Prediction Tool for White Men and Women Without Known Susceptibility. *Journal of Clinical Oncology* **2009**, 27 (5), 686--693 DOI: 10.1200/JCO.2008.17.4797.
52. Ma, Y.; Yang, Y.; Wang, F.; Zhang, P.; Shi, C.; Zou, Y.; Qin, H. Obesity and Risk of Colorectal Cancer: A Systematic Review of Prospective Studies. *PLoS ONE* **2013**, 8 (1), e53916 DOI: 10.1371/journal.pone.0053916.
53. Brenner, H.; Kloor, M.; Pox, C. P. Colorectal cancer. *Lancet* **2014**, 383 (9927), 1490--1502 DOI: 10.1016/S0140-6736(13)61649-9.
54. Aran, V.; Victorino, A. P.; Thuler, L. C.; Ferreira, C. G. Colorectal Cancer: Epidemiology,

Disease Mechanisms and Interventions to Reduce Onset and Mortality. *Clinical Colorectal Cancer* **2016**, 15 (3), 195--203 DOI: 10.1016/j.clcc.2016.02.008.

55. Huerta, S.; Goulet, E. J.; Livingston, E. H. Colon cancer and apoptosis. *The American Journal of Surgery* **2006**, 191 (4), 517--526 DOI: 10.1016/j.amjsurg.2005.11.009.

56. Sen, K.; Banerjee, S.; Mandal, M. Dual drug loaded liposome bearing apigenin and 5-Fluorouracil for synergistic therapeutic efficacy in colorectal cancer. *Colloids and Surfaces B: Biointerfaces* **2019**, 180 (April), 9--22 DOI: 10.1016/j.colsurfb.2019.04.035.

57. Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **2003**, 22 (47), 7265.

58. Longley, D. B.; Harkin, D. P.; Johnston, P. G. 5-fluorouracil: mechanisms of action and clinical strategies. *Nature reviews cancer* **2003**, 3 (5), 330.

59. Javle, M.; Hsueh, C.-T. Updates in Gastrointestinal Oncology – insights from the 2008 44th annual meeting of the American Society of Clinical Oncology. *Journal of Hematology & Oncology* **2009**, 2 (1), 9 DOI: 10.1186/1756-8722-2-9.

60. Hussain, S. A.; Sulaiman, A. A.; Balch, C.; Chauhan, H.; Alhadidi, Q. M.; Tiwari, A. K. Natural polyphenols in cancer chemoresistance. *Nutrition and cancer* **2016**, 68 (6), 879-891.

61. Kohne, C. H.; Lorenz, M.; Herrmann, R. Colorectal cancer liver metastasis: Local treatment for a systemic disease? *Annals of Oncology* **1998**, 9 (9), 967--971 DOI: 10.1023/A:1008463712683.

62. Kohne, C. H.; van Cutsem, E.; Wils, J.; Bokemeyer, C.; El-Serafi, M.; Lutz, M. P.; Lorenz, M.; Reichardt, P.; Rckle-Lanz, H.; Frickhofen, N.; Fuchs, R.; Mergenthaler, H. G.; Langenbuch, T.; Vanhoefer, U.; Rougier, P.; Voigtmann, R.; Mller, L.; Genicot, B.; Anak; Nordlinger, B. Phase III Study of Weekly High-Dose Infusional Fluorouracil Plus Folinic Acid With or Without Irinotecan in Patients With Metastatic Colorectal Cancer: European Organisation for Research and Treatment of Cancer Gastrointestinal Group Study 40986. *Journal of Clinical Oncology* **2005**, 23 (22), 4856--4865 DOI: 10.1200/JCO.2005.05.546.

63. Gourdier, I. a. Drug specific resistance to oxaliplatin is associated with apoptosis defect in a cellular model of colon carcinoma. *FEBS Letters* **2002**, 529 (2-3), 232--236 DOI: 10.1016/S0014-5793(02)03347-1.

64. Parasramka, M. A.; Gupta, S. V. Synergistic effect of garcinol and curcumin on antiproliferative and apoptotic activity in pancreatic cancer cells. *Journal of Oncology* **2012**, 2012, DOI: 10.1155/2012/709739.
65. Madunić, J.; Madunić, I. V.; Gajski, G.; Popić, J.; Garaj-Vrhovac, V. Apigenin: A dietary flavonoid with diverse anticancer properties. *Cancer Letters* **2018**, 413, 11-22.
66. Eng, C. Toxic effects and their management: daily clinical challenges in the treatment of colorectal cancer. *Nature Reviews Clinical Oncology* **2009**, 6 (4), 207--218 DOI: 10.1038/nrclinonc.2009.16.
67. Davatgaran-Taghipour, Y.; Masoomzadeh, S.; Farzaei, M. H.; Bahramsoltani, R.; Karimi-Soureh, Z.; Rahimi, R.; Abdollahi, M. Polyphenol nanoformulations for cancer therapy: Experimental evidence and clinical perspective. *International Journal of Nanomedicine* **2017**, 12, 2689--2702 DOI: 10.2147/IJN.S131973.
68. Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **2008**, 454 (7203), 428--435 DOI: 10.1038/nature07201.
69. Ashley, N. T.; Weil, Z. M.; Nelson, R. J. Inflammation: Mechanisms, Costs, and Natural Variation. *Annual Review of Ecology, Evolution, and Systematics* **2012**, 43 (1), 385--406 DOI: 10.1146/annurev-ecolsys-040212-092530.
70. Hougee, S.; Sanders, A.; Faber, J.; Graus, Y. M. F. a. Decreased pro-inflammatory cytokine production by LPS-stimulated PBMC upon in vitro incubation with the flavonoids apigenin, luteolin or chrysin, due to selective elimination of monocytes/macrophages. *Biochemical Pharmacology* **2005**, 69 (2), 241--248 DOI: 10.1016/j.bcp.2004.10.002.
71. Ambriz-Pérez, D. L.; Leyva-López, N.; Gutierrez-Grijalva, E. P.; Heredia, J. B. Phenolic compounds: Natural alternative in inflammation treatment. A Review. *Cogent Food & Agriculture* **2016**, 2 (1), 1131412.
72. Leyva-Lopez, N.; Gutierrez-Grijalva, E. P.; Ambriz-Perez, D. L. a. Flavonoids as cytokine modulators: A possible therapy for inflammation-related diseases. *International Journal of Molecular Sciences* **2016**, 17 (6), DOI: 10.3390/ijms17060921.
73. Landskron, G.; De la Fuente, M.; Thuwajit, P.; Thuwajit, C.; Hermoso, M. A. Chronic inflammation and cytokines in the tumor microenvironment. *Journal of immunology research*

2014, 2014.

74. Guo, W.; Kong, E. H.; Meydani, M. Dietary polyphenols, inflammation, and cancer. *Nutrition and cancer* **2009**, 61 (6), 807--810 DOI: 10.1080/01635580903285098.
75. Gabay, C. Interleukin-6 and chronic inflammation. *Arthritis Research and Therapy* **2006**, 8 (SUPPL. 2), 1--6 DOI: 10.1186/ar1917.
76. Yoon, J.-H.; Baek, S. J. Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei medical journal* **2005**, 46 (5), 585-596.
77. Norling, L. V.; Serhan, C. N. Profiling in resolving inflammatory exudates identifies novel anti-inflammatory and pro-resolving mediators and signals for termination. *Journal of Internal Medicine* **2010**, no--no DOI: 10.1111/j.1365-2796.2010.02235.x.
78. Netea, M. G.; Balkwill, F.; Chonchol, M.; Cominelli, F.; Donath, M. Y.; Giamarellos-Bourboulis, E. J.; Golenbock, D.; Gresnigt, M. S.; Heneka, M. T.; Hoffman, H. M.; Hotchkiss, R.; Joosten, L. A. B.; Kastner, D. L.; Korte, M.; Latz, E.; Libby, P.; Mandrup-Poulsen, T.; Mantovani, A.; Mills, K. H. G.; Nowak, K. L.; O'Neill, L. A.; Pickkers, P.; van der Poll, T.; Ridker, P. M.; Schalkwijk, J.; Schwartz, D. A.; Siegmund, B.; Steer, C. J.; Tilg, H.; van der Meer, J. W. M.; van de Veerdonk, F. L.; Dinarello, C. A. A guiding map for inflammation. *Nature Immunology* **2017**, 18 (8), 826--831 DOI: 10.1038/ni.3790.
79. Ma, K. C.; Schenck, E. J.; Pabon, M. A.; Choi, A. M. K. The Role of Danger Signals in the Pathogenesis and Perpetuation of Critical Illness. *American Journal of Respiratory and Critical Care Medicine* **2018**, 197 (3), 300--309 DOI: 10.1164/rccm.201612-2460PP.
80. Herwald, H.; Egesten, A. On PAMPs and DAMPs. *Journal of Innate Immunity* **2016**, 8 (5), 427--428 DOI: 10.1159/000448437.
81. Janeway, C. A.; Medzhitov, R. Innate immune recognition. *Annual Review of Immunology* **2002**, 20 (1), 197-216 DOI: 10.1146/annurev.immunol.20.083001.084359.
82. Yi, Y.-S. Caspase-11 non-canonical inflammasome: a critical sensor of intracellular lipopolysaccharide in macrophage-mediated inflammatory responses. *Immunology* **2017**, 152 (2), 207--217 DOI: 10.1111/imm.12787.
83. Delneste, Y.; Beauvillain, C.; Jeannin, P. Immunit e naturelle. *m e decine/sciences* **2007**, 23 (1), 67--74 DOI: 10.1051/medsci/200723167.

84. Kumar, K. S.; Sabu, V.; Sindhu, G.; Rauf, A. A.; Helen, A. Isolation, identification and characterization of apigenin from *Justicia gendarussa* and its anti-inflammatory activity. *International Immunopharmacology* **2018**, 59 (April), 157--167 DOI: 10.1016/j.intimp.2018.04.004.
85. Yi, Y.-S.; Son, Y.-J.; Ryou, C.; Sung, G.-H.; Kim, J.-H.; Cho, J. Y. Functional Roles of Syk in Macrophage-Mediated Inflammatory Responses. *Mediators of Inflammation* **2014**, 2014, 1--12 DOI: 10.1155/2014/270302.
86. Byeon, S. E.; Yi, Y.-S.; Oh, J.; Yoo, B. C.; Hong, S.; Cho, J. Y. The Role of Src Kinase in Macrophage-Mediated Inflammatory Responses. *Mediators of Inflammation* **2012**, 2012, 1--18 DOI: 10.1155/2012/512926.
87. Takeuchi, O.; Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* **2010**, 140 (6), 805--820 DOI: 10.1016/j.cell.2010.01.022.
88. Yi, Y. S. Regulatory roles of flavonoids on inflammasome activation during inflammatory responses. *Molecular nutrition & food research* **2018**, 62 (13), 1800147.
89. Joseph, S. V.; Edirisinghe, I.; Burton-Freeman, B. M. Fruit Polyphenols: A Review of Anti-inflammatory Effects in Humans. *Critical Reviews in Food Science and Nutrition* **2016**, 56 (3), 419--444 DOI: 10.1080/10408398.2013.767221.
90. Gupta, S. C.; Kunnumakkara, A. B.; Aggarwal, S.; Aggarwal, B. B. Inflammation, a Double-Edge Sword for Cancer and Other Age-Related Diseases. *Frontiers in immunology* **2018**, 9 (September), 2160 DOI: 10.3389/fimmu.2018.02160.
91. Zhuang, J.; Peng, W.; Li, H.; Lu, Y.; Wang, K.; Fan, F.; Li, S.; Xu, Y. Inhibitory Effects of Vinpocetine on the Progression of Atherosclerosis Are Mediated by Akt/NF- κ B Dependent Mechanisms in apoE^{-/-} Mice. *PLoS ONE* **2013**, 8 (12), e82509 DOI: 10.1371/journal.pone.0082509.
92. Tak, P. P.; Firestein, G. S. NF- κ B: a key role in inflammatory diseases. *Journal of Clinical Investigation* **2001**, 107 (1), 7--11 DOI: 10.1172/JCI11830.
93. Hoesel, B.; Schmid, J. A. The complexity of NF- κ B signaling in inflammation and cancer. *Molecular Cancer* **2013**, 12 (1), 86 DOI: 10.1186/1476-4598-12-86.
94. Ben-Neriah, Y.; Karin, M. Inflammation meets cancer, with NF- κ B as the matchmaker.

Nature Immunology **2011**, 12 (8), 715--723 DOI: 10.1038/ni.2060.

95. Disis, M. L. Immune Regulation of Cancer. *Journal of Clinical Oncology* **2010**, 28 (29), 4531--4538 DOI: 10.1200/JCO.2009.27.2146.

96. Aggarwal, B. B. Nuclear factor- κ B. *Cancer Cell* **2004**, 6 (3), 203--208 DOI: 10.1016/j.ccr.2004.09.003.

97. Puangpraphant, S.; Berhow, M. A.; Vermillion, K.; Potts, G. a. Dicafeoylquinic acids in Yerba mate (*Ilex paraguariensis* St. Hilaire) inhibit NF- κ B nucleus translocation in macrophages and induce apoptosis by activating caspases-8 and -3 in human colon cancer cells. *Molecular Nutrition and Food Research* **2011**, 55 (10), 1509--1522 DOI: 10.1002/mnfr.201100128.

98. Saw, C. L. L.; Huang, Y.; Kong, A.-N. Synergistic anti-inflammatory effects of low doses of curcumin in combination with polyunsaturated fatty acids: docosahexaenoic acid or eicosapentaenoic acid. *Biochemical pharmacology* **2010**, 79 (3), 421-430.

99. Xu, R.; Ma, S.; Lin, P.; Yu, B.; Zhou, F.; Liu, W. High strength astringent hydrogels using protein as the building block for physically cross-linked multi-network. *ACS applied materials & interfaces* **2017**, 10 (9), 7593-7601.

100. Federico, A.; Morgillo, F.; Tuccillo, C.; Ciardiello, F.; Loguercio, C. Chronic inflammation and oxidative stress in human carcinogenesis. *International Journal of Cancer* **2007**, 121 (11), 2381--2386 DOI: 10.1002/ijc.23192.

101. Wingo, P. A.; Ries, L. A. G.; Giovino, G. A.; Miller, D. S.; Rosenberg, H. M.; Shopland, D. R.; Thun, M. J.; Edwards, B. K. Annual Report to the Nation on the Status of Cancer, 1973-1996, With a Special Section on Lung Cancer and Tobacco Smoking. *JNCI Journal of the National Cancer Institute* **1999**, 91 (8), 675--690 DOI: 10.1093/jnci/91.8.675.

102. Mantovani, A. Inflammation by remote control. *Nature* **2005**, 435 (7043), 752--753 DOI: 10.1038/435752a.

103. Peter, S.; Beglinger, C. Helicobacter pylori and Gastric Cancer: The Causal Relationship. *Digestion* **2007**, 75 (1), 25--35 DOI: 10.1159/000101564.

104. Asquith, M.; Powrie, F. An innately dangerous balancing act: intestinal homeostasis, inflammation, and colitis-associated cancer: Figure 1. *The Journal of Experimental Medicine*

2010, 207 (8), 1573--1577 DOI: 10.1084/jem.20101330.

105. Ullman, T. A.; Itzkowitz, S. H. Intestinal Inflammation and Cancer. *Gastroenterology* **2011**, 140 (6), 1807--1816.e1 DOI: 10.1053/j.gastro.2011.01.057.

106. Coussens, L. M.; Werb, Z. Inflammation and cancer. *Nature* **2002**, 420 (6917), 860--867 DOI: 10.1038/nature01322.

107. Sethi, G.; Shanmugam, M. K.; Ramachandran, L.; Kumar, A. P.; Tergaonkar, V. Multifaceted link between cancer and inflammation. *Bioscience Reports* **2012**, 32 (1), 1--15 DOI: 10.1042/BSR20100136.

108. Xu, Y.; Xin, Y.; Diao, Y.; Lu, C.; Fu, J.; Luo, L.; Yin, Z. Synergistic effects of apigenin and paclitaxel on apoptosis of cancer cells. *PLoS ONE* **2011**, 6 (12), DOI: 10.1371/journal.pone.0029169.

109. Nakao, N.; Kurokawa, T.; Nonami, T.; Tumurkhuu, G.; Koide, N.; Yokochi, T. Hydrogen peroxide induces the production of tumor necrosis factor- α in RAW 264.7 macrophage cells via activation of p38 and stress-activated protein kinase. *Innate Immunity* **2008**, 14 (3), 190--196 DOI: 10.1177/1753425908093932.

110. Zhang, H.; Tsao, R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science* **2016**, 8, 33-42.

111. Singh, S.; Singh, R. P. In vitro methods of assay of antioxidants: An overview. *Food Reviews International* **2008**, 24 (4), 392--415 DOI: 10.1080/87559120802304269.

112. Hernández-Ledesma, B.; Hsieh, C.-C.; Ben, O. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochemical and biophysical research communications* **2009**, 390 (3), 803-808.

113. Roy, M. K.; Koide, M.; Rao, T. P.; Okubo, T.; Ogasawara, Y.; Juneja, L. R. ORAC and DPPH assay comparison to assess antioxidant capacity of tea infusions: Relationship between total polyphenol and individual catechin content. *International Journal of Food Sciences and Nutrition* **2010**, 61 (2), 109--124 DOI: 10.3109/09637480903292601.

114. Singh, R. P.; Sharad, S.; Kapur, S. Free radicals and oxidative stress in neurodegenerative diseases: relevance of dietary antioxidants. *J Indian Acad Clin Med* **2004**, 5 (3), 218--225.

115. Khan, N.; Afaq, F.; Mukhtar, H. Cancer Chemoprevention Through Dietary Antioxidants:

Progress and Promise. *Antioxidants & Redox Signaling* **2008**, 10 (3), 475--510 DOI: 10.1089/ars.2007.1740.

116. Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences* **1993**, 90 (17), 7915--7922 DOI: 10.1073/pnas.90.17.7915.

117. Guo, S.; Qiu, P.; Xu, G.; Wu, X.; Dong, P.; Yang, G.; Zheng, J.; McClements, D. J.; Xiao, H. Synergistic anti-inflammatory effects of nobiletin and sulforaphane in lipopolysaccharide-stimulated RAW 264.7 cells. *Journal of agricultural and food chemistry* **2012**, 60 (9), 2157--2164 DOI: 10.1021/jf300129t.

118. Kundu, J. K.; Surh, Y. J. Inflammation: Gearing the journey to cancer. *Mutation Research - Reviews in Mutation Research* **2008**, 659 (1-2), 15--30 DOI: 10.1016/j.mrrev.2008.03.002.

119. Santangelo, C.; Var. Polyphenols, intracellular signalling and inflammation. *Annali dell'Istituto Superiore di Sanita* **2007**, 43 (4), 394--405.

120. Sade-Feldman, M.; Kanterman, J.; Ish-Shalom, E.; Elnekave, M.; Horwitz, E.; Baniyash, M. Tumor necrosis factor- α blocks differentiation and enhances suppressive activity of immature myeloid cells during chronic inflammation. *Immunity* **2013**, 38 (3), 541-554.

121. Feghali, C. A.; Wright, T. M. Cytokines in acute and chronic inflammation. *Frontiers in bioscience : a journal and virtual library* **1997**, 2 (February 1997), DOI: 10.2741/a171.

122. Oz, H. S. Chronic inflammatory diseases and green tea polyphenols. *Nutrients* **2017**, 9 (6), 1--14 DOI: 10.3390/nu9060561.

123. Popa, C.; Netea, M. G. a. The role of TNF- α in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. *Journal of lipid research* **2007**, 48 (4), 751-762.

124. Balkwill, F.; Mantovani, A. Inflammation and cancer: back to Virchow? *The Lancet* **2001**, 357 (9255), 539--545 DOI: 10.1016/S0140-6736(00)04046-0.

125. Burke, F.; Relf, M.; Negus, R.; Balkwill, F. A Cytokine Profile of Normal and Malignant Ovary. *Cytokine* **1996**, 8 (7), 578--585 DOI: 10.1006/cyto.1996.0077.

126. Naylor, M. S.; Stamp, G. W.; Foulkes, W. D.; Eccles, D.; Balkwill, F. R. Tumor necrosis factor and its receptors in human ovarian cancer. Potential role in disease progression.

Journal of Clinical Investigation **1993**, 91 (5), 2194--2206 DOI: 10.1172/JCI116446.

127. Montesano, R. Tumour necrosis factor confers an invasive, transformed phenotype on mammary epithelial cells. *Journal of Cell Science* **2005**, 118 (15), 3487--3500 DOI: 10.1242/jcs.02467.

128. Sade-Feldman, M.; Kanterman, J.; Ish-Shalom, E.; Elnekave, M.; Horwitz, E.; Baniyash, M. Tumor Necrosis Factor- α Blocks Differentiation and Enhances Suppressive Activity of Immature Myeloid Cells during Chronic Inflammation. *Immunity* **2013**, 38 (3), 541--554 DOI: 10.1016/j.immuni.2013.02.007.

129. Zamarron, B. F.; Chen, W. Dual Roles of Immune Cells and Their Factors in Cancer Development and Progression. *International Journal of Biological Sciences* **2011**, 7 (5), 651--658 DOI: 10.7150/ijbs.7.651.

130. Yao, X.; Huang, J.; Zhong, H.; Shen, N.; Faggioni, R.; Fung, M.; Yao, Y. Targeting interleukin-6 in inflammatory autoimmune diseases and cancers. *Pharmacology & Therapeutics* **2014**, 141 (2), 125--139 DOI: 10.1016/j.pharmthera.2013.09.004.

131. Vasilopoulos, Y.; Sourli, F.; Zafiriou, E.; Klimi, E.; Ioannou, M.; Mamuris, Z.; Simos, G.; Koukoulis, G.; Roussaki-Schulze, A. High serum levels of HIF-1 α in psoriatic patients correlate with an over-expression of IL-6. *Cytokine* **2013**, 62 (1), 38--39 DOI: 10.1016/j.cyto.2013.02.029.

132. Scheller, J.; Ohnesorge, N.; Rose-John, S. Interleukin-6 Trans-Signalling in Chronic Inflammation and Cancer. *Scandinavian Journal of Immunology* **2006**, 63 (5), 321--329 DOI: 10.1111/j.1365-3083.2006.01750.x.

133. Zhen, L.; Fan, D.; Yi, X.; Cao, X.; Chen, D.; Wang, L. Curcumin inhibits oral squamous cell carcinoma proliferation and invasion via EGFR signaling pathways. *International journal of clinical and experimental pathology* **2014**, 7 (10), 6438.

134. Stow, J. L. a. Cytokine secretion in macrophages and other cells: Pathways and mediators. *Immunobiology* **2009**, 214 (7), 601--612 DOI: 10.1016/j.imbio.2008.11.005.

135. Grisham, M. B.; Jourdain, D.; Wink, D. A. I. Physiological chemistry of nitric oxide and its metabolites: implications in inflammation. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **1999**, 276 (2), G315--G321 DOI:

10.1152/ajpgi.1999.276.2.G315.

136. Ben, P.; Liu, J.; Lu, C.; Xu, Y.; Xin, Y.; Fu, J.; Huang, H.; Zhang, Z.; Gao, Y.; Luo, L.; Yin, Z. Curcumin promotes degradation of inducible nitric oxide synthase and suppresses its enzyme activity in RAW 264.7 cells. *International Immunopharmacology* **2011**, 11 (2), 179--186 DOI: 10.1016/j.intimp.2010.11.013.

137. Jaiswal, M.; LaRusso, N. F.; Burgart, L. J.; Gores, G. J. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. *Cancer Res.* **2000**, 60 (1), 184--190.

138. Schetter, A. J.; Heegaard, N. H. H.; Harris, C. C. Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* **2009**, 31 (1), 37--49.

139. Villegas, I.; Sanchez-Fidalgo, S. a. New mechanisms and therapeutic potential of curcumin for colorectal cancer. *Molecular Nutrition and Food Research* **2008**, 52 (9), 1040--1061 DOI: 10.1002/mnfr.200700280.

140. Li, Q.; Verma, I. M. NF- κ B regulation in the immune system. *Nature Reviews Immunology* **2002**, 2 (10), 725--734 DOI: 10.1038/nri910.

141. Jaiswal, M.; LaRusso, N. F.; Gores, G. J. Nitric oxide in gastrointestinal epithelial cell carcinogenesis: linking inflammation to oncogenesis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2001**, 281 (3), G626--G634 DOI: 10.1152/ajpgi.2001.281.3.G626.

142. Chen, T.; Rose, M. E.; Hwang, H.; Nines, R. G.; Stoner, G. D. Black raspberries inhibit N-nitrosomethylbenzylamine (NMBA)-induced angiogenesis in rat esophagus parallel to the suppression of COX-2 and iNOS. *Carcinogenesis* **2006**, 27 (11), 2301--2307 DOI: 10.1093/carcin/bgl109.

143. Wang, D.; DuBois, R. N. Eicosanoids and cancer. *Nature Reviews Cancer* **2010**, 10 (3), 181--193 DOI: 10.1038/nrc2809.

144. Liu, X.-H.; Kirschenbaum, A.; Yao, S.; Stearns, M. E.; Holland, J. F.; Claffey, K.; Levine, A. C. Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human

- prostate cancer cell line. *Clinical & experimental metastasis* **1999**, 17 (8), 687--694.
145. Sheng, H.; Shao, J.; Morrow, J. D.; Beauchamp, R. D.; DuBois, R. N. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res.* **1998**, 58 (2), 362--366.
 146. Tsujii, M.; Kawano, S.; DuBois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proceedings of the National Academy of Sciences* **1997**, 94 (7), 3336--3340.
 147. Morceau, F.; Chateauvieux, S.; Gaigneaux, A.; Dicato, M.; Diederich, M. Long and Short Non-Coding RNAs as Regulators of Hematopoietic Differentiation. *International Journal of Molecular Sciences* **2013**, 14 (7), 14744--14770 DOI: 10.3390/ijms140714744.
 148. Pahwa, R.; Jialal, I., Chronic inflammation. In *StatPearls [Internet]*, StatPearls Publishing: 2018.
 149. Amin, A. R.; Vyas, P.; Attur, M.; Leszczynska-Piziak, J.; Patel, I. R.; Weissmann, G.; Abramson, S. B. The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. *Proceedings of the National Academy of Sciences* **1995**, 92 (17), 7926--7930 DOI: 10.1073/pnas.92.17.7926.
 150. Zhang, H.; Sun, X.-F. Overexpression of cyclooxygenase-2 correlates with advanced stages of colorectal cancer. *The American Journal of Gastroenterology* **2002**, 97 (4), 1037--1041 DOI: 10.1111/j.1572-0241.2002.05625.x.
 151. Hawkey, C. J.; Langman, M. J. S. Non-steroidal anti-inflammatory drugs: overall risks and management. Complementary roles for COX-2 inhibitors and proton pump inhibitors. *Gut* **2003**, 52 (4), 600--608 DOI: 10.1136/gut.52.4.600.
 152. Juni, P.; Nartey, L.; Reichenbach, S.; Sterchi, R.; Dieppe, P. A.; Egger, M. Risk of cardiovascular events and rofecoxib: cumulative meta-analysis. *The Lancet* **2004**, 364 (9450), 2021--2029 DOI: 10.1016/S0140-6736(04)17514-4.
 153. Rasouli, H.; Farzaei, M. H.; Khodarahmi, R. Polyphenols and their benefits: A review. *International Journal of Food Properties* **2017**, 20 (sup2), 1700-1741.
 154. Lambert, J. D.; Hong, J.; Yang, G.-y.; Liao, J.; Yang, C. S. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. *The American journal of clinical*

nutrition **2005**, 81 (1), 284S-291S.

155. Banerjee, K.; Banerjee, S.; Das, S.; Mandal, M. Probing the potential of apigenin liposomes in enhancing bacterial membrane perturbation and integrity loss. *Journal of colloid and interface science* **2015**, 453, 48-59.

156. Murphy, N.; Achaintre, D.; Zamora-Ros, R.; Jenab, M.; Boutron-Ruault, M. C.; Carbonnel, F.; Savoye, I.; Kaaks, R.; Khn, T.; Boeing, H.; Aleksandrova, K.; Tjnneland, A.; Kyr. A prospective evaluation of plasma polyphenol levels and colon cancer risk. *International Journal of Cancer* **2018**, 143 (7), 1620--1631 DOI: 10.1002/ijc.31563.

157. Abbas, M.; Saeed, F.; Anjum, F. M.; Afzaal, M.; Tufail, T.; Bashir, M. S.; Ishtiaq, A.; Hussain, S.; Suleria, H. A. R. Natural polyphenols: An overview. *International Journal of Food Properties* **2017**, 20 (8), 1689--1699 DOI: 10.1080/10942912.2016.1220393.

158. Zamora-Ros, R.; Touillaud, M.; Rothwell, J. A.; Romieu, I.; Scalbert, A. Measuring exposure to the polyphenol metabolome in observational epidemiologic studies: current tools and applications and their limits. *The American Journal of Clinical Nutrition* **2014**, 100 (1), 11--26 DOI: 10.3945/ajcn.113.077743.

159. Manach, C.; Scalbert, A.; Morand, C.; Rmsy, C.; Jimnez, L. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* **2004**, 79 (5), 727--747 DOI: 10.1093/ajcn/79.5.727.

160. Chang, H.; Lei, L.; Zhou, Y.; Ye, F.; Zhao, G. Dietary flavonoids and the risk of colorectal cancer: An updated meta-analysis of epidemiological studies. *Nutrients* **2018**, 10 (7), DOI: 10.3390/nu10070950.

161. Ramos, S. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Molecular nutrition & food research* **2008**, 52 (5), 507--526.

162. Nijveldt, R. J. a. Flavonoids: a review of probable mechanisms of action and potential applications. *The American journal of clinical nutrition* **2001**, 74 (4), 418--425.

163. Raffa, D.; Maggio, B.; Raimondi, M. V.; Plescia, F.; Daidone, G. Recent discoveries of anticancer flavonoids. *European Journal of Medicinal Chemistry* **2017**, 142, 213--228 DOI: 10.1016/j.ejmech.2017.07.034.

164. González, R.; Ballester, I.; López-Posadas, R.; Suárez, M.; Zarzuelo, A.; Martinez-

Augustin, O.; Medina, F. S. D. Effects of flavonoids and other polyphenols on inflammation. *Critical reviews in food science and nutrition* **2011**, 51 (4), 331-362.

165. Barnes, S. Soy isoflavones-phytoestrogens and what else? *The Journal of nutrition* **2004**, 134 (5), 1225S----1228S.

166. Charepalli, V. R. Polyphenol-rich Foods as Inhibitors of Colon Cancer Stem Cells. **2017**.

167. Janakiram, N. B.; Mohammed, A.; Madka, V.; Kumar, G.; Rao, C. V. Prevention and treatment of cancers by immune modulating nutrients. *Molecular nutrition \& food research* **2016**, 60 (6), 1275--1294 DOI: 10.1002/mnfr.201500884.

168. Pan, M. H.; Lai, C. S.; Wu, J. C.; Ho, C. T. Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds. *Molecular nutrition & food research* **2011**, 55 (1), 32-45.

169. Kumar, G.; Tajpara, P.; Maru, G. Dietary turmeric post-treatment decreases DMBA-induced hamster buccal pouch tumor growth by altering cell proliferation and apoptosis-related markers. *Journal of Environmental Pathology, Toxicology and Oncology* **2012**, 31 (4).

170. Watson, R. R.; Preedy, V. R., Bioactive foods and extracts: Cancer treatment and prevention. CRC Press: 2010.

171. Churchill, M.; Chadburn, A.; Bilinski, R. T.; Bertagnolli, M. M. Inhibition of intestinal tumors by curcumin is associated with changes in the intestinal immune cell profile. *Journal of Surgical Research* **2000**, 89 (2), 169--175.

172. Li, Y.; Zhang, T. Targeting cancer stem cells by curcumin and clinical applications. *Cancer letters* **2014**, 346 (2), 197-205.

173. Bhattacharyya, S.; Hossain, D. M. S.; Mohanty, S.; Sen, G. S.; Chattopadhyay, S.; Banerjee, S.; Chakraborty, J.; Das, K.; Sarkar, D.; Das, T.; et al. Curcumin reverses T cell-mediated adaptive immune dysfunctions in tumor-bearing hosts. *Cellular \& molecular immunology* **2010**, 7 (4), 306.

174. Hope, C.; Planutis, K.; Planutiene, M.; Moyer, M. P.; Johal, K. S.; Woo, J.; Santoso, C.; Hanson, J. A.; Holcombe, R. F. Low concentrations of resveratrol inhibit Wnt signal throughput in colon - derived cells: Implications for colon cancer prevention. *Molecular*

nutrition & food research **2008**, 52 (S1), S52-S61.

175. Engelbrecht, A. M.; Mattheyse, M.; Ellis, B.; Loos, B.; Thomas, M.; Smith, R.; Peters, S.; Smith, C.; Myburgh, K. Proanthocyanidin from grape seeds inactivates the PI3-kinase/PKB pathway and induces apoptosis in a colon cancer cell line. *Cancer Letters* **2007**, 258 (1), 144--153 DOI: 10.1016/j.canlet.2007.08.020.

176. Tang, F. Y.; Shih, C. J.; Cheng, L. H.; Ho, H. J.; Chen, H. J. Lycopene inhibits growth of human colon cancer cells via suppression of the Akt signaling pathway. *Molecular nutrition & food research* **2008**, 52 (6), 646-654.

177. Hollman, P. C. H. Unravelling of the health effects of polyphenols is a complex puzzle complicated by metabolism. *Archives of Biochemistry and Biophysics* **2014**, 559, 100--105 DOI: 10.1016/j.abb.2014.04.013.

178. Scalbert, A.; Johnson, I. T.; Saltmarsh, M. Polyphenols: antioxidants and beyond. *The American Journal of Clinical Nutrition* **2005**, 81 (1), 215S--217S DOI: 10.1093/ajcn/81.1.215S.

179. Kehler, J. P. Free radicals in biology: sources, reactivities, and roles in the etiology of human diseases. *Natural antioxidants in human health and disease* **1994**, 25--62.

180. Zhu, F.; Du, B.; Xu, B. Anti-inflammatory effects of phytochemicals from fruits, vegetables, and food legumes: A review. *Critical reviews in food science and nutrition* **2018**, 58 (8), 1260-1270.

181. Guo, W.; Kong, E.; Meydani, M. Dietary polyphenols, inflammation, and cancer. *Nutrition and cancer* **2009**, 61 (6), 807-810.

182. Gerhäuser, C.; Klimo, K.; Heiss, E.; Neumann, I.; Gamal-Eldeen, A.; Knauff, J.; Liu, G.-Y.; Sitthimonchai, S.; Frank, N. Mechanism-based in vitro screening of potential cancer chemopreventive agents. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **2003**, 523, 163-172.

183. Wheeler, D. S.; Catravas, J. D.; Odoms, K.; Denenberg, A.; Malhotra, V.; Wong, H. R. Epigallocatechin-3-gallate, a green tea-derived polyphenol, inhibits IL-1 β -dependent proinflammatory signal transduction in cultured respiratory epithelial cells. *The Journal of nutrition* **2004**, 134 (5), 1039-1044.

184. Chen, Y.-C.; Shen, S.-C.; Lee, W.-R.; Hou, W.-C.; Yang, L.-L.; Lee, T. J. F. Inhibition

of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. *Journal of Cellular Biochemistry* **2001**, 82 (4), 537--548 DOI: 10.1002/jcb.1184.

185. Zhang, L.; Zhu, W.; Yang, C.; Guo, H.; Yu, A.; Ji, J.; Gao, Y.; Sun, M.; Zhai, G. A novel folate-modified self-microemulsifying drug delivery system of curcumin for colon targeting. *International journal of nanomedicine* **2012**, 7, 151.

186. Wang, X.; Jiang, Y.; Wang, Y.-W.; Huang, M.-T.; Ho, C.-T.; Huang, Q. Enhancing anti-inflammation activity of curcumin through O/W nanoemulsions. *Food chemistry* **2008**, 108 (2), 419--424 DOI: 10.1016/j.foodchem.2007.10.086.

187. Luca, S. V.; Macovei, I.; Bujor, A.; Miron, A.; Skalicka-Woniak, K.; Aprotosoaie, A. C.; Trifan, A. Bioactivity of dietary polyphenols: The role of metabolites. *Critical Reviews in Food Science and Nutrition* **2019**, 0 (0), 1--34 DOI: 10.1080/10408398.2018.1546669.

188. Joye, I. J.; Davidov-Pardo, G.; McClements, D. J. Nanotechnology for increased micronutrient bioavailability. *Trends in Food Science and Technology* **2014**, 40 (2), 168--182 DOI: 10.1016/j.tifs.2014.08.006.

189. Wang, B.; Wang, J.; Zhao, X. H. In vitro Activities of the Four Structurally Similar Flavonols Weakened by the Prior Thermal and Oxidative Treatments to a Human Colorectal Cancer Line. *Journal of Food Biochemistry* **2017**, 41 (2), 1--12 DOI: 10.1111/jfbc.12310.

190. Boon, C. S.; McClements, D. J.; Weiss, J.; Decker, E. A. Factors Influencing the Chemical Stability of Carotenoids in Foods. *Critical Reviews in Food Science and Nutrition* **2010**, 50 (6), 515--532 DOI: 10.1080/10408390802565889.

191. Wang, Y.-J.; Pan, M.-H.; Cheng, A.-L.; Lin, L.-I.; Ho, Y.-S.; Hsieh, C.-Y.; Lin, J.-K. Stability of curcumin in buffer solutions and characterization of its degradation products. *Journal of pharmaceutical and biomedical analysis* **1997**, 15 (12), 1867--1876.

192. Tonnesen, H. H.; Karlsen, J.; van Henegouwen, G. B. Studies on curcumin and curcuminoids VIII. Photochemical stability of curcumin. *Zeitschrift f \ u r Lebensmittel-Untersuchung und Forschung* **1986**, 183 (2), 116--122.

193. Pathak, K.; Raghuvanshi, S. Oral Bioavailability: Issues and Solutions via

Nanoformulations. *Clinical Pharmacokinetics* **2015**, 54 (4), 325--357 DOI: 10.1007/s40262-015-0242-x.

194. Niedzwiecki, A.; Roomi, M. W.; Kalinovsky, T.; Rath, M. Anticancer efficacy of polyphenols and their combinations. *Nutrients* **2016**, 8 (9), DOI: 10.3390/nu8090552.

195. Davidov-Pardo, G.; McClements, D. J. Nutraceutical delivery systems: resveratrol encapsulation in grape seed oil nanoemulsions formed by spontaneous emulsification. *Food chemistry* **2015**, 167, 205--212.

196. McClements, D. J.; Xiao, H. Excipient foods: designing food matrices that improve the oral bioavailability of pharmaceuticals and nutraceuticals. *Food & Function* **2014**, 5 (7), 1320--1333.

197. Núñez - Sánchez, M. A.; González - Sarrías, A.; Romo - Vaquero, M.; García - Villalba, R.; Selma, M. V.; Tomás - Barberán, F. A.; García - Conesa, M. T.; Espín, J. C. Dietary phenolics against colorectal cancer—From promising preclinical results to poor translation into clinical trials: Pitfalls and future needs. *Molecular nutrition & food research* **2015**, 59 (7), 1274-1291.

198. Kim, H. P.; Mani, I.; Iversen, L.; Ziboh, V. A. Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **1998**, 58 (1), 17--24 DOI: 10.1016/S0952-3278(98)90125-9.

199. Gupta, S.; Afaq, F.; Mukhtar, H. Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. *Oncogene* **2002**, 21 (23), 3727.

200. Zheng, P.-W.; Chiang, L.-C.; Lin, C.-C. Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. *Life sciences* **2005**, 76 (12), 1367--1379.

201. Vargo, M. A.; Voss, O. H.; Poustka, F.; Cardounel, A. J.; Grotewold, E.; Doseff, A. I. Apigenin-induced-apoptosis is mediated by the activation of PKC δ and caspases in leukemia cells. *Biochemical pharmacology* **2006**, 72 (6), 681-692.

202. Khan, T. H.; Sultana, S. Apigenin induces apoptosis in Hep G2 cells: possible role of TNF- α and IFN- γ . *Toxicology* **2006**, 217 (2-3), 206--212.

203. Brusselmans, K.; Vrolix, R.; Verhoeven, G.; Swinnen, J. V. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. *Journal of Biological Chemistry* **2005**, 280 (7), 5636--5645.
204. Lee, Y.; Sung, B.; Kang, Y. J.; Kim, D. H.; Jang, J.-Y.; Hwang, S. Y.; Kim, M.; Lim, H. S.; Yoon, J.-H.; Chung, H. Y. Apigenin-induced apoptosis is enhanced by inhibition of autophagy formation in HCT116 human colon cancer cells. *International journal of oncology* **2014**, 44 (5), 1599-1606.
205. Kuo, M.-L.; Lee, K.-C.; Lin, J.-K. Genotoxicities of nitropyrenes and their modulation by apigenin, tannic acid, ellagic acid and indole-3-carbinol in the Salmonella and CHO systems. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1992**, 270 (2), 87-95.
206. Van Dross, R. T.; Hong, X.; Pelling, J. C. Inhibition of TPA - induced cyclooxygenase - 2 (COX - 2) expression by apigenin through downregulation of Akt signal transduction in human keratinocytes. *Molecular Carcinogenesis: Published in cooperation with the University of Texas MD Anderson Cancer Center* **2005**, 44 (2), 83-91.
207. Xu, M.; Wang, S.; Song, Y.; Yao, J.; Huang, K.; Zhu, X. Apigenin suppresses colorectal cancer cell proliferation, migration and invasion via inhibition of the Wnt/ β -catenin signaling pathway. *Oncology letters* **2016**, 11 (5), 3075-3080.
208. Dai, J.; Van Wie, P. G.; Fai, L. Y.; Kim, D.; Wang, L.; Poyil, P.; Luo, J.; Zhang, Z. Downregulation of NEDD9 by apigenin suppresses migration, invasion, and metastasis of colorectal cancer cells. *Toxicology and applied pharmacology* **2016**, 311, 106-112.
209. Wang, W.; Heideman, L.; Chung, C. S.; Pelling, J. C.; Koehler, K. J.; Birt, D. F. Cell - cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Molecular Carcinogenesis: Published in cooperation with the University of Texas MD Anderson Cancer Center* **2000**, 28 (2), 102--110.
210. Thompson, L. U.; Boucher, B. A.; Liu, Z.; Cotterchio, M.; Thompson, L. U.; Boucher, B. A.; Liu, Z.; Cotterchio, M.; Thompson, L. U.; Boucher, B. A.; Liu, Z.; Cotterchio, M.; Kreiger, N. Phytoestrogen Content of Foods Consumed in Canada , Including Isoflavones , Lignans , and Coumestan Phytoestrogen Content of Foods Consumed in Canada , Including

Isoflavones , Lignans , and Coumestan. **2009**, 5581, DOI: 10.1207/s15327914nc5402.

211. Kowalski, J.; Samojedny, A.; Paul, M.; Pietsz, G.; Wilczok, T. Effect of apigenin, kaempferol and resveratrol on the expression of interleukin-1beta and tumor necrosis factor-alpha genes in J774.2 macrophages. *Pharmacological reports : PR* **57** (3), 390-4 DOI: 15985724.

212. Lee, J.-H.; Zhou, H. Y.; Cho, S. Y.; Kim, Y. S.; Lee, Y. S.; Jeong, C. S. Anti-inflammatory mechanisms of apigenin: inhibition of cyclooxygenase-2 expression, adhesion of monocytes to human umbilical vein endothelial cells, and expression of cellular adhesion molecules. *Archives of Pharmacal Research* **2007**, 30 (10), 1318--1327 DOI: 10.1007/BF02980273.

213. Patil, R. H.; Babu, R. L. a. Apigenin inhibits PMA-induced expression of pro-inflammatory cytokines and AP-1 factors in A549 cells. *Molecular and Cellular Biochemistry* **2015**, 403 (1-2), 95--106 DOI: 10.1007/s11010-015-2340-3.

214. Ai, X.-Y.; Qin, Y.; Liu, H.-J.; Cui, Z.-H.; Li, M.; Yang, J.-H.; Zhong, W.-L.; Liu, Y.-R.; Chen, S.; Sun, T. Apigenin inhibits colonic inflammation and tumorigenesis by suppressing STAT3-NF- κ B signaling. *Oncotarget* **2017**, 8 (59), 100216.

215. Márquez-Flores, Y. K.; Villegas, I.; Cárdeno, A.; Rosillo, M. Á.; Alarcón-de-la-Lastra, C. Apigenin supplementation protects the development of dextran sulfate sodium-induced murine experimental colitis by inhibiting canonical and non-canonical inflammasome signaling pathways. *The Journal of nutritional biochemistry* **2016**, 30, 143-152.

216. Li, B.; Robinson, D. H.; Birt, D. F. Evaluation of properties of apigenin and G-3H apigenin and analytic method development. *Journal of pharmaceutical sciences* **1997**, 86 (6), 721--725.

217. Zhang, J.; Liu, D.; Huang, Y.; Gao, Y.; Qian, S. Biopharmaceutics classification and intestinal absorption study of apigenin. *International journal of pharmaceutics* **2012**, 436 (1), 311-317.

218. Havsteen, B. H. The biochemistry and medical significance of the flavonoids. *Pharmacology & therapeutics* **2002**, 96 (2-3), 67--202.

219. Hu, M. Commentary: Bioavailability of Flavonoids and Polyphenols: Call to Arms.

Molecular Pharmaceutics **2007**, 4 (6), 803--806 DOI: 10.1021/mp7001363.

220. Liu, R. H. Dietary bioactive compounds and their health implications. *Journal of Food Science* **2013**, 78 (SUPPL.1), DOI: 10.1111/1750-3841.12101.

221. Phan, M. A. T.; Paterson, J.; Bucknall, M.; Arcot, J. Interactions between phytochemicals from fruits and vegetables: Effects on bioactivities and bioavailability. *Critical Reviews in Food Science and Nutrition* **2018**, 58 (8), 1310--1329 DOI: 10.1080/10408398.2016.1254595.

222. Chou, T.-C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacological reviews* **2006**, 58 (3), 621--681.

223. Wagner, H. Synergy research: Approaching a new generation of phytopharmaceuticals. *Fitoterapia* **2011**, 82 (1), 34--37 DOI: 10.1016/j.fitote.2010.11.016.

224. Saldanha, S. N.; Tollefsbol, T. O. The Role of Nutraceuticals in Chemoprevention and Chemotherapy and Their Clinical Outcomes. *Journal of Oncology* **2012**, 2012, 1--23 DOI: 10.1155/2012/192464.

225. Wang, P.; Heber, D.; Henning, S. M. Quercetin Increased the Antiproliferative Activity of Green Tea Polyphenol (-)-Epigallocatechin Gallate in Prostate Cancer Cells. *Nutrition and Cancer* **2012**, 64 (4), 580--587 DOI: 10.1080/01635581.2012.661514.

226. Wang, S.; Chen, R.; Zhong, Z.; Shi, Z.; Chen, M.; Wang, Y. Epigallocatechin-3-Gallate Potentiates the Effect of Curcumin in Inducing Growth Inhibition and Apoptosis of Resistant Breast Cancer Cells. *The American Journal of Chinese Medicine* **2014**, 42 (05), 1279--1300 DOI: 10.1142/S0192415X14500803.

227. Wei, D. Z.; Yang, J. Y.; Liu, J. W.; Tong, W. Y. Inhibition of Liver Cancer Cell Proliferation and Migration by a Combination of (-)-Epigallocatechin-3- Gallate and Ascorbic Acid. *Journal of Chemotherapy* **2003**, 15 (6), 591--595 DOI: 10.1179/joc.2003.15.6.591.

228. Cruz-Correa, M.; Shoskes, D. A.; Sanchez, P.; Zhao, R.; Hyland, L. M.; Wexner, S. D.; Giardiello, F. M. Combination Treatment With Curcumin and Quercetin of Adenomas in Familial Adenomatous Polyposis. *Clinical Gastroenterology and Hepatology* **2006**, 4 (8), 1035--1038 DOI: 10.1016/j.cgh.2006.03.020.

229. Iwuchukwu, O. F.; Tallarida, R. J.; Nagar, S. Resveratrol in combination with other dietary polyphenols concomitantly enhances antiproliferation and UGT1A1 induction in Caco-2 cells. *Life Sciences* **2011**, 88 (23-24), 1047--1054 DOI: 10.1016/j.lfs.2011.03.016.
230. Radhakrishnan, S.; Reddivari, L.; Sclafani, R.; Das, U. N.; Vanamala, J. Resveratrol potentiates grape seed extract induced human colon cancer cell apoptosis. *Frontiers in Bioscience* **2011**, E3 (4), 352 DOI: 10.2741/e352.
231. Funaro, A.; Wu, X.; Song, M.; Zheng, J.; Guo, S.; Rakariyatham, K.; Rodriguez-Estrada, M. T.; Xiao, H. Enhanced Anti-Inflammatory Activities by the Combination of Luteolin and Tangeretin. *Journal of Food Science* **2016**, 81 (5), H1320--H1327 DOI: 10.1111/1750-3841.13300.
232. Hadad, N.; Levy, R. The synergistic anti-inflammatory effects of lycopene, lutein, β -carotene, and carnosic acid combinations via redox-based inhibition of NF- κ B signaling. *Free Radical Biology and Medicine* **2012**, 53 (7), 1381-1391.
233. Liu, Z.; Luo, Z.; Jia, C.; Wang, D.; Li, D. Synergistic Effects of *Potentilla fruticosa* L. Leaves Combined with Green Tea Polyphenols in a Variety of Oxidation Systems. *Journal of Food Science* **2016**, 81 (5), C1091--C1101 DOI: 10.1111/1750-3841.13292.
234. Bohm, V.; Bitsch, R. Intestinal absorption of lycopene from different matrices and interactions to other carotenoids, the lipid status, and the antioxidant capacity of human plasma. *European Journal of Nutrition* **1999**, 38 (3), 118--125 DOI: 10.1007/s003940050052.
235. Kale, A.; Gawande, S.; Kotwal, S.; Netke, S.; Roomi, W.; Ivanov, V.; Niedzwiecki, A.; Rath, M. Studies on the effects of oral administration of nutrient mixture, quercetin and red onions on the bioavailability of epigallocatechin gallate from green tea extract. *Phytotherapy research* **2010**, 24 (S1), S48----S55.
236. Nakagawa, K.; Miyazawa, T. Chemiluminescence--high-performance liquid chromatographic determination of tea catechin,(-)-epigallocatechin 3-gallate, at picomole levels in rat and human plasma. *Analytical biochemistry* **1997**, 248 (1), 41--49.
237. Lambert, J. D.; Kwon, S.-J.; Ju, J.; Bose, M.; Lee, M.-J.; Hong, J.; Hao, X.; Yang, C. S. Effect of genistein on the bioavailability and intestinal cancer chemopreventive activity of (-)-epigallocatechin-3-gallate. *Carcinogenesis* **2008**, 29 (10), 2019--2024.

238. Lambert, J. D.; Hong, J.; Kim, D. H.; Mishin, V. M.; Yang, C. S. Piperine enhances the bioavailability of the tea polyphenol (-)-epigallocatechin-3-gallate in mice. *The Journal of nutrition* **2004**, 134 (8), 1948--1952.
239. Wang, S.; Su, R.; Nie, S.; Sun, M.; Zhang, J.; Wu, D.; Moustaid-Moussa, N. Application of nanotechnology in improving bioavailability and bioactivity of diet-derived phytochemicals. *The Journal of nutritional biochemistry* **2014**, 25 (4), 363-376.
240. Kale, S. N.; Deore, S. L. Emulsion micro emulsion and nano emulsion: a review. *Systematic Reviews in Pharmacy* **2017**, 8 (1), 39.
241. McClements, D. J.; Rao, J. Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical reviews in food science and nutrition* **2011**, 51 (4), 285-330.
242. Fang, Z.; Bhandari, B. Encapsulation of polyphenols—a review. *Trends in Food Science & Technology* **2010**, 21 (10), 510-523.
243. McClements, D. J.; Decker, E. A.; Park, Y.; Weiss, J. Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical reviews in food science and nutrition* **2009**, 49 (6), 577-606.
244. Jafari, S. M.; McClements, D. J., Nanotechnology approaches for increasing nutrient bioavailability. In *Advances in food and nutrition research*, Elsevier: 2017; Vol. 81, pp 1-30.
245. Singh, H. Nanotechnology applications in functional foods; opportunities and challenges. *Preventive nutrition and food science* **2016**, 21 (1), 1.
246. Assadpour, E.; Mahdi Jafari, S. A systematic review on nanoencapsulation of food bioactive ingredients and nutraceuticals by various nanocarriers. *Critical reviews in food science and nutrition* **2019**, 59 (19), 3129-3151.
247. Li, J.; Hwang, I.-C.; Chen, X.; Park, H. J. Effects of chitosan coating on curcumin loaded nano-emulsion: Study on stability and in vitro digestibility. *Food Hydrocolloids* **2016**, 60, 138-147.
248. Mohammadi, A.; Jafari, S. M.; Assadpour, E.; Esfanjani, A. F. Nano-encapsulation of olive leaf phenolic compounds through WPC–pectin complexes and evaluating their release rate. *International journal of biological macromolecules* **2016**, 82, 816-822.

249. Mohammadi, A.; Jafari, S. M.; Esfanjani, A. F.; Akhavan, S. Application of nano-encapsulated olive leaf extract in controlling the oxidative stability of soybean oil. *Food chemistry* **2016**, 190, 513-519.
250. Chatzidaki, M. D.; Mitsou, E.; Yaghmur, A.; Xenakis, A.; Papadimitriou, V. Formulation and characterization of food-grade microemulsions as carriers of natural phenolic antioxidants. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2015**, 483, 130-136.
251. Chen, Y.; SHOU, T. X.; ZHANG, Q. Y.; Li, D. Bioavailability of diacylglycerol microemulsion. *Journal of Food Biochemistry* **2013**, 37 (2), 144-150.
252. Puri, A.; Loomis, K.; Smith, B.; Lee, J.-H.; Yavlovich, A.; Heldman, E.; Blumenthal, R. Lipid-based nanoparticles as pharmaceutical drug carriers: from concepts to clinic. *Critical Reviews™ in Therapeutic Drug Carrier Systems* **2009**, 26 (6).
253. Rafiee, Z.; Nejatian, M.; Daeihamed, M.; Jafari, S. M. Application of different nanocarriers for encapsulation of curcumin. *Critical reviews in food science and nutrition* **2019**, 59 (21), 3468-3497.
254. McClements, D. J. Nanoscale Nutrient Delivery Systems for Food Applications: Improving Bioactive Dispersibility, Stability, and Bioavailability. *Journal of Food Science* **2015**, 80 (7), N1602--N1611 DOI: 10.1111/1750-3841.12919.
255. Jennings, V.; Lippacher, A.; Gohla, S. Medium scale production of solid lipid nanoparticles (SLN) by high pressure homogenization. *Journal of microencapsulation* **2002**, 19 (1), 1-10.
256. Souto, E. B.; Severino, P.; Santana, M. H. A.; Pinho, S. C. Solid lipid nanoparticles: classical methods of lab production. *Quimica Nova* **2011**, 34 (10), 1762-1769.
257. Weiss, J.; Decker, E. A.; McClements, D. J.; Kristbergsson, K.; Helgason, T.; Awad, T. Solid lipid nanoparticles as delivery systems for bioactive food components. *Food Biophysics* **2008**, 3 (2), 146-154.
258. Ramalingam, P.; Yoo, S. W.; Ko, Y. T. Nanodelivery systems based on mucoadhesive polymer coated solid lipid nanoparticles to improve the oral intake of food curcumin. *Food Research International* **2016**, 84, 113-119.

259. Jose, S.; Anju, S.; Cinu, T.; Aleykutty, N.; Thomas, S.; Souto, E. In vivo pharmacokinetics and biodistribution of resveratrol-loaded solid lipid nanoparticles for brain delivery. *International journal of pharmaceutics* **2014**, 474 (1-2), 6-13.
260. Üner, M. Preparation, characterization and physico-chemical properties of solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC): their benefits as colloidal drug carrier systems. *Die pharmazie-an international journal of pharmaceutical sciences* **2006**, 61 (5), 375-386.
261. Müller, R.; Radtke, M.; Wissing, S. Nanostructured lipid matrices for improved microencapsulation of drugs. *International journal of pharmaceutics* **2002**, 242 (1-2), 121-128.
262. Gokce, E. H.; Korkmaz, E.; Deller, E.; Sandri, G.; Bonferoni, M. C.; Ozer, O. Resveratrol-loaded solid lipid nanoparticles versus nanostructured lipid carriers: evaluation of antioxidant potential for dermal applications. *International journal of nanomedicine* **2012**, 7, 1841.
263. Chen, P.; Zhang, H.; Cheng, S.; Zhai, G.; Shen, C. Development of curcumin loaded nanostructured lipid carrier based thermosensitive in situ gel for dermal delivery. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2016**, 506, 356-362.
264. Chen-yu, G.; Chun-fen, Y.; Qi-lu, L.; Qi, T.; Yan-wei, X.; Wei-na, L.; Guang-xi, Z. Development of a quercetin-loaded nanostructured lipid carrier formulation for topical delivery. *International journal of pharmaceutics* **2012**, 430 (1-2), 292-298.
265. Langer, R. New methods of drug delivery. *Science* **1990**, 249 (4976), 1527-1533.
266. Mozafari, M.; Pardakhty, A.; Azarmi, S.; Jazayeri, J.; Nokhodchi, A.; Omri, A. Role of nanocarrier systems in cancer nanotherapy. *Journal of liposome research* **2009**, 19 (4), 310-321.
267. Pripem, A.; Watanatorn, J.; Sutthiparinyanont, S.; Phachonpai, W.; Muchimapura, S. Anxiety and cognitive effects of quercetin liposomes in rats. *Nanomedicine: Nanotechnology, Biology and Medicine* **2008**, 4 (1), 70-78.
268. Banerjee, K.; Banerjee, S.; Mandal, M. Enhanced chemotherapeutic efficacy of apigenin liposomes in colorectal cancer based on flavone-membrane interactions. *Journal of Colloid and Interface Science* **2017**, 491, 98--110 DOI: 10.1016/j.jcis.2016.12.025.

269. Sahari, M. A.; Moghimi, H. R.; Hadian, Z.; Barzegar, M.; Mohammadi, A. Improved physical stability of docosahexaenoic acid and eicosapentaenoic acid encapsulated using nanoliposome containing α - tocopherol. *International Journal of Food Science & Technology* **2016**, 51 (5), 1075-1086.
270. Rasti, B.; Jinap, S.; Mozafari, M.; Yazid, A. Comparative study of the oxidative and physical stability of liposomal and nanoliposomal polyunsaturated fatty acids prepared with conventional and Mozafari methods. *Food chemistry* **2012**, 135 (4), 2761-2770.
271. Liu, W.; Zhai, Y.; Heng, X.; Che, F. Y.; Chen, W.; Sun, D.; Zhai, G. Oral bioavailability of curcumin: problems and advancements. *Journal of drug targeting* **2016**, 24 (8), 694-702.
272. Cuomo, F.; Cofelice, M.; Venditti, F.; Ceglie, A.; Miguel, M.; Lindman, B.; Lopez, F. In-vitro digestion of curcumin loaded chitosan-coated liposomes. *Colloids and Surfaces B: Biointerfaces* **2018**, 168, 29-34.
273. Cai, X.; Fang, Z.; Dou, J.; Yu, A.; Zhai, G. Bioavailability of quercetin: problems and promises. *Current medicinal chemistry* **2013**, 20 (20), 2572-2582.
274. Zhai, Y.; Guo, S.; Liu, C.; Yang, C.; Dou, J.; Li, L.; Zhai, G. Preparation and in vitro evaluation of apigenin-loaded polymeric micelles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2013**, 429, 24-30.
275. Letchford, K.; Liggins, R.; Burt, H. Solubilization of hydrophobic drugs by methoxy poly (ethylene glycol) - block - polycaprolactone diblock copolymer micelles: Theoretical and experimental data and correlations. *Journal of pharmaceutical sciences* **2008**, 97 (3), 1179-1190.
276. Tan, B.-J.; Liu, Y.; Chang, K.-L.; Lim, B. K.; Chiu, G. N. Perorally active nanomicellar formulation of quercetin in the treatment of lung cancer. *International journal of nanomedicine* **2012**, 7, 651.
277. Paredes, A. J.; Asensio, C. M.; Llabot, J. M.; Allemandi, D. A.; Palma, S. D. Nanoencapsulation in the food industry: manufacture, applications and characterization. **2016**.
278. Lee, S. H.; Zhang, Z.; Feng, S.-S. Nanoparticles of poly (lactide)—tocopheryl polyethylene glycol succinate (PLA-TPGS) copolymers for protein drug delivery. *Biomaterials* **2007**, 28 (11), 2041-2050.

279. Patel, A.; Hu, Y.; Tiwari, J. K.; Velikov, K. P. Synthesis and characterisation of zein–curcumin colloidal particles. *Soft Matter* **2010**, 6 (24), 6192-6199.
280. Haham, M.; Ish-Shalom, S.; Nodelman, M.; Duek, I.; Segal, E.; Kustanovich, M.; Livney, Y. D. Stability and bioavailability of vitamin D nanoencapsulated in casein micelles. *Food & Function* **2012**, 3 (7), 737-744.
281. Kumari, A.; Yadav, S. K.; Pakade, Y. B.; Singh, B.; Yadav, S. C. Development of biodegradable nanoparticles for delivery of quercetin. *Colloids and Surfaces B: Biointerfaces* **2010**, 80 (2), 184-192.
282. Das, S.; Das, J.; Samadder, A.; Paul, A.; Khuda-Bukhsh, A. R. Strategic formulation of apigenin-loaded PLGA nanoparticles for intracellular trafficking, DNA targeting and improved therapeutic effects in skin melanoma in vitro. *Toxicology letters* **2013**, 223 (2), 124-138.
283. Werle, M.; Takeuchi, H. Chitosan–aprotinin coated liposomes for oral peptide delivery: development, characterisation and in vivo evaluation. *International journal of pharmaceutics* **2009**, 370 (1-2), 26-32.
284. Werle, M.; Takeuchi, H.; Bernkop-Schnürch, A. Modified chitosans for oral drug delivery. *Journal of pharmaceutical sciences* **2009**, 98 (5), 1643-1656.
285. Hu, B.; Ting, Y.; Yang, X.; Tang, W.; Zeng, X.; Huang, Q. Nanochemoprevention by encapsulation of (–)-epigallocatechin-3-gallate with bioactive peptides/chitosan nanoparticles for enhancement of its bioavailability. *Chemical communications* **2012**, 48 (18), 2421-2423.
286. Ezhilarasi, P.; Karthik, P.; Chhanwal, N.; Anandharamakrishnan, C. Nanoencapsulation techniques for food bioactive components: a review. *Food and Bioprocess Technology* **2013**, 6 (3), 628-647.
287. Crini, G. A history of cyclodextrins. *Chemical reviews* **2014**, 114 (21), 10940-10975.
288. Pápay, Z. E.; Balogh, E.; Zariwala, M. G.; Somavarapu, S.; Antal, I. Drug delivery approaches for apigenin: A review. *APIGENIN AND NARINGENIN* **2015**, 1.
289. Kim, H.-M.; Kim, H.-W.; Jung, S.-H. Aqueous solubility enhancement of some flavones by complexation with cyclodextrins. *Bulletin of the Korean Chemical Society* **2008**, 29 (3), 590-594.

290. Kale, R.; Saraf, M.; Juvekar, A.; Tayade, P. Decreased B16F10 melanoma growth and impaired tumour vascularization in BDF1 mice with quercetin - cyclodextrin binary system. *Journal of Pharmacy and Pharmacology* **2006**, 58 (10), 1351-1358.
291. Yallapu, M. M.; Jaggi, M.; Chauhan, S. C. β -Cyclodextrin-curcumin self-assembly enhances curcumin delivery in prostate cancer cells. *Colloids and Surfaces B: Biointerfaces* **2010**, 79 (1), 113-125.
292. Keck, C. M.; Muller, R. H., SmartCrystals—Review Of The Second Generation Of Drug Nanocrystals. In *Handbook of Materials for Nanomedicine*, Pan Stanford Publishing: 2010; pp 555-579.
293. Al Shaal, L.; Müller, R.; Shegokar, R. smartCrystal combination technology—scale up from lab to pilot scale and long term stability. *Die Pharmazie-An International Journal of Pharmaceutical Sciences* **2010**, 65 (12), 877-884.
294. Hecq, J.; Deleers, M.; Fanara, D.; Vranckx, H.; Amighi, K. Preparation and characterization of nanocrystals for solubility and dissolution rate enhancement of nifedipine. *International journal of pharmaceutics* **2005**, 299 (1-2), 167--177.
295. Sun, M.; Gao, Y.; Pei, Y.; Guo, C.; Li, H.; Cao, F.; Yu, A.; Zhai, G. Development of nanosuspension formulation for oral delivery of quercetin. *Journal of biomedical nanotechnology* **2010**, 6 (4), 325-332.
296. Patravale, V.; Date, A. A.; Kulkarni, R. Nanosuspensions: a promising drug delivery strategy. *Journal of pharmacy and pharmacology* **2004**, 56 (7), 827-840.
297. Elzoghby, A. O.; Samy, W. M.; Elgindy, N. A. Protein-based nanocarriers as promising drug and gene delivery systems. *Journal of controlled release* **2012**, 161 (1), 38-49.
298. Elzoghby, A. O.; El-Fotoh, W. S. A.; Elgindy, N. A. Casein-based formulations as promising controlled release drug delivery systems. *Journal of controlled release* **2011**, 153 (3), 206--216.
299. Elzoghby, A. O.; Elgohary, M. M.; Kamel, N. M., Implications of protein-and Peptide-based nanoparticles as potential vehicles for anticancer drugs. In *Advances in protein chemistry and structural biology*, Elsevier: 2015; Vol. 98, pp 169-221.
300. Głąb, T. K.; Boratyński, J. Potential of casein as a carrier for biologically active agents.

Topics in Current Chemistry **2017**, 375 (4), 71.

301. Fathi, M.; Donsi, F.; McClements, D. J. Protein - Based Delivery Systems for the Nanoencapsulation of Food Ingredients. *Comprehensive reviews in food science and food safety* **2018**, 17 (4), 920-936.

302. ABD EL - SALAM, M. H.; EL - SHIBINY, S. Formation and potential uses of milk proteins as nano delivery vehicles for nutraceuticals: a review. *International journal of dairy technology* **2012**, 65 (1), 13-21.

303. Yildirim-Elikoglu, S.; Erdem, Y. K. Interactions between milk proteins and polyphenols: Binding mechanisms, related changes, and the future trends in the dairy industry. *Food reviews international* **2018**, 34 (7), 665-697.

304. Livney, Y. D. Milk proteins as vehicles for bioactives. *Current Opinion in Colloid & Interface Science* **2010**, 15 (1), 73-83.

305. Horne, D. S. Casein micelle structure: models and muddles. *Current Opinion in Colloid & Interface Science* **2006**, 11 (2-3), 148-153.

306. Gebhardt, R.; Vendrely, C.; Kulozik, U. Structural characterization of casein micelles: shape changes during film formation. *Journal of Physics: Condensed Matter* **2011**, 23 (44), 444201.

307. Jollès, P. The carbohydrate portions of milk glycoproteins. *Journal of Dairy Research* **1979**, 46 (2), 187-191.

308. De Keuif, C.; Holl, C. Caesin micelle structure, function and interactions. *Advanced Dairy Chemistry: Proteins, Part A* **2003**, 233-276.

309. Downey, W.; Murphy, R. The temperature-dependent dissociation of β -casein from bovine casein micelles and complexes. *Journal of Dairy Research* **1970**, 37 (3), 361-372.

310. Pan, K.; Luo, Y.; Gan, Y.; Baek, S. J.; Zhong, Q. pH-driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity. *Soft Matter* **2014**, 10 (35), 6820-6830.

311. Huppertz, T.; De Kruif, C. G. Disruption and reassociation of casein micelles under high pressure: influence of milk serum composition and casein micelle concentration. *Journal of agricultural and food chemistry* **2006**, 54 (16), 5903-5909.

312. O'Connell, J. E.; Kelly, A. L.; Auty, M. A.; Fox, P. F.; de Kruif, K. G. Ethanol-dependent heat-induced dissociation of casein micelles. *Journal of agricultural and food chemistry* **2001**, 49 (9), 4420-4423.
313. Pan, K.; Zhong, Q.; Baek, S. J. Enhanced dispersibility and bioactivity of curcumin by encapsulation in casein nanocapsules. *Journal of agricultural and food chemistry* **2013**, 61 (25), 6036-6043.
314. Mehravar, R.; Jahanshahi, M.; Saghatoleslami, N. Production of biological nanoparticles from α -lactalbumin for drug delivery and food science application. *African Journal of Biotechnology* **2009**, 8 (24).
315. Lohcharoenkal, W.; Wang, L.; Chen, Y. C.; Rojanasakul, Y. Protein nanoparticles as drug delivery carriers for cancer therapy. *BioMed research international* **2014**, 2014.
316. Shafaei, Z.; Ghalandari, B.; Vaseghi, A.; Divsalar, A.; Haertle, T.; Saboury, A. A.; Sawyer, L. β -Lactoglobulin: An efficient nanocarrier for advanced delivery systems. *Nanomedicine: Nanotechnology, Biology and Medicine* **2017**, 13 (5), 1685-1692.
317. Ko, S.; Gunasekaran, S. Preparation of sub-100-nm β -lactoglobulin (BLG) nanoparticles. *Journal of microencapsulation* **2006**, 23 (8), 887-898.
318. Gunasekaran, S.; Xiao, L.; Ould Eleya, M. Whey protein concentrate hydrogels as bioactive carriers. *Journal of Applied Polymer Science* **2006**, 99 (5), 2470-2476.
319. Kelly, P.; Woonton, B.; Smithers, G., Improving the sensory quality, shelf-life and functionality of milk. In *Functional and speciality beverage technology*, Elsevier: 2009; pp 170-231.
320. Li, J.; Yao, P. Self-assembly of ibuprofen and bovine serum albumin– dextran conjugates leading to effective loading of the drug. *Langmuir* **2009**, 25 (11), 6385-6391.
321. Fee, C.; Billakanti, J.; Saufi, S., Methods for purification of dairy nutraceuticals. In *Separation, Extraction and Concentration Processes in the Food, Beverage and Nutraceutical Industries*, Elsevier: 2013; pp 450-482.
322. Edwards, P. B.; Creamer, L. K.; Jameson, G. B., Structure and stability of whey proteins In: *Milk Proteins from Expression to Food* Thompson A, Boland M, Singh H eds. 2009.
323. Augustin, M. A.; Hemar, Y. Nano-and micro-structured assemblies for encapsulation

of food ingredients. *Chemical society reviews* **2009**, 38 (4), 902-912.

324. Kimpel, F.; Schmitt, J. J. Milk proteins as nanocarrier systems for hydrophobic nutraceuticals. *Journal of food science* **2015**, 80 (11), R2361-R2366.

325. Yuksel, Z.; Avci, E.; Erdem, Y. K. Characterization of binding interactions between green tea flavanoids and milk proteins. *Food chemistry* **2010**, 121 (2), 450-456.

326. Williamson, M. P. The structure and function of proline-rich regions in proteins. *Biochemical journal* **1994**, 297 (Pt 2), 249.

327. Moeiniafshari, A.-A.; Zarrabi, A.; Bordbar, A.-K. Exploring the interaction of naringenin with bovine beta-casein nanoparticles using spectroscopy. *Food hydrocolloids* **2015**, 51, 1-6.

328. Forrest, S. A.; Yada, R. Y.; Rousseau, D. Interactions of vitamin D3 with bovine β -lactoglobulin A and β -casein. *Journal of agricultural and food chemistry* **2005**, 53 (20), 8003-8009.

329. Zimet, P.; Rosenberg, D.; Livney, Y. D. Re-assembled casein micelles and casein nanoparticles as nano-vehicles for ω -3 polyunsaturated fatty acids. *Food hydrocolloids* **2011**, 25 (5), 1270-1276.

330. Jameson, G. B.; Adams, J. J.; Creamer, L. K. Flexibility, functionality and hydrophobicity of bovine β -lactoglobulin. *International Dairy Journal* **2002**, 12 (4), 319-329.

331. Pérez, O. E.; David-Birman, T.; Kesselman, E.; Levi-Tal, S.; Lesmes, U. Milk protein–vitamin interactions: Formation of beta-lactoglobulin/folic acid nano-complexes and their impact on in vitro gastro-duodenal proteolysis. *Food Hydrocolloids* **2014**, 38, 40-47.

332. Liang, L.; Line, V. L. S.; Remondetto, G. E.; Subirade, M. In vitro release of α -tocopherol from emulsion-loaded β -lactoglobulin gels. *International Dairy Journal* **2010**, 20 (3), 176-181.

333. Shpigelman, A.; Israeli, G.; Livney, Y. D. Thermally-induced protein–polyphenol co-assemblies: beta lactoglobulin-based nanocomplexes as protective nanovehicles for EGCG. *Food Hydrocolloids* **2010**, 24 (8), 735--743 DOI: 10.1016/j.foodhyd.2010.03.015.

334. Mohammadi, F.; Moeeni, M. Analysis of binding interaction of genistein and kaempferol with bovine α -lactalbumin. *Journal of functional foods* **2015**, 12, 458-467.

335. Damodaran, S.; Parkin, K. L., *Fennema's food chemistry*. CRC press: 2017.
336. Esmaili, M.; Ghaffari, S. M.; Moosavi-Movahedi, Z.; Atri, M. S.; Sharifizadeh, A.; Farhadi, M.; Yousefi, R.; Chobert, J.-M.; Haertlé, T.; Moosavi-Movahedi, A. A. Beta casein-micelle as a nano vehicle for solubility enhancement of curcumin; food industry application. *LWT-food science and technology* **2011**, 44 (10), 2166-2172.
337. Mehranfar, F.; Bordbar, A.-K.; Amiri, R. A. In vitro cytotoxic activity and binding properties of curcumin in the presence of β -casein micelle nanoparticles. *Biomacromolecular Journal* **2015**, 1 (1), 69-79.
338. Semo, E.; Kesselman, E.; Danino, D.; Livney, Y. D. Casein micelle as a natural nano-capsular vehicle for nutraceuticals. *Food hydrocolloids* **2007**, 21 (5), 936-942.
339. Sáiz-Abajo, M.-J.; González-Ferrero, C.; Moreno-Ruiz, A.; Romo-Hualde, A.; González-Navarro, C. J. Thermal protection of β -carotene in re-assembled casein micelles during different processing technologies applied in food industry. *Food chemistry* **2013**, 138 (2-3), 1581-1587.
340. Diarrassouba, F.; Remondetto, G.; Garrat, G.; Alvarez, P.; Beyssac, E.; Subirade, M. Self-assembly of β -lactoglobulin and egg white lysozyme as a potential carrier for nutraceuticals. *Food chemistry* **2015**, 173, 203-209.
341. Ipsen, R.; Otte, J. Self-assembly of partially hydrolysed α -lactalbumin. *Biotechnology advances* **2007**, 25 (6), 602-605.
342. Damodaran, S., *Food Proteins and Their Applications*. CRC Press: 1997; Vol. 80.
343. Nag, A.; Han, K.-S.; Singh, H. Microencapsulation of probiotic bacteria using pH-induced gelation of sodium caseinate and gellan gum. *International Dairy Journal* **2011**, 21 (4), 247-253.
344. Heidebach, T.; Först, P.; Kulozik, U. Transglutaminase-induced caseinate gelation for the microencapsulation of probiotic cells. *International Dairy Journal* **2009**, 19 (2), 77-84.
345. Silva, N. F. N.; Saint-Jalmes, A.; de Carvalho, A. n. F.; Gaucheron, F. d. r. Development of casein microgels from cross-linking of casein micelles by genipin. *Langmuir* **2014**, 30 (34), 10167-10175.
346. LaClair, C. E. Purification and use of whey proteins for improved health. **2008**.

347. Chen, L.; Subirade, M. Alginate–whey protein granular microspheres as oral delivery vehicles for bioactive compounds. *Biomaterials* **2006**, 27 (26), 4646-4654.
348. Hébrard, G.; Blanquet, S.; Beyssac, E.; Remondetto, G.; Subirade, M.; Alric, M. Use of whey protein beads as a new carrier system for recombinant yeasts in human digestive tract. *Journal of biotechnology* **2006**, 127 (1), 151-160.
349. Walstra, P. In P. Walstra & R. Jenness. *Dairy chemistry and physics* **1984**, 98-122.
350. Singh, H. Functional properties of milk proteins. **2016**.
351. Cornacchia, L.; Roos, Y. H. Stability of β -carotene in protein-stabilized oil-in-water delivery systems. *Journal of agricultural and food chemistry* **2011**, 59 (13), 7013-7020.
352. Mao, Y.; Dubot, M.; Xiao, H.; McClements, D. J. Interfacial engineering using mixed protein systems: Emulsion-based delivery systems for encapsulation and stabilization of β -carotene. *Journal of agricultural and food chemistry* **2013**, 61 (21), 5163-5169.
353. Ye, A. Complexation between milk proteins and polysaccharides via electrostatic interaction: principles and applications—a review. *International journal of food science & technology* **2008**, 43 (3), 406-415.
354. Zhang, J.; Liu, X.; Subirade, M.; Zhou, P.; Liang, L. A study of multi-ligand beta-lactoglobulin complex formation. *Food chemistry* **2014**, 165, 256-261.
355. Esmaili, M.; Ghaffari, S. M.; Moosavi-Movahedi, Z.; Atri, M. S.; Sharifizadeh, A.; Farhadi, M.; Yousefi, R.; Chobert, J.-M.; Haertl. Beta casein-micelle as a nano vehicle for solubility enhancement of curcumin; food industry application. *LWT-food science and technology* **2011**, 44 (10), 2166-2172.
356. Oancea, A.-M.; Aprodu, I.; Ghinea, I. O.; Barbu, V.; Ioniță, E.; Bahrim, G.; Râpeanu, G.; Stănciuc, N. A bottom-up approach for encapsulation of sour cherries anthocyanins by using β -lactoglobulin as matrices. *Journal of Food Engineering* **2017**, 210, 83-90.
357. Abbasi, A.; Emam-Djomeh, Z.; Mousavi, M. A. E.; Davoodi, D. Stability of vitamin D3 encapsulated in nanoparticles of whey protein isolate. *Food chemistry* **2014**, 143, 379-383.

Appendix

Table 1-1. Composition of proteins in bovine milk^{1, 2}

Protein		Content in milk (g/l)	Molecular weight (kDa)
Caseins	α_{s1} -casein	12-15	22.1-23.7
	α_{s2} -casein	3-4	25.2-25.4
	β -casein	9-11	23.9-24.1
	κ -casein	3-4	19.0
Whey proteins	β -lactoglobulin	2-4	18.3
	α -lactalbumin	1-1.5	14.2
	Bovine serum albumin (BSA)	0.1-0.4	66
	Immunoglobulins (mainly IgG, IgM)	0.6-1.0	150, 900
	Lactoferrin	~0.1	80
Fat globule membrane proteins		~0.4	13-200
Total milk proteins		30-35	

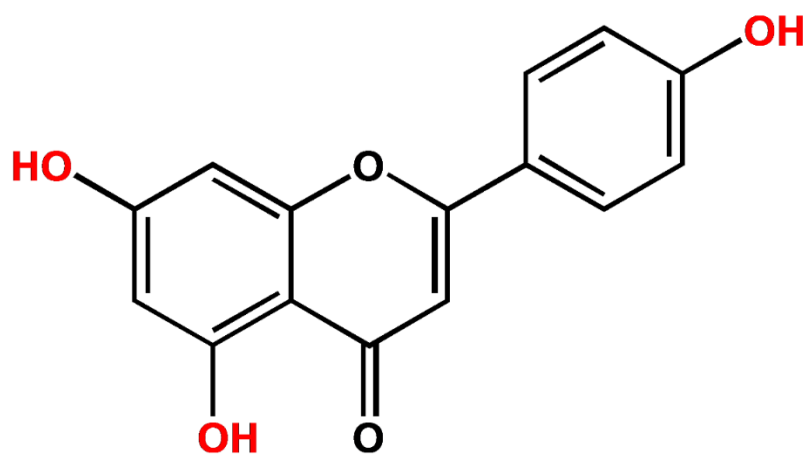


Figure 1-1. Molecular structure of apigenin (4',5,7-trihydroxyflavone).

**Chapter 2. Nanoencapsulation of apigenin with whey protein isolate:
physicochemical properties and *in vitro* activities against colorectal
cancer cells**

2.1 Abstract

Incorporating lipophilic phytochemicals with anti-cancer activities in functional beverages requires an appropriate nanoencapsulation technology. The present objective was to encapsulate apigenin, a compound present in many fruits and vegetables, with whey protein isolate (WPI), utilizing a pH-cycle method, and subsequently characterize physicochemical properties of the nanoencapsulated apigenin and the *in vitro* anticancer activities against human colorectal cancer cells (HCT-116 & HT-29). Up to 2.0 mg/mL of apigenin was nanoencapsulated with 1.0% w/v WPI successfully, with an encapsulation efficiency of up to 98.38% and loading capacity of up to 195.78 mg/g-WPI. Nanodispersions maintained stable after 30-day ambient storage with the particle hydrodynamic diameter of 240 nm. The obtained nanoparticles exhibited near-spherical morphology under atomic force microscopy. Results from differential scanning calorimetry showed amorphous structures of apigenin verifying encapsulation. Hydrogen bonds and hydrophobic interactions between apigenin and WPI were confirmed in Fourier transform infrared spectroscopy. Anti-proliferative activities against HCT-116 and HT-29 were studied with 5, 10 and 20 μ M of nanoencapsulated or dimethyl sulfoxide (DMSO)-dissolved apigenin, before and after *in vitro* digestions. Results showed that nanoencapsulation and *in vitro* digestion did not negatively affect the growth inhibitory activity of apigenin. Cellular uptake of apigenin was quantified using HPLC after lysing cells. Apigenin accumulated in HCT-116 and HT-29 treated with 20 μ M DMSO-dissolved-apigenin was 0.37 and 0.08 μ g/mg-protein, respectively, which was enhanced to 2.51 and 2.75 μ g/mg-protein for the 20 μ M nanoencapsulated apigenin. Cell cycle distribution and apoptotic cells were further

analyzed by flow cytometry. Results suggested that the growth inhibitory activity of apigenin was possibly due to the induction of cell apoptosis, and nanoencapsulation enhanced the pro-apoptotic effects. Therefore, the studied scalable nanoencapsulation system may be significant to incorporate lipophilic phytochemicals in functional beverages for disease prevention.

Keywords: apigenin, whey protein isolate, pH-cycle, nanoencapsulation, *in vitro* anticancer activity

2.2 Introduction

Natural polyphenols, occurring as the secondary plant metabolites, have attracted increasing attentions for decades due to their versatile biological activities.^{3,4} Polyphenols from edible plants, when studied at a physiologically relevant concentration, have been reported for low or no toxicity on human organs and cells.⁵ Apigenin (4',5,7-trihydroxyflavone) is one of the most investigated polyphenols and is abundantly presented in numerous dietary plants such as celery, parsley, onions, chamomile.⁶ Apigenin is believed to exert a broad spectrum of health beneficial effects, including free radical scavenging properties, and anti-inflammatory activities.⁷ More importantly, owing to non-mutagenesis and relatively low toxicity, apigenin has shown promising preventive and therapeutic potential against cancers.⁸ For example, the potential chemotherapeutic effects of apigenin have been reported for numerous human colon cancer cell lines via inhibition of cell proliferation,⁹ promotion of cell cycle arrest,¹⁰ and induction of cell apoptosis.¹¹ However, the high hydrophobicity of apigenin results in a very low water solubility, with the highest aqueous solubility of only 2.16 µg/mL at pH = 7.5,¹² leading to a poor absorption in the gastrointestinal tract. In addition, the rapid metabolism and elimination via glucuronidation and sulfation *in vivo* lead to a low bioavailability of apigenin, which limits the therapeutic efficacy.¹³ Therefore, improvement of solubility and bioavailability is of primary significance for further development and application of apigenin.

Nanotechnology is a potential approach to improve solubility and enhance bioavailability and bioactivity of lipophilic polyphenols. Recently, numerous studies set their aims at developing appropriate delivery systems to address the limitations of apigenin, such as

liposomes prepared by lipid film hydration method,¹⁴ polymeric micelles made from Pluronic P123 and Solutol HS 15,¹⁵ nanosuspensions prepared by high-pressure homogenization,¹⁶ nanocrystals fabricated using supercritical antisolvent process,¹⁷ and poly (lactic-co-glycolic acid) (PLGA)-based nanoparticles.¹⁸ The usage of synthetic surfactants, the residual organic solvent, and the presence of synthetic polymers in these studies may cause chronic toxicity and be impractical in food applications. Besides, processes used to fabricate nanostructures may be costly or difficult to scale up. To overcome these challenges, efforts to develop low-cost, and low-energy processes and non-toxic delivery systems are needed to further exert the pharmacological functions and therapeutic efficacy of apigenin.

A pH-cycle method, by first increasing a mixture to alkaline pH followed by lowering pH to neutral conditions, has been successfully applied to encapsulate curcumin in sodium caseinate in our research group.¹⁹ In addition, the high biodegradability, biocompatibility, non-toxicity, abundance, sustainability, low-cost and unique physicochemical properties of dairy proteins have made them suitable as nanocarriers for delivering various bioactives.^{1, 20, 21} However, dairy proteins have not been studied to incorporate apigenin to enhance the solubility and bioavailability.

The first objective of the current study was firstly to apply the pH-cycle method to fabricate a low-cost, low energy, and organic solvent-free nanodelivery system of apigenin, using whey protein isolate (WPI), and characterize physicochemical properties of the optimized nanoparticles. The *in vitro* anticancer activities of nanoencapsulated apigenin were investigated using human colon cancer cells (HCT-116 and HT-29) in the second objective.

2.3 Materials and methods

2.3.1 Materials

Apigenin (>98%) was purchased from Indofine Chemical Co. Inc (Hillsborough, NJ, USA). WPI was purchased from Hilmar Ingredients (Hilmar, CA, USA). Human colon cancer cell lines HCT-116 and HT-29 were purchased from American Type Culture Collection (Manassas, VA, USA). Growth media MEM 1X was purchased from Corning Inc. (Corning, NY, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA, USA). All other chemicals were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated.

2.3.2 Nanoencapsulation of apigenin

Nanoencapsulation of apigenin followed our previous work with some modifications.¹⁹ Experimentally, 1% w/v WPI was hydrated in deionized water overnight at 4 °C, followed by adding 0.6-2.0 mg/mL apigenin and adjusting pH to 13.0 using 2.0 M KOH solution. After stirring for 5 min at ambient temperature (21 °C) and adjusting the pH to 10.0, samples were stirred for another 30 min at 21 °C before subsequent titration to pH 7.0 with 1.0 M HCl. The unencapsulated apigenin was removed by centrifugation at 10,000 g for 10 min (Sorvall LYNX 6000 Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA), and the supernatant was collected as the nanodispersion. The precipitate was dissolved in DMSO to quantify the encapsulation efficiency and loading capacity based on the absorbance at 337 nm and a calibration curve that was established using standard solutions with various concentrations of apigenin in DMSO.

$$EE \% = \frac{\text{total amount of AP (mg)} - \text{amount of AP in precipitate (mg)}}{\text{total amount of AP (mg)}} \times 100$$

$$\text{Loading capacity (mg/g)} = \frac{\text{total amount of AP (mg)} - \text{amount of AP in precipitate (mg)}}{\text{total amount of WPI (g)}}$$

Lyophilized samples (Model Advantage Plus EL-85, VirTis Company, Inc., Gardiner, NY, USA) were additionally prepared for following experiments.

2.3.3 Physicochemical characterizations of nanoencapsulated apigenin

2.3.3.1 Dynamic light scattering (DLS) and zeta-potential

Particle size distributions of pH 7.0 dispersions added with 0.2% w/v sodium azide to prevent microorganisms during 30-day ambient storage (21 °C) were measured using a Zetasizer Nano-ZS series instrument (Malvern Instruments, Ltd., Worcestershire, UK). Zeta-potential of nanodispersions and WPI solution that was also processed by the same pH-cycle were also measured using the same instrument.

2.3.3.2 Atomic force microscopy (AFM)

The morphology of nanoparticles was studied using AFM. The nanodispersion prepared with 2.0 mg/mL apigenin was diluted 200 times in deionized water, to an equivalent WPI concentration of 50 µg/mL. After spreading 10 µL of the diluted sample on a freshly cleaved mica sheet adhered on a sample disk, samples were dried overnight at ambient conditions (21 °C). The rectangular cantilever had an aluminum reflective coating on the backside (ScanAsyst, Bruker Corp., Billerica, MA, USA). Topographical images were collected at the scan area of 1.0 × 1.0 µm using a scanning speed of 1 Hz (multimode VIII microscope, Bruker AXS, Billerica, MA, USA).

2.3.3.3 Differential scanning calorimetry (DSC)

Thermal properties of lyophilized nanodispersion prepared with 2 mg/mL apigenin, lyophilized WPI powder with the pH-cycle, and pristine apigenin were characterized with a model Q2000 calorimeter (TA Instruments, New Castle, DE, USA). Around 3 mg powder was placed in a hermetic aluminum pan that was then sealed before heating from 20 to 400 °C followed by cooling from 400 to 25 °C at a constant rate of 10 °C/min, with nitrogen as a purge gas at 50 mL/min.²²

2.3.3.5 Fourier transform infrared (FTIR) spectroscopy

Possible intermolecular interactions between apigenin and WPI were investigated using FTIR spectroscopy.²³ The FTIR spectra of freeze-dried samples were recorded by performing 64 scans over 400-4000 cm⁻¹ with a resolution of 4 cm⁻¹ (Spectrum Spotlight, PerkinElmer Inc., Waltham, MA, USA).

2.3.4 In vitro gastrointestinal digestion

In vitro gastrointestinal digestion was conducted following a previously reported method with modifications.²⁴ Specifically, 200 µL of apigenin pre-dissolved in DMSO at 20 mM, 500 µL of the nanodispersion prepared with 2.0 mg/mL apigenin, 500 µL of 1% w/v WPI processed by the pH-cycle or 500 µL of 1% w/v unprocessed WPI was mixed with 6 mL of the simulated gastric fluid, containing 1.5 mg/mL pepsin. After adjusting to pH 2.0 and incubating at 37 °C in a water bath shaker (New Brunswick Scientific Co Inc., Edison, NJ, USA) for 2h (150 rpm), the mixture was added with 4 mL of simulated intestinal fluid, containing 5 mg/mL pancreatin and 5 mg/mL bile salts, adjusted to pH 7.0 with 1 M NaOH, and incubated for 4 h in the shaking

water bath at 37 °C. The digestion was terminated by heating at 75 °C for 20 min to inactivate enzymes. At the end of 2-h peptic and 4-h pancreatic digestions, 1 mL aliquots were sampled for gel electrophoresis. The mixture samples after terminating the digestions were further used in cell work.

2.3.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Unprocessed 1% w/v WPI, pH-cycle treated 1% w/v WPI, the nanodispersion prepared with 2 mg/mL apigenin, and digested samples were mixed with an equal volume of Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 5% β -mercaptoethanol and heated in boiling water for 5 min. Samples were loaded into wells of a 4–20% Mini-Protean TGX gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and run in Bio-Rad Tris/Glycine/SDS buffer for 30 min at 200 volts. After washing twice for 10 min each with deionized (DI) water, the gel was incubated and stained with 50 mL of Bio-Safe™ Coomassie G-250 blue dye for 1 h with gentle shaking. Thereafter, the gel was washed and destained with DI water until the background became clear.

2.3.6 Cell culture and cell proliferation assay

Anti-proliferative activities of free or nanoencapsulated apigenin against human colorectal cancer cells HCT-116 and HT-29 both before and after the simulated digestions were evaluated. Human colorectal HCT-116 and HT-29 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in minimum essential medium (MEM) (Corning Inc., NY, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Life Tech, Carlsbad, CA, USA) and 1% v/v penicillin/streptomycin at 37 °C under a

humidified atmosphere of 5% CO₂. The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used. After seeding cells at a density of 1000 cells/well in 96-well plates in 200 µL complete medium, cells were allowed to attach overnight before challenging with 0-20 µM of DMSO-dissolved or nanoencapsulated apigenin. After incubation for 72 h, growth media were removed followed by adding 100 µL MEM containing 10% v/v 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS). Plates were incubated at 37 °C for 3 h and absorbance was recorded at 490 nm. The growth inhibitory activity was expressed as the percentage with respect to the absorbance of wells with untreated control cells. All experiments were performed in at least three trials with four replicates per trail.

2.3.7 Cellular uptake studied with high-performance liquid chromatography (HPLC)

Cellular uptake of free and nanoencapsulated apigenin analyzed using HPLC was modified from a method reported by Chen et al.²⁵ Briefly, the nanodispersion prepared at 2 mg/mL apigenin was diluted to 5, 10 and 20 µM using the growth medium. Both HCT-116 and HT-29 were seeded at 2×10^5 per well in 6-well plates and incubated overnight at 37 °C for attachment. Cells were treated with 5, 10 and 20 µM of DMSO-dissolved or nanoencapsulated apigenin for 24 h. After treatment, cells were washed twice with cold PBS, lysed, and harvested by Radio immunoprecipitation assay (RIPA) buffer containing 1% v/v protease inhibitor cocktail. The cell lysates were vortexed for 5 min at 4 °C and an aliquot of 5 µL lysates was used to determine the protein concentration by Bradford assay, using bovine serum albumin as the standard protein. The remaining lysates were mixed with 2 parts (v : v) of methanol and

vortexed for 10 min in a cold room (4 °C), followed by 30 min ultrasonic treatment (Branson 1510 Ultrasonic Cleaner, MO, USA). Supernatant was collected after centrifugation (Sorvall Legend Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA) at 13,800 g for 10 min at 4 °C. The apigenin concentration in the cell lysates was analyzed using a reserved phase HPLC system (1200 series, Agilent Technologies, Waldbronn, Germany) under the detection wavelength of 340 nm. A binary solvent mixture of water with 4% formic acid (solvent A) and acetonitrile (solvent B) at a gradient volume ratio from 88:12 to 50:50 was used as the mobile phase. The concentration of apigenin was quantified with a calibration curve that was established using standard solutions with 0-2.5 µg/mL apigenin dissolved in DMSO ($y = 76.262x$, $R^2 = 0.9997$). The cellular uptake was then calculated as follows:

$$\text{Uptake of AP } (\mu\text{g/mg}) = \frac{C_{AP} (\mu\text{g/mL})}{C_P (\text{mg/mL})}$$

Where C_{AP} and C_P represent concentrations of apigenin and protein in the cell lysates, respectively.

2.3.8 Cell cycle analysis by flow cytometry

Analyses of cell-cycle distribution and apoptotic ratio were performed using flow cytometry.²⁶ Cells were prepared similarly to cellular uptake experiments by seeding at a density of 2×10^5 per well in 6-well plates and were treated with DMSO-dissolved or nanoencapsulated apigenin (5 µM, 10 µM, 20 µM) for 24 h. After which, cells were washed twice with cold PBS and harvested with trypsinization. After centrifugation at 1,000 g for 5 min at 4 °C, the collected cell pellet was re-suspended in 60 µL cold PBS and fixed in 1mL ice-cold 70% ethanol. After being left to stand overnight, the permeabilized cells were collected

by centrifugation (Sorvall Legend Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA) at 1,000 g for 10 min, 4 °C, and re-suspended in 1mL PBS containing 16 µg/mL propidium iodide (PI) and 30 µg/mL RNase A, and incubated at 37 °C for 60 min in the dark. Fluorescence emitted from the PI-DNA complex was acquired at an excitation wavelength of 488 nm using MACSQuant flow cytometer from Miltenyi Biotec Inc. (Bergisch Gladbach, Germany). A total of 10,000 events were acquired for each sample. Results were reported as the percentage of cells in each phase of cell cycle, and apoptotic ratios were reported as relative folds to control cells. The analysis was performed independently in triplicates.

2.3.9 Statistical analysis

Data were expressed as mean $\pm$ SD ($n \geq 3$). Results were analyzed by one-way ANOVA using the IBM SPSS Statistics version 25.0 software (SPSS Inc., Chicago, IL, USA). Significant difference were compared using Tukey's test for multiple comparisons, and $p < 0.05$ was considered significantly different.

2.4 Results and discussion

2.4.1 Physicochemical characteristics of nanoencapsulated apigenin

When various concentrations of apigenin were studied, the encapsulation efficiency (EE) was 93.44-98.38%, and the corresponding loading capacity (LC) was 57.19-195.78 mg-apigenin/g-WPI (Figure 2-1A). These results were found to be higher than some previous studies,^{14, 15, 27} and are important in drug delivery since low EE and LC may result in consuming a larger amount of nanocarriers which may cause adverse effects.²⁸ Zeta potential is the

indicator of the surface charge of nanoparticles.²⁸ Table 2-1 shows that WPI was negatively charged after the pH-cycle treatment under the same encapsulation conditions. Zeta-potentials of nanodispersions prepared with different initial apigenin concentrations were similar to the pH-treated WPI, which suggested that encapsulation of apigenin with WPI has no significant influence on the protein charge.

Particle size distributions of nanodispersions prepared with 0.6, 1.0, 1.5 and 2.0 mg/mL apigenin were studied during 30-day ambient storage. With the elevated concentration of apigenin, hydrodynamic diameters of nanodispersions increased from 180 nm to 240 nm, which is possibly due to an increased loading capacity. The nanosized dimension enables the obtained nanoparticles as effective carrier to deliver apigenin since most of the biological processes occur at the nanoscale.²⁹ Additionally, particle size distributions of all nanodispersions maintained stable after 30-day storage ($p > 0.05$) (Figure 2-1B). The AFM image (Figure 2-1C) showed near-spherical shape and uniform of nanoparticles, which is consistent with the DLS results.

DSC was carried out to qualitatively study changes in the physical state of apigenin. As shown in Figure 2-2A, the broad endothermic peak observed at around 100 °C of the pH-treated WPI and nanocapsule sample was attributed to the evaporation of bound water.³⁰ The sharp endothermic peak at 366 °C represented the melting point of pristine apigenin, which was absent in the thermogram of the nanocapsules. Therefore, apigenin existed in amorphism after encapsulation in the protein matrix.

FTIR was applied to study the potential molecular interactions between apigenin and WPI.

As shown in Figure 2-2B, the absorption peak at around 3278-3282 cm^{-1} of all samples is due to the hydroxy stretching vibration (O-H) associated with intermolecular hydrogen bonds.³¹ Bands at the 1700-1500 cm^{-1} region correspond to the amide I (1700-1600 cm^{-1}) and amide II (1600-1500 cm^{-1}) groups of proteins, attributing to the stretching vibrations of C-O groups and the bending vibration of N-H groups, respectively.³² Hence, the peak of pristine apigenin at 1652 cm^{-1} shifting to 1631 cm^{-1} after encapsulation was possibly caused by the binding between apigenin and amide I group of WPI. Besides, compared with the native WPI, there was a shift of the absorbance peak from 1520 to 1532 cm^{-1} for the nanocapsules. This shift was indicative of hydrophobic interactions, ascribing to structural changes of WPI after encapsulation of apigenin using pH-cycle treatment, which was consistent with the peak in the pH-cycle treated WPI. These results indicate non-covalent interactions between apigenin and protein molecules.

Protein profiles of unprocessed WPI, WPI after the pH-cycle treatment and the nanodispersion prepared with 2 mg/mL apigenin and their pepsin hydrolysis products after simulated gastric digestion as well as pancreatin hydrolysates after intestinal digestion were analyzed using SDS-PAGE. As shown in Figure 2-3, compared with the unprocessed WPI, no significant differences were detected after the pH-cycle treatment and encapsulation with apigenin, indicating no hydrolysis after the pH-cycle treatment. After pepsin hydrolysis, high molecular weight whey proteins were hydrolyzed significantly, while β -lactoglobulin was only partially hydrolyzed, which shows resistance to peptic digestion of β -lactoglobulin.³³ The remaining β -lactoglobulin was completely hydrolyzed in the subsequent intestinal digestion. These observations suggest that encapsulating apigenin with WPI can possibly enhance the

solubility of apigenin in the gastric tract thus obtain a higher release in the intestine tract, which may enhance the bioavailability of apigenin.

Taken together, the possible principle of encapsulating apigenin in WPI under the studied conditions can be summarized in Figure 2-4. Apigenin is hardly dissolved in water at neutral pH; however, it is deprotonated when pH increases to 13, which makes it negatively charged and solubilized. Meanwhile, the globular whey proteins are unfolded, expose hydrophobic amino acid residues to bind with apigenin and become loose under the alkaline pH.³⁴ During acidification to pH 7, apigenin loses charges and solubility due to protonation; the unfolded whey proteins become refolded.³⁵ The increased hydrophobicity of apigenin results in aggregation together with bound proteins, which forms nanocapsules with multiple whey proteins and a core enriched of apigenin.

2.4.2 *In vitro* anti-proliferative activity

Uncontrolled cell proliferation is known to be a hallmark of cancers. Thus, the growth inhibitory activities of free (pre-dissolved in DMSO) and nanoencapsulated apigenin against human colorectal cancer cells HCT-116 and HT-29 before and after simulated digestions were evaluated.

As shown in Figure 2-5, both the free apigenin and encapsulated apigenin dose dependently inhibited the proliferation of HCT-116 and HT-29. At 5 and 10 μM , apigenin before and after nanoencapsulation showed similar anti-proliferative activities in both cell lines ($p > 0.05$). However, at 20 μM , nanoencapsulated apigenin treatments exerted significantly lower anti-proliferative effects, the viability of HCT-116 and HT-29 cells was reduced by 29.63%

and 37.70%, respectively, after 72h incubation at 37 °C, while the administration of apigenin pre-dissolved in DMSO, with respective viability reductions of 48.21% and 54.00% ($p < 0.05$). These results may result from the protective effect of WPI which entrapped apigenin by binding them with the hydrophobic residues in the protein molecules, and decreased the direct interactions between apigenin and cells, with a less numbers of apigenin molecules that exposed directly to cells.

In order to investigate the influence of digestion on the anti-proliferative activity of both free and encapsulated apigenin, samples of 10 and 20 μM were further studied after the simulated 2 h-gastric and 4 h-intestinal digestions. Figure 2-6 shows that both undigested and digested samples dose-dependently inhibited HCT-116 and HT-29 after 72 h incubation at 37 °C. At 10 μM , no significant difference of the anti-proliferative activity was induced by the digestion process for both free and nanoencapsulated apigenin; while at 20 μM , the treatment of nanoencapsulated apigenin became significantly more cytotoxic after digestion ($p < 0.05$), which was not found for free apigenin. WPI is completely hydrolyzed after the sequential gastric and intestinal digestion (Figure 2-3), and the released apigenin can be dissolved by bile surfactant micelle, which may be responsible for the enhanced inhibitory activity after digestion. At 10 μM , apigenin was not enough to inhibit significant cell viability, thus the digestion effect may become insignificant. Therefore, the simulated digestions did not negatively affect the anti-proliferative activity of free apigenin ($p > 0.05$), but significantly enhanced the ability of nanoencapsulated apigenin ($p < 0.05$).

2.4.3 Cellular uptake of apigenin

Cellular uptake of apigenin pre-dissolved in DMSO (free) and nanoencapsulated in WPI was assessed with HPLC after lysing cells. As presented in Figure 2-7A for HCT-116, after 24 h incubation, no significant difference between free apigenin and nanoencapsulated apigenin treatments was observed at 5 and 10 μM . However, apigenin accumulated in HCT-116 reached to 2.51 $\mu\text{g-apigenin/mg-protein}$ for the 20 μM nanoencapsulated apigenin treatment, which was significantly higher than the 0.37 $\mu\text{g-apigenin/mg-protein}$ in cells that treated with 20 μM free apigenin. For HT-29 cells (Figure 2-7B), cellular concentration of apigenin treated with 5 and 10 μM of free apigenin was not detectable, and 0.08 $\mu\text{g-apigenin/mg-protein}$ was detected for the 20 μM free apigenin treatment. For nanoencapsulated apigenin, the trend observed for HT-29 cells was similar to that of HCT-116, reaching 2.75 $\mu\text{g-apigenin/mg-protein}$ for the treatment of 20 μM . It has been reported that particle dimension plays important roles in cell endocytosis, as smaller particles can cross cellular membranes and permeate into cell cytoplasm more easily.³⁶ Nanoencapsulation prevent precipitation of apigenin in the cell culture medium, to contribute to the enhanced cellular uptake when compared to free apigenin. The insignificant difference of cellular uptake between free and encapsulated apigenin at 5 and 10 μM (Figure 2-7) agrees with no difference in cell proliferation (Figure 2-5). No correlation between the cellular uptake and cytotoxicity at 20 μM is probably due to the different mechanisms among these measurements to indicate anticancer activity.

2.4.4 Effects of apigenin on cell cycle distribution and apoptotic ratio

To identify the possible mechanisms involved in cell growth inhibited by apigenin, cell cycle progression and pre-apoptotic ratio were evaluated using flow cytometry after free and nanoencapsulated apigenin treatments. The apoptotic ratio was calculated as relative folds that of control cells. As shown in Figure 2-8A, both free and nanoencapsulated apigenin dose-dependently induced apoptosis of HCT-116 after 24 h treatment. Incubation with 20 μ M of free apigenin resulted in 2.77 folds of apoptotic cells compared with control cells, while 20 μ M of nanoencapsulated apigenin induced 3.33 folds of apoptotic cells. Similarly, administration of 20 μ M nanoencapsulated apigenin induced a much higher apoptotic ratio of HT-29 cells, with 3.10 folds that of control cells, while free apigenin at the same dosage resulted in 2.16 folds of apoptosis (Figure 2-9A). The enhanced cell apoptotic ratio is consistent with the cellular uptake results (Figure 2-7). Correspondingly, the percentage of cells distributed in the cell cycle phase decreased as the treatments caused significant apoptotic cells (Figure 2-8B, C; Figure 2-9B, C). These results indicate that apigenin affected cell proliferation, at least in part, by inducing cell apoptosis rather than arresting cell cycle progression, and nanoencapsulation potentially enhanced the apoptosis induction.

2.5 Conclusions

In summary, the current study demonstrated a low-cost, low-energy, and organic solvent-free process to use whey protein isolate to fabricate a nanodelivery system of apigenin. With 0.6-2.0 mg/mL apigenin, the studied conditions resulted in high encapsulation efficiencies of

93.44-98.38% and the corresponding loading capacity of 57.19-195.78 mg-apigenin/g-WPI. The nanodispersions had hydrodynamic diameters of 180-240 nm and exhibited good storage stability. The improved dispersibility and small dimension of nanoencapsulated apigenin enhanced the cellular uptake of apigenin in human colon cancer cells of HCT-116 and HT-29. As a result, cell apoptosis was significantly enhanced by administration of nanodispersion, which partially contributed to the growth inhibitory activity of apigenin. The present study suggests the potential of the nanodelivered apigenin in the development of functional beverages for disease prevention. However, further studies remain to be conducted to elucidate the effect against various cancer cells *in vitro* and *in vivo*.

References

1. Livney, Y. D. Milk proteins as vehicles for bioactives. *Current Opinion in Colloid & Interface Science* **2010**, 15 (1), 73-83.
2. Butler, J. E. Bovine immunoglobulins: A review. *Journal of Dairy Science* **1969**, 52 (12), 1895-1909.
3. D'Archivio, M.; Filesi, C.; Di Benedetto, R.; Gargiulo, R.; Giovannini, C.; Masella, R. Polyphenols, dietary sources and bioavailability. *Annali-Istituto Superiore di Sanita* **2007**, 43 (4), 348.
4. Scalbert, A.; Johnson, I. T.; Saltmarsh, M. Polyphenols: antioxidants and beyond. *The American journal of clinical nutrition* **2005**, 81 (1), 215S-217S.
5. Xie, J.; Yang, Z.; Zhou, C.; Zhu, J.; Lee, R. J.; Teng, L. Nanotechnology for the delivery of phytochemicals in cancer therapy. *Biotechnology advances* **2016**, 34 (4), 343-353.
6. Zhou, X.; Wang, F.; Zhou, R.; Song, X.; Xie, M. Apigenin: A current review on its beneficial biological activities. *Journal of Food Biochemistry* **2017**, 41 (4), e12376.
7. Zhang, X.; Wang, G.; Gurley, E. C.; Zhou, H. Flavonoid apigenin inhibits lipopolysaccharide-induced inflammatory response through multiple mechanisms in macrophages. *PLoS One* **2014**, 9 (9), e107072.
8. Kim, H.; Mani, I.; Iversen, L.; Ziboh, V. Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostaglandins, leukotrienes and essential fatty acids* **1998**, 58 (1), 17-24.
9. Xu, M.; Wang, S.; Song, Y.; Yao, J.; Huang, K.; Zhu, X. Apigenin suppresses colorectal cancer cell proliferation, migration and invasion via inhibition of the Wnt/ β -catenin signaling pathway. *Oncology letters* **2016**, 11 (5), 3075-3080.
10. Gupta, S.; Afaq, F.; Mukhtar, H. Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. *Oncogene* **2002**, 21 (23), 3727.
11. Lee, Y.; Sung, B.; Kang, Y. J.; Kim, D. H.; Jang, J.-Y.; Hwang, S. Y.; Kim, M.; Lim, H. S.; Yoon, J.-H.; Chung, H. Y. Apigenin-induced apoptosis is enhanced by inhibition of autophagy

formation in HCT116 human colon cancer cells. *International journal of oncology* **2014**, 44 (5), 1599-1606.

12. Zhang, J.; Liu, D.; Huang, Y.; Gao, Y.; Qian, S. Biopharmaceutics classification and intestinal absorption study of apigenin. *International journal of pharmaceutics* **2012**, 436 (1), 311-317.

13. Hu, M., Commentary: bioavailability of flavonoids and polyphenols: call to arms. ACS Publications: 2007.

14. Banerjee, K.; Banerjee, S.; Das, S.; Mandal, M. Probing the potential of apigenin liposomes in enhancing bacterial membrane perturbation and integrity loss. *Journal of colloid and interface science* **2015**, 453, 48-59.

15. Zhai, Y.; Guo, S.; Liu, C.; Yang, C.; Dou, J.; Li, L.; Zhai, G. Preparation and in vitro evaluation of apigenin-loaded polymeric micelles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2013**, 429, 24-30.

16. Al Shaal, L.; Shegokar, R.; Müller, R. H. Production and characterization of antioxidant apigenin nanocrystals as a novel UV skin protective formulation. *International journal of pharmaceutics* **2011**, 420 (1), 133-140.

17. Zhang, J.; Huang, Y.; Liu, D.; Gao, Y.; Qian, S. Preparation of apigenin nanocrystals using supercritical antisolvent process for dissolution and bioavailability enhancement. *European Journal of Pharmaceutical Sciences* **2013**, 48 (4-5), 740-747.

18. Park, J. H.; Lee, S.; Kim, J.-H.; Park, K.; Kim, K.; Kwon, I. C. Polymeric nanomedicine for cancer therapy. *Progress in polymer science* **2008**, 33 (1), 113-137.

19. Pan, K.; Luo, Y.; Gan, Y.; Baek, S. J.; Zhong, Q. pH-driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity. *Soft Matter* **2014**, 10 (35), 6820-6830.

20. Elgindy, N. A.; Samy, W. A.; Elzoghby, A. O. Casein-based micelles: a novel vector for delivery of the poorly soluble anticancer drug, flutamide? *Therapeutic delivery* **2014**, 5 (1), 7-9.

21. Elzoghby, A. O.; El-Fotoh, W. S. A.; Elgindy, N. A. Casein-based formulations as promising controlled release drug delivery systems. *Journal of controlled release* **2011**, 153

(3), 206-216.

22. Pan, K.; Zhong, Q. Low energy, organic solvent-free co-assembly of zein and caseinate to prepare stable dispersions. *Food Hydrocolloids* **2016**, 52, 600-606.

23. Sun, C.; Gao, Y.; Zhong, Q. Properties of Ternary Biopolymer Nanocomplexes of Zein, Sodium Caseinate, and Propylene Glycol Alginate and Their Functions of Stabilizing High Internal Phase Pickering Emulsions. *Langmuir* **2018**, 34 (31), 9215-9227.

24. Oancea, A.-M.; Aprodu, I.; Ghinea, I. O.; Barbu, V.; Ioniță, E.; Bahrim, G.; Râpeanu, G.; Stănciuc, N. A bottom-up approach for encapsulation of sour cherries anthocyanins by using β -lactoglobulin as matrices. *Journal of Food Engineering* **2017**, 210, 83-90.

25. Chen, H.; Guan, Y.; Baek, S. J.; Zhong, Q. Caffeic Acid Phenethyl Ester Loaded in Microemulsions: Enhanced In Vitro Activity against Colon and Breast Cancer Cells and Possible Cellular Mechanisms. *Food biophysics* **2019**, 14 (1), 80-89.

26. Lim, S.; Xu, J.; Kim, J.; Chen, T. Y.; Su, X.; Standard, J.; Carey, E.; Griffin, J.; Herndon, B.; Katz, B. Role of anthocyanin - enriched purple - fleshed sweet potato p40 in colorectal cancer prevention. *Molecular nutrition & food research* **2013**, 57 (11), 1908-1917.

27. Banerjee, K.; Banerjee, S.; Mandal, M. Enhanced chemotherapeutic efficacy of apigenin liposomes in colorectal cancer based on flavone-membrane interactions. *Journal of Colloid and Interface Science* **2017**, 491, 98--110 DOI: 10.1016/j.jcis.2016.12.025.

28. Wang, S.; Su, R.; Nie, S.; Sun, M.; Zhang, J.; Wu, D.; Moustaid-Moussa, N. Application of nanotechnology in improving bioavailability and bioactivity of diet-derived phytochemicals. *The Journal of nutritional biochemistry* **2014**, 25 (4), 363-376.

29. Zhang, L.; Gu, F.; Chan, J.; Wang, A.; Langer, R.; Farokhzad, O. Nanoparticles in medicine: therapeutic applications and developments. *Clinical pharmacology & therapeutics* **2008**, 83 (5), 761-769.

30. Pereira, R. N.; Souza, B. W.; Cerqueira, M. A.; Teixeira, J. A.; Vicente, A. A. Effects of electric fields on protein unfolding and aggregation: influence on edible films formation. *Biomacromolecules* **2010**, 11 (11), 2912-2918.

31. Cerqueira, M.; Bourbon, A.; Pinheiro, A.; Martins, J.; Souza, B.; Teixeira, J.; Vicente, A. Galactomannans use in the development of edible films/coatings for food applications. *Trends*

in Food Science & Technology **2011**, 22 (12), 662-671.

32. Hoque, M. S.; Benjakul, S.; Prodpran, T. Effects of partial hydrolysis and plasticizer content on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. *Food Hydrocolloids* **2011**, 25 (1), 82-90.

33. Ko, S.; Gunasekaran, S. Preparation of sub-100-nm β -lactoglobulin (BLG) nanoparticles. *Journal of microencapsulation* **2006**, 23 (8), 887-898.

34. Chen, W.; Wang, W.; Ma, X.; Lv, R.; Watharkar, R. B.; Ding, T.; Ye, X.; Liu, D. Effect of pH-shifting treatment on structural and functional properties of whey protein isolate and its interaction with (–)-epigallocatechin-3-gallate. *Food chemistry* **2019**, 274, 234-241.

35. Jiang, J.; Wang, Q.; Xiong, Y. L. A pH shift approach to the improvement of interfacial properties of plant seed proteins. *Current Opinion in Food Science* **2018**, 19, 50-56.

36. Jin, X.; Yang, Q.; Zhang, Y. Synergistic apoptotic effects of apigenin TPGS liposomes and tyroservatide: implications for effective treatment of lung cancer. *International journal of nanomedicine* **2017**, 12, 5109.

Appendix

Table 2-1. Zeta-potential of pH 7.0 nanodispersions with 1% w/v and different amount of apigenin after the pH-cycle treatment

Initial apigenin con. (mg/mL)	Zeta-potential (mV)
0 (pH-treated WPI)	-20.43 ± 1.41^a
0.6	-20.10 ± 1.57^a
1.0	-20.50 ± 1.44^a
1.5	-21.53 ± 1.52^a
2.0	-21.60 ± 1.43^a

Numbers are mean $\pm$ standard deviation (n = 3). The same letters indicate no significant difference ($p > 0.05$).

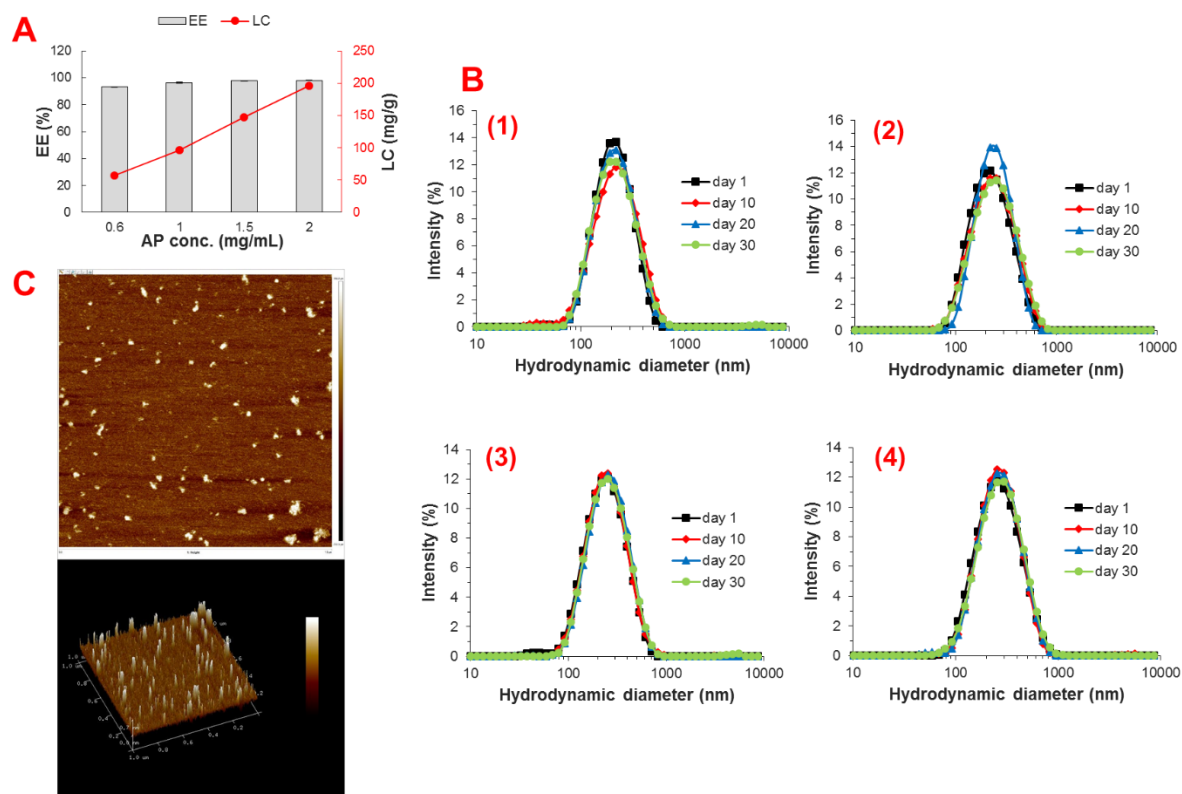


Figure 2-1. (A) Encapsulation efficiency and loading capacity as affected by the amount of apigenin (AP); (B) Particle size distributions of nanodispersions prepared with (1) 0.6, (2) 1.0, (3) 1.5, or (4) 2.0 mg/mL AP during 30-day storage at 21 °C; (C) AFM image of nanodispersion prepared with 2.0 mg/mL AP in height channel (top) and 3-dimensional view (bottom);

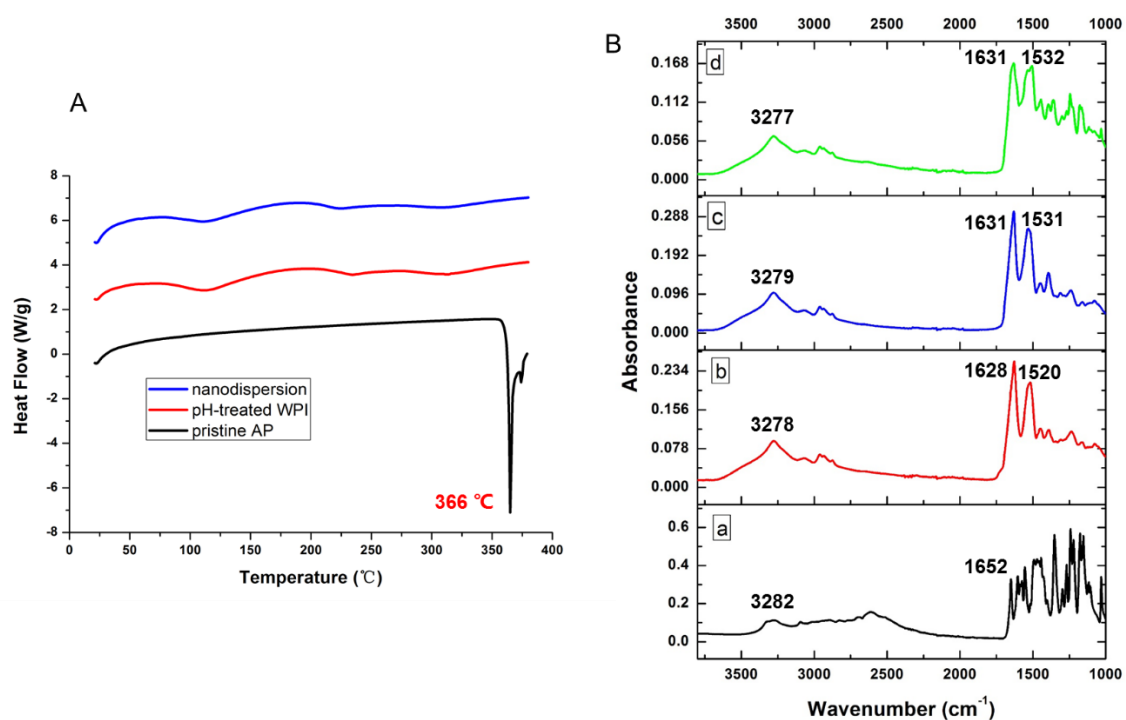


Figure 2-2. (A) DSC thermograms of pristine AP, pH-treated WPI and powder lyophilized from nanodispersion sample with 2.0 mg/mL AP; (B) FTIR spectra of (a) pristine AP, (b) unprocessed WPI, (c) pH-treated WPI and (d) powder freeze-dried from nanodispersion with 2.0 mg/mL AP.

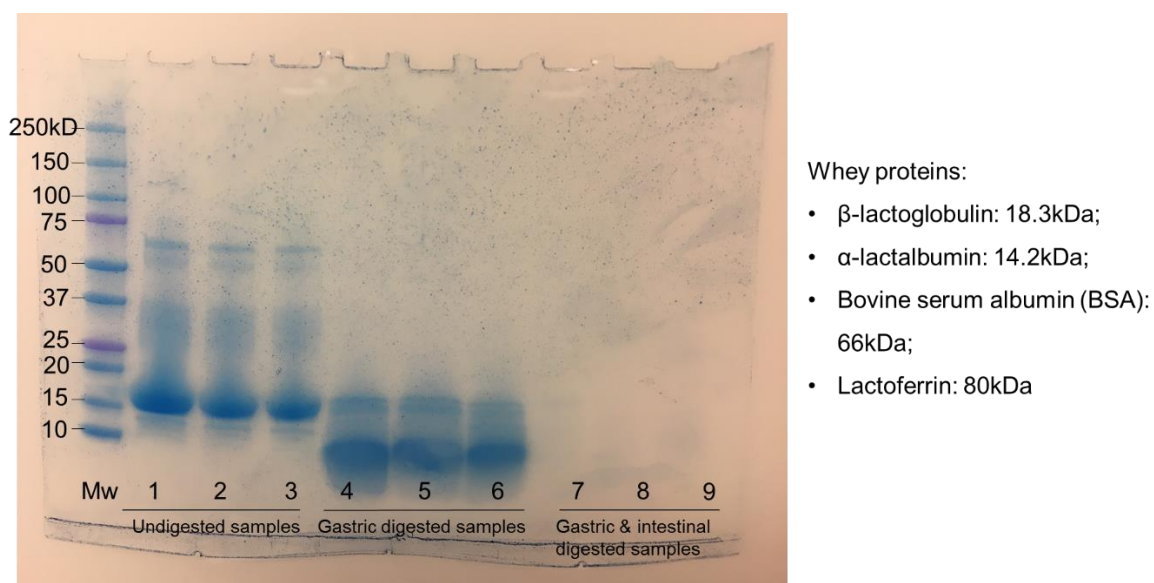


Figure 2-3. Electrophoretic profile of (1) Native WPI, (2) pH-treated WPI, (3) nanodispersion with 2 mg/mL AP; (4) native WPI, (5) pH-treated WPI, and (6) nanodispersion after simulated gastric digestion; (7) native WPI, (8) pH-treated WPI, and (9) nanodispersion after simulated gastric and intestinal digestions. Lane Mw represents the molecular weight marker.

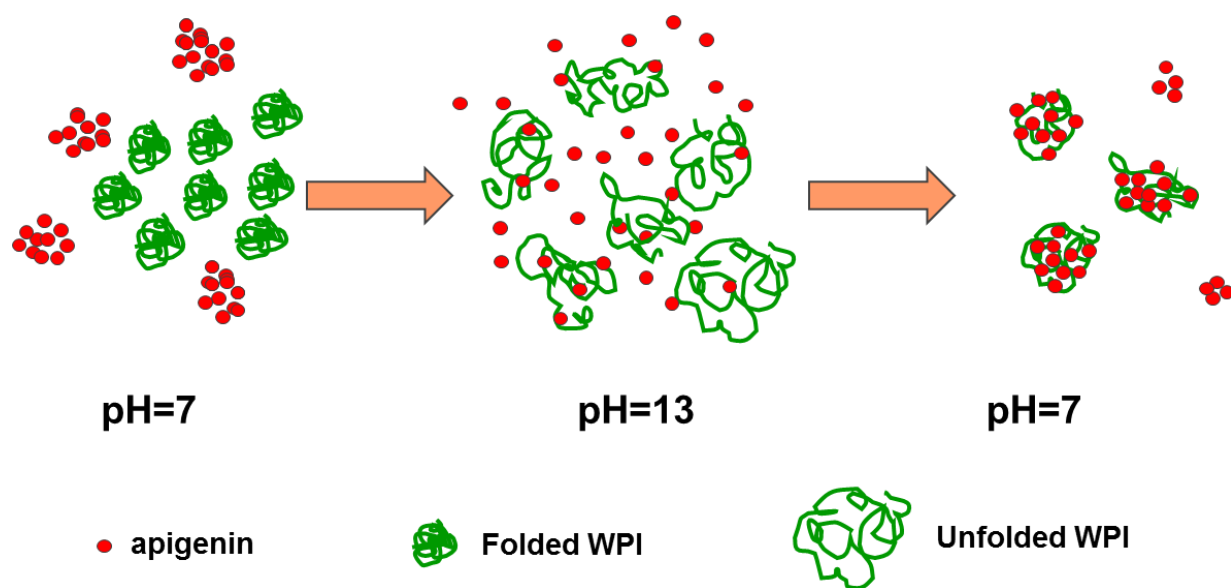


Figure 2-4. Proposed structural changes leading to nanoencapsulation of apigenin in WPI during the pH-cycle.

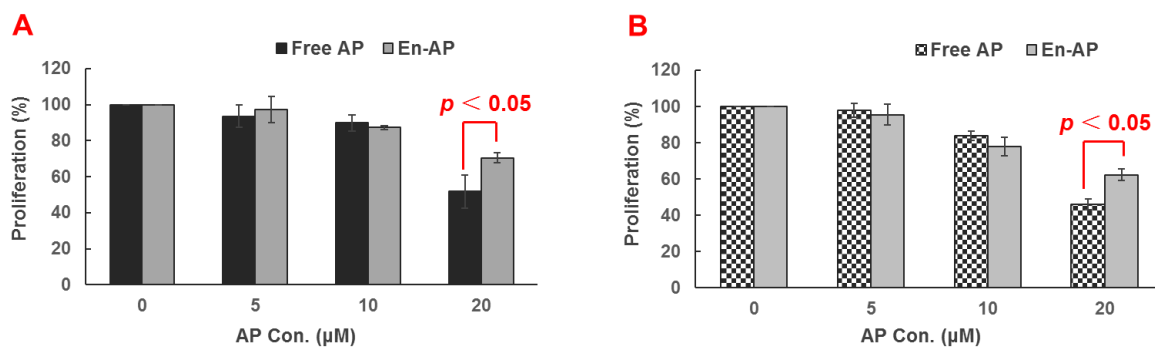


Figure 2-5. Anti-proliferative activity of 0, 5, 10, and 20 μM of free (pre-dissolved in DMSO) apigenin (AP) and nanoencapsulated AP (En-AP) against (A) HCT-116 and (B) HT-29 colon cancer cells after 72-h incubation at 37 °C. Error bars are standard deviations ($n \geq 3$). Treatments labeled with $p < 0.05$ represent a significant difference between each other.

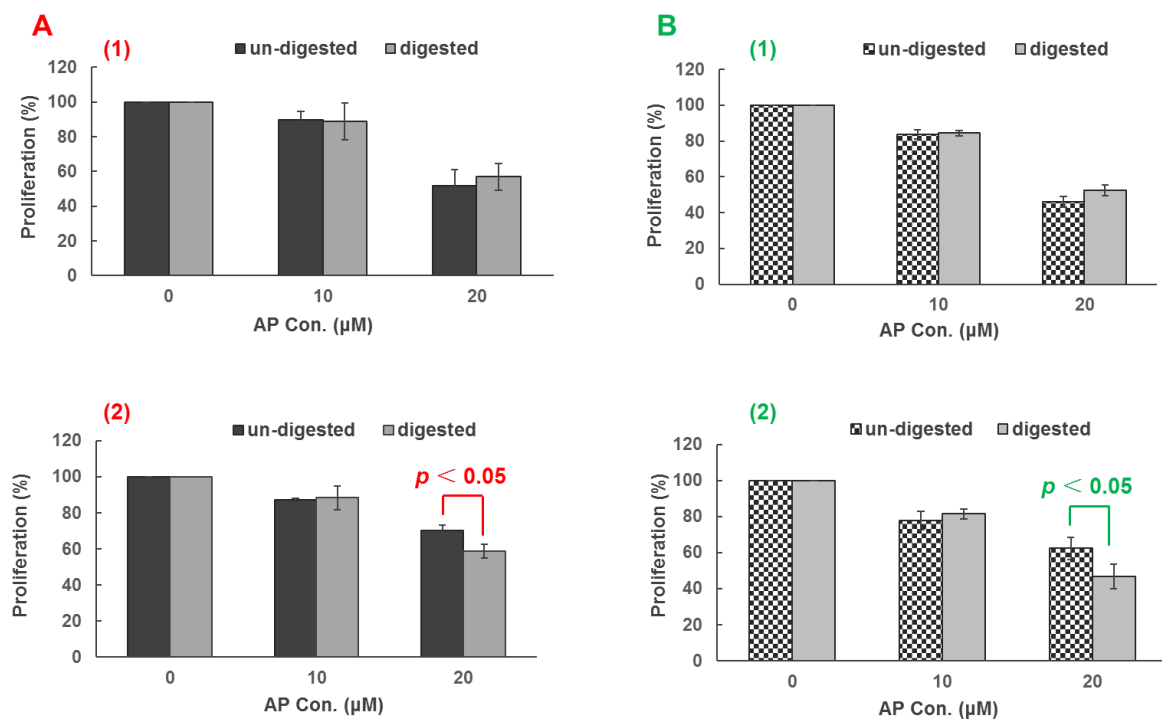


Figure 2-6. Anti-proliferative activity of 0, 10, and 20 μM of (1) free (pre-dissolved in DMSO) apigenin (AP) and (2) nanoencapsulated AP (En-AP) against (A) HCT-116 and (B) HT-29 colon cancer cells before and after *in vitro* digestions after 72-h incubation at 37 °C. Error bars are standard deviations ($n \geq 3$). Treatments labeled with $p < 0.05$ represent a significant difference between each other.

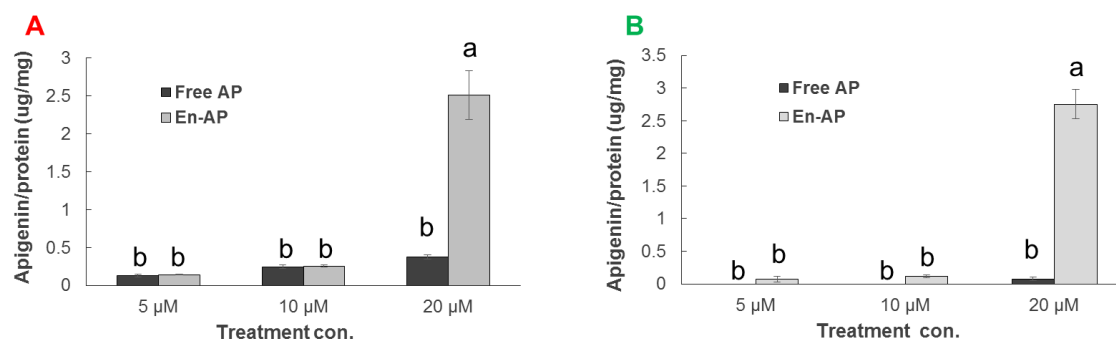


Figure 2-7. Cellular uptake of 5, 10, and 20 μM of free (pre-dissolved in DMSO) apigenin (AP) and nanoencapsulated AP (En-AP) in (A) HCT-116 and (B) HT-29 colon cancer cells after 24-h incubation at 37 °C. Error bars are standard deviations ($n = 3$). Different letters above columns indicate significant differences ($p < 0.05$).

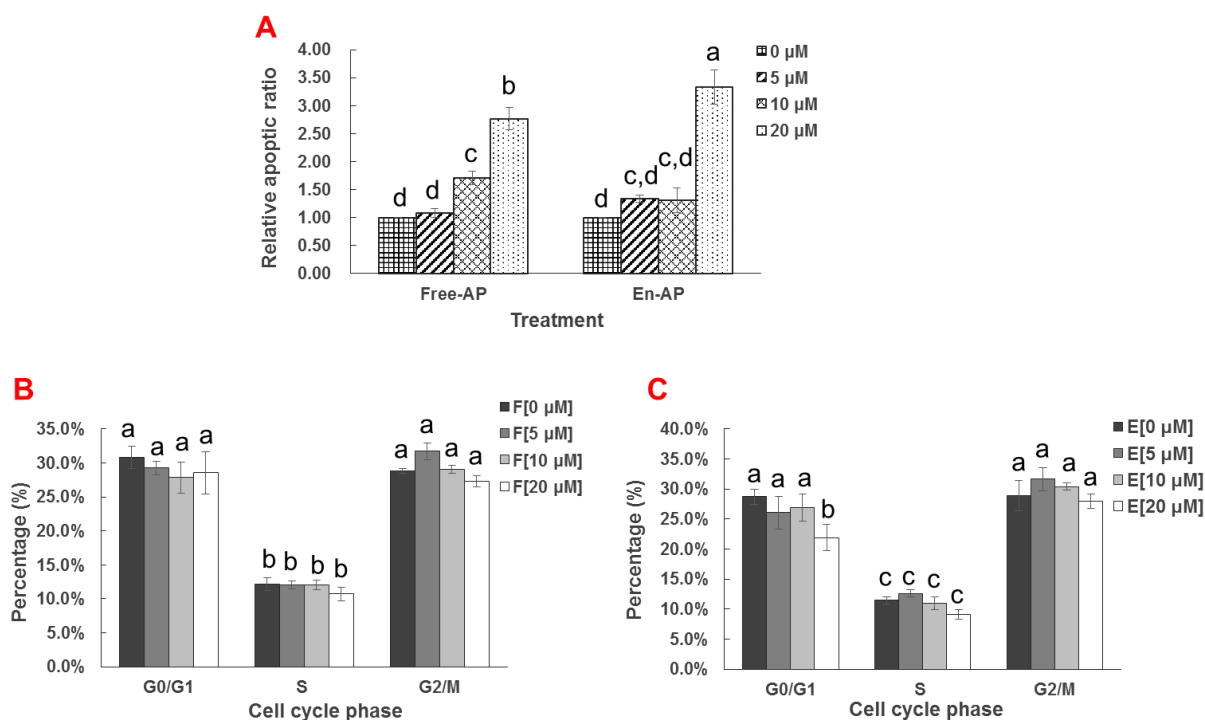


Figure 2-8. Flow cytometry analysis of relative apoptotic ratio (A) and the percentage of (B, C) cell cycle distribution of HCT-116 cell line treated by 0, 5, 10, and 20 μM of free (pre-dissolved in DMSO) apigenin (AP) and nanoencapsulated AP (En-AP) for 24 h at 37 $^{\circ}\text{C}$. Relative apoptotic ratio was calculated with reference to control cells without AP treatment. Error bars are standard deviations ($n = 3$). Different letters above columns indicate significant differences ($p < 0.05$).

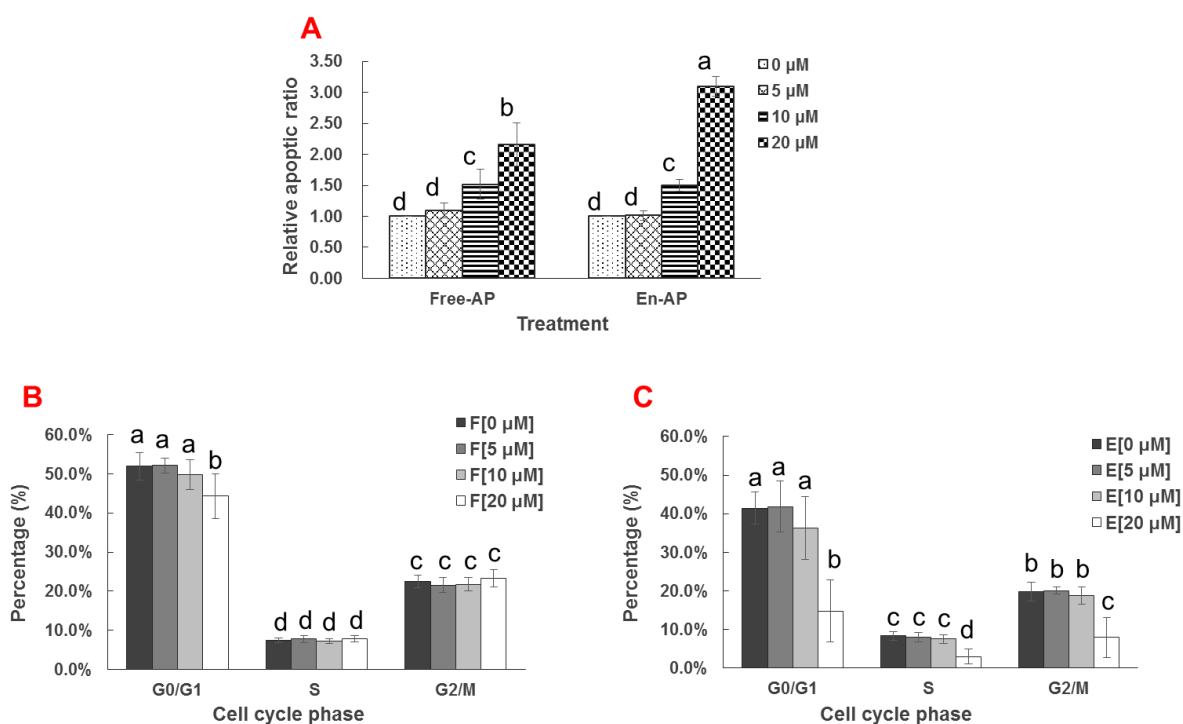


Figure 2-9. Flow cytometry analysis of relative apoptotic ratio (A) and the percentage of (B, C) cell cycle distribution of HT-29 cell line treated by 0, 5, 10, and 20 μ M of free (pre-dissolved in DMSO) apigenin (AP) and nanoencapsulated AP (En-AP) for 24 h at 37 °C. Relative apoptotic ratio was calculated with reference to control cells without AP treatment. Error bars are standard deviations ($n = 3$). Different letters above columns indicate significant differences ($p < 0.05$)

**Chapter 3. Oral bioavailability and *in vivo* anti-colorectal cancer effects
of apigenin nanoencapsulated with whey protein isolate**

3.1 Abstract

Apigenin is a food-sourced lipophilic polyphenol with anti-cancer activities. In chapter 2, stable aqueous dispersions were prepared after mixing apigenin and whey protein isolate (WPI) at alkaline pH followed by titrating to pH 7.0. The present objective was to characterize bioavailability of nanoencapsulated apigenin and *in vivo* anti-colorectal cancer activities. Two groups of C57BL/6J mice were orally administered with 50 mg/kg-mice-body-mass of free or nanoencapsulated apigenin. Blood and colon mucosa were collected at 0, ~1, ~6, and ~24 hr after oral gavage and euthanization of mice. After HPLC analysis, apigenin concentration in serum and colon mucosa of mice treated with nanoencapsulated apigenin was found to be 1.39 and 1.41 times that of free apigenin, respectively, implying the absorption of apigenin was enhanced by the nanodelivery system. Additionally, four groups of 12 Apc^{Min+} mice were each administered with vehicle (0.5% w/v methylcellulose), 50 mg/kg-mice-body-weight free or nanoencapsulated apigenin, and a solution with WPI amount equivalent to the apigenin nanodispersion by oral gavage for 21 times every other day to investigate the anticancer efficacy of apigenin. The entire intestinal tract was removed to evaluate the tumorigenesis and apoptosis after euthanization. However, neither free apigenin nor nanoencapsulated apigenin significantly affected polyps numbers ($p > 0.05$). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay showed no significant difference of apoptosis in mouse intestine within different treatments. Tumors isolated from colonic tissues were analyzed for COX-2, β -catenin, c-myc and cyclin D1, and no significant difference of relative expression was observed between treatments. Effects of apigenin in inflammatory response *in vivo* were

studied by analyzing cytokines (TNF- α , IL-1 β and IL-6) both in the serum and colon mucosa. None of these cytokines was significantly affected by different treatments. The current study understanding the *in vivo* fate of nanoencapsulated apigenin may assist the advancement and formulation of functional foods incorporated with phytochemicals.

Keywords: apigenin, nanoencapsulation, *in vivo*, bioavailability, anticancer activity

3.2 Introduction

Colorectal cancer (CRC) has continued to be one of the most commonly diagnosed cancers in the world. In 2018, over 881,000 deaths were estimated among the total 1.8 million new CRC cases. The alarming mortality has made CRC a big threat to human health as it has been the second most prevalent cause of the cancer-related death in the overall population.¹ While only about 5-10% of CRC cases are hereditary, approximately 90% CRC cases are sporadic, involving genetic mutations that lead to the conversions of normal functional epithelial cells to adenocarcinoma and carcinoma.^{2, 3} In addition to genetic alterations, CRC is highly influenced by poor lifestyle, such as low physical exercise, obesity, cigarette smoking, alcohol abuse, and overconsumption of fat or red meat.^{4, 5} The long-standing chronic inflammatory bowel diseases including ulcerative colitis and Crohn's disease also account for the increasing risk of CRC.^{6, 7} Since the current therapeutic treatments involving a combination of surgery, radiation, and chemotherapy still have not completely controlled the incidence and outcome of CRC and have severe adverse effects, many researchers have turned to dietary factors to seek potential alternative therapeutic interventions.⁸⁻¹⁰

Mounting evidence have suggested an inverse association of a high intake of fruits and vegetables with the reduced risk of developing CRC, on account of the high content of dietary fiber and polyphenols.¹¹ Dietary polyphenols are the most important bioactive phytochemicals, occurring as the secondary metabolites of plants, which help plants to adapt to their environment and protect them against environmental stimulators and stress such as reactive oxygen species, photosynthetic stress and others.^{12, 13} Apigenin (4',5,7-trihydroxyflavone), a

compound found most commonly in parsley, onion, orange, paper mulberry, and chamomile,^{14, 15} has been studied extensively for possible cancer preventive effects.^{8, 16, 17} Potential mechanisms are recognized by targeting diverse molecules and kinase pathways, including promoting cell cycle arrest¹⁸ and apoptosis,^{19, 20} inhibition of mutagenesis,²¹ and suppression of signal transduction.^{22, 23} However, studies regarding *in vivo* inhibitory effects of apigenin against CRC are limited and inconclusive. Free apigenin has shown potential anti-tumorigenic activity by decreasing polyps numbers and tumor load in *Min/+* mice,²⁴ whereas in another study, apigenin failed to inhibit adenoma formation in the same mice model.²⁵

In order to exert therapeutic efficacy, phenolic compounds must be absorbed by human body after ingestion, and have to reach a considerable level that is high enough to exert health beneficial effects in the blood stream or to the target organ site.²⁶ Many lipophilic polyphenols are questionable in applications due to their poor bioavailability that refers to the proportion of an ingested bioactive component that is absorbed into systemic circulation and becomes available at the required site.²⁷ Apigenin is reported as a quite hydrophobic drug with very low water solubility and poor bioavailability.^{15, 28} In Chapter 2, a pH-cycle method was successfully applied to nanoencapsulate apigenin with whey protein isolate (WPI) to improve the dispersibility. The obtained stable aqueous dispersions had promising *in vitro* anticancer activities against human CRC cells, HCT-116 and HT-29. However, to date, the *in vivo* anti-CRC efficacy of nanodelivered apigenin remains unexamined. To this end, the aim of this study was to evaluate the oral bioavailability of nanoencapsulated apigenin and the *in vivo* effects on intestinal tumorigenesis in *Apc^{Min+}* mouse, a model carrying heritable mutant adenomatous

polyposis coli (APC) gene and is frequently employed to study colorectal carcinogenesis in the preclinical selection of chemopreventive agents for further development.^{29, 30}

3.3 Materials and methods

3.3.1 Materials

Apigenin (>98%) was purchased from Indofine Chemical Co. Inc (Hillsborough, NJ, USA). WPI was purchased from Hilmar Ingredients (Hilmar, CA, USA). Primary antibodies (β -catenin 51067-2-AP, cyclin D1 60186-1-Ig, COX-2 66351-1-Ig, c-myc 10828-1-AP and β -actin 66009-1-Ig) were purchased from Proteintech (Rosemont, IL, USA). Secondary antibodies (goat anti-mouse and goat anti-rabbit) were purchased from Li-Cor Biosciences Inc. (Lincoln, NE, USA). ELISA kits (mouse TNF- α , IL-6 and IL-1 β) were purchased from BioLegend (San Diego, CA, USA). All other chemicals were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated.

3.3.2 Preparation of nanoencapsulated apigenin

Nanoencapsulated apigenin was prepared similarly as described in Chapter 2. Briefly, 4% w/v WPI was hydrated in deionized water overnight at 4 °C, followed by mixing with 8 mg/mL apigenin and adjusting pH to 13.0 using 2.0 M KOH solution. The mixture was adjusted to pH 10.0 after stirring for 5 min at 21 °C. The sample was stirred for another 30 min before titration to pH 7.0 with 1.0 M HCl. After centrifugation at 10,000 g for 10 min (Sorvall LYNX 6000 Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA), the supernatant was collected as the nanoencapsulated apigenin.

3.3.3 *In vivo* bioavailability of nanodelivered apigenin

3.3.3.1 *Animals*

All Animal experiments were carried out under the approval of the University of Tennessee Animal Care and Use Committee (Protocol #2369-0615) and were in compliance with the National Institute of Health guidelines for the care and use of laboratory animals.

Thirty C57BL/6J mice (strain for Apc^{Min+} mice) of 7-week old (Jackson Laboratories, Harbor, ME, USA), including both males and females, were randomly housed in standard mouse cages (23 ± 2 °C, 30-70% relative humidity, 12 h light and 12 h dark cycle). Mice were provided with rodent diet and water ad libitum. Mice were randomly separated into two groups after one week acclimatization, and fed with free apigenin that was suspended in 0.5% w/v methylcellulose (vehicle) or nanoencapsulated apigenin in accordance with the dosage of 50 mg/kg apigenin by oral gavage, respectively. After oral administration, mice were euthanized by CO₂ inhalation at 0, ~1, ~6, and ~24 hr, blood was then obtained by cardiac puncture and colon mucosa was collected. Blood samples were centrifuged at 900 g for 15 min (Sorvall Legend Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA), and the serum was separated, collected, and stored at -80 °C until analysis.

3.3.3.2 *Measurement of apigenin from mouse colon mucosa and serum*

Fifty microliters of the serum sample were mixed with 100 µL methanol to extract apigenin. The mixture was vortexed for 3 min at 4 °C and was then ultrasonically treated (Branson 1510 Ultrasonic Cleaner, MO, USA) for 20 min. The supernatant was obtained after centrifugation at 13,800 g for 10 min (Sorvall Legend Micro 21R Microcentrifuge, Thermo

Fisher Scientific, Waltham, MA, USA).

To extract apigenin from mice colon, colon mucosa was rinsed with PBS, mixed with 3 mL clean PBS and homogenized at 8,000 rpm twice for 30s each (Kinematica Polytron PT 10/35 GT Homogenizer, Fisher Scientific, Hampton, NH, USA). After centrifugation for 10 min at 1,900 g (Sorvall Legend Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA), the supernatant was processed under the same procedure as mice serum to extract apigenin. One milliliter of DMSO was added into the remainder mucosa precipitate to extract apigenin from the precipitation by vortexing at the ambient temperature for 1 h and the supernatant was collected after centrifugation for 10 min at 14,000 g (Sorvall Legend Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA). Apigenin distributed in both mice serum and colon mucosa was quantified using HPLC analysis as in Chapter 2.

3.3.4 *In vivo* anticancer activities

3.3.4.1 *Animal studies*

Procedures in animal studies followed the National Institute of Health guidelines and were approved by the University of Tennessee Animal Care and Use Committee (Protocol #2369-0615). Forty-eight Apc^{Min+} mice of 8-week age (Jackson Laboratories, Harbor, ME, USA), including both males and females, were randomly assigned into four treatment groups of 12 mice each with 6 males and 6 females. Mice were administered by oral gavage with vehicle (0.5% w/v methylcellulose), 50 mg/kg of free apigenin suspended in 0.5% w/v methylcellulose, encapsulated apigenin equivalent to 50 mg/kg apigenin, and WPI solution treated with the same pH-cycle at an amount equivalent to the WPI amount in the apigenin nanodispersion for 21

times every other day. At the termination of the study at around ~100 days of age, mice were euthanized by CO₂ inhalation, blood was collected by cardiac puncture, and blood serum was collected after centrifugation (Sorvall Legend Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA) at 900 g for 15 min for cytokine analysis. The entire intestinal tract was excised, flushed with cold PBS, and opened longitudinally. The number, size and location of polyps in the small intestine were recorded from proximal to distal parts ³⁰. The middle parts were swiss rolled and fixed in 4% neutral buffered formalin for tissue apoptosis study. The remainder tissues were frozen immediately in liquid nitrogen and stored at -80 °C for further analysis.

3.3.4.2 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The TUNEL assay was used to study apoptosis in the middle part of small intestine. Tumors embedded in paraffin were first deparaffinized, and the apoptotic tissues were stained by green fluorescence using a DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) based on manufacturer's protocol. For each sample, 10 fields with 10× magnification were chosen randomly, and ImageJ software (NIH, Bethesda, MD, USA) was used to quantify the green fluorescence intensity.

3.3.4.3 Protein extraction and Western blotting analysis

To further characterize the mechanisms involved, lysates of colon tumors and normal colonic tissues from different treatment groups were subjected to western blot to examine the molecular expression of different tumor markers including cyclooxygenase-2 (COX-2), β -catenin, c-myc and cyclin D1 (ProteinTech, Chicago, IL, USA) that related to proliferation and

apoptosis.³¹⁻³⁴ Tumors were isolated from colonic tissues. The total cellular lysates of both neoplastic tumors and normal tissues were prepared according to a method reported previously with some modifications³⁰. Specifically, separated tumors and normal colonic mucosa counterparts were homogenized at 3,400 rpm (HT Mini Microtube Homogenizer, OPS Diagnostics, Lebanon, NJ, USA) for 1 min at 4 °C after addition of RIPA buffer containing 1% v/v protease inhibitor cocktail. After centrifugation (14,000 g, 4 °C, 10 min) twice (Sorvall Legend Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA), the supernatants were combined, a proportion of the sample was used to determine protein concentration using the Bradford assay (Thermo Scientific Inc., Pittsburgh, PA, USA), and the rest of the sample was mixed with an equal volume of Laemmli Buffer with 5% v/v β -mercaptoethanol and boiled at 100 °C for 5 min. Western blotting analysis was conducted according to a previous protocol.³⁵ Equal amounts of protein (25 μ g) were loaded to 4-20% Mini-Protean TGX gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gel electrophoresis was run at 200V for 35 min at ambient temperature (21 °C). After which, the separated proteins were transferred to a PVDF membrane (GE Healthcare Life Sciences, Pittsburgh PA, USA) using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was then blocked with 5% non-fat dry milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at 21 °C, followed by washing with TBST three times for 5min each time. After incubation with primary antibodies against COX-2, β -catenin, and c-myc at dilution of 1:1000 in 1% non-fat dry milk of TBST, cyclin D1 and GAPDH at dilution of 1:5000 of overnight at 4 °C, membranes were washed three time for 10

min with TBST, and incubated for 1.5 h at room temperature with anti-rabbit or anti-mouse secondary antibody at 1:5000 dilution. Following washing with TBST three time for 10 min, expressions of proteins were imaged using a C-Digit blot scanner (Li-Cor Biosciences, Lincoln, NE, USA) with Bio-Rad ClarityTM Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The intensity of bands was quantified using ImageJ software (NIH, Bethesda, MD, USA).

3.3.4.4 Inflammatory cytokines assays

The blood serum as well as colonic extracts were used to measure the expression of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β) using ELISA kits according to manufacturer's instructions (BioLegend, San Diego, CA, USA). To extract cytokines from colon mucosa, 50 mg tissues were homogenized (TissueLyser II, QIAGEN, Hilden, Germany) for 6 min at a frequency of 30/second by mixing with 1 mL extracting reagent (0.05 mL 1 M Tris-HCl, 0.03 mL 5 M NaCl solution, 0.01 mL Tween 20, 0.91 mL deionized water) supplemented with 0.5% v/v protease inhibitor cocktail. After centrifugation for 15 min at 20,000 g, 4 °C twice (Sorvall Legend Micro 21R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA), 5 μ L of the supernatant was used to measure protein concentration based on the Bradford assay, and the rest of the supernatant was analyzed for cytokine levels. Cytokines were expressed as pg of cytokines per milliliter of serum and pg of cytokines per microgram protein in colonic extracts. All standards and samples were measured in two independent replicates.

3.3.5 Statistical analysis

Data were expressed as mean $\pm$ SD ($n \geq 3$). Results were analyzed by one-way ANOVA

with application of the IBM SPSS Statistics version 25.0 software (SPSS Inc., Chicago, IL, USA). Significant difference among results were compared using Tukey's test multiple comparisons, and $p < 0.05$ was considered as significant difference. Graphpad Prism 7 software (GraphPad Software, Inc., La Jolla, USA) was used for statistical analyses for tumorigenesis evaluation.

3.4 Results and discussion

3.4.1 *In vivo* bioavailability of apigenin influenced by nanoencapsulation

The apigenin concentration in mice serum and colon mucosa is shown in Figure 3-1. It was found that the bioavailability of apigenin in mice treated with nanoencapsulated apigenin was always higher than that of mice treated with free apigenin at the same dosage (Figure 3-1A). One hour after gavage, the maximum apigenin concentration in the serum was observed for both encapsulated and free apigenin, which was 28.70 and 19.05 μM , respectively. The oral bioavailability of nanoencapsulated apigenin, determined from the area under curve based on the determined time points, was found to be 1.39 times that of free apigenin. Moreover, the concentration of apigenin in mouse colon mucosa of the nanodispersion treatment was 1.41 folds that of free apigenin (Figure 3-1B). These findings imply that the absorption of apigenin was enhanced by the nanodelivery system.

However, the apigenin concentration rapidly declined in both mice serum and colon mucosa 6 hours after gavage, and became undetectable after 24 hours. As noted in Chapter 2, WPI is hydrolyzed in the gastrointestinal environment, especially in the intestinal tract, which

may account for the quick elimination of the absorbed apigenin, similarly to free apigenin. On the other hand, although some previous studies showed higher enhancement of apigenin bioavailability after encapsulation with poloxamer using liquid antisolvent precipitation technique or loading into mixed micelles made from Soluplus and Pluronic F127 with ethanol thin-film hydration,^{36,37} it is worth to mention that the usage of organic solvent and high amount of surfactant impede the further application of these nanocapsules in food systems.

3.4.2 *In vivo* anticancer studies

3.4.2.1 *Effects of apigenin on intestinal tumorigenesis in Apc^{Min/+} mice*

Apc^{Min/+} mice were oral administrated with free or nanoencapsulated apigenin under the same dosage of 50 mg/kg-body weight, to assess the anti-tumorigenesis activity of apigenin. Both number and size of polyps in different intestinal sections and colon were recorded. As shown in Figure 3-2A, B, Apc^{Min/+} mouse polyps were mostly located in the medial and distal sections of the small intestine, with the primary size ranging from 1-2 mm. Only very few, mostly large, lesions were located in the colon. The total polyps number was further compared within different groups. As shown in Figure 3-2 C, compared with control groups, neither free apigenin nor nanoencapsulated apigenin significantly affected the number of polyps ($p > 0.05$). A previous study also reported that apigenin failed to inhibit adenoma formation in the *Min* mice model, but reduced the aberrant crypt foci formation (ACF) which leads to early preneoplastic lesions and development of adenoma and polyps.²⁵ Therefore, the anti-tumorigenesis activity of apigenin may vary greatly over different stages of CRC development.

3.4.2.2 Effects of apigenin on apoptosis in intestinal tissue

The intestinal tumor development in $Apc^{Min/+}$ mice was evaluated for the level of apoptosis by the TUNEL staining of sections from middle small intestine Swiss rolls. Apoptotic tissues were defined as green fluorescence while the DAPI-stained nuclei were blue (Figure 3-3A). Green intensity from 10 randomly selected fields with each section was calculated to determine the pro-apoptotic effects of apigenin. As shown in Figure 3-3B, the quantified green fluorescence showed a slightly lower intensity in mice tissues after apigenin treatments, however, the differences of apoptosis were not statistically different among four treatment groups.

Expressions of COX-2, β -catenin, c-myc, and cyclin D1 were analyzed to further address the effects of apigenin on intestinal tumorigenesis in molecular level. Figure 3-4A presents higher expressions of all the studied proteins in tumors compared with their normal colonic mucosa counterparts as expected. Since only one mouse in the WPI treatment group developed tumor in colon and was not statistically meaningful, relative expression of each protein was quantified and compared between vehicle, free apigenin and nanoencapsulated apigenin treatment groups. As shown in Figure 3-4B, both free and encapsulated apigenin slightly suppressed the expressions of c-myc and β -catenin in tumors, however, the difference was not significant ($p > 0.05$). On the other hand, apigenin treatments failed to inhibit the expression of cyclin D1, which may suggest an inactive role of apigenin in regulating cell cycle. Nevertheless, relative expressions of all the above tumor markers showed no significant difference between vehicle and apigenin (both free and nanoencapsulated) treatment groups

($p > 0.05$), which is consistent with results obtained in the TUNEL assay (Figure 3-3). More comprehensive studies involving other proteins and signaling pathways are to be conducted to elucidate the potential roles of apigenin on cell proliferation, apoptosis, and tumor progressions.

3.4.2.3 Effects of apigenin on expression of inflammatory cytokines

Chronic inflammation plays a crucial role in regulating immune responses relevant to the initiation or promotion of CRC development.³⁸ Cytokines are essential mediators that contribute to modulating immune and inflammatory responses during infections and tissue injuries; cytokines also promote tumor progression by inhibiting cell apoptosis at the site of inflammation.^{39, 40} Hence, in this study, levels of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6, both in mice serum and colonic extracts were analyzed using ELISA to determine the effects of apigenin in inflammatory response *in vivo*. As presented in Figure 3-5B, none of the detected cytokines was significantly affected by apigenin (both free and encapsulated) treatments in colonic extracts and serum with respect to vehicle group ($p > 0.05$).

In Chapter 2, nanoencapsulation enhanced cellular uptake of apigenin and the anti-proliferative activity against human colon cancer cells was also enhanced after simulated digestions. Nanoencapsulation enhanced the oral bioavailability of apigenin in mice serum (Figure 3-1A), leading to possibly a smaller amount in the colon. Although apigenin concentration in the colon mucosa was also enhanced after nanoencapsulation (Figure 3-1B), this concentration may not be enough to inhibit tumor growth.

Many studies reported contrary results of dietary polyphenols *in vitro* and *in vivo*.^{41, 42}

These disagreements may result from many factors. One reason for this discrepancy is that concentrations used in most *in vitro* and *in vivo* studies are different, which makes it difficult to link the physiological relevance with *in vitro* studies.⁴³ Although the concentration of absorbed apigenin in mice serum (Figure 3-1A) in the current study is relevant to that in the cell culture studies in Chapter 2, the relevance of apigenin concentration in colon mucosa (Figure 3-1B) remained unknown. In addition, the rapid elimination and metabolism of apigenin (Figure 3-1) may be responsible for the ineffectiveness of apigenin *in vivo*. Besides, once polyphenols are absorbed into the systemic circulation, they are capable of binding with soluble proteins in the blood and tissues. As the “free drug” hypothesis suggests, only compounds unbound to plasma proteins exert biological activity.⁴⁴ Apigenin has been reported to bind strongly to human serum albumin,⁴⁵ which on the other hand, may explain the non-significant anti-cancer activity *in vivo* although the absorbed concentration in the serum has reached to the cell culture dosage. Moreover, despite that apigenin was easily to access colon, the accumulated apigenin in colon mucosa may act on the whole inner wall of colon, instead of targeting and specifically interacting with colonic polyps and tumors, which may largely attenuate the biological activities of apigenin.

Additionally, it cannot be discarded that derivatives of bioactive compounds may be formed during experimental incubation times *in vitro*, which might not be taking place *in vivo* or differ from derived metabolites that are produced by gut microbiota *in vivo*, and the activity of these derived products remains unknown. Apart from these, although *Min* mice are frequently used to study colon carcinogenesis, the small intestine is the place of most adenomas

in the *Min* mice, with none or only a few adenomas found in the colon; thus the adenomas in the *Min* mouse study are developed in a microenvironment different from that of the colon,^{25, 46} which makes it impossible for apigenin that accumulated in colon mucosa to affect the adenomas in the small intestine.

Lastly, the different location of the APC mutation in the *Min* mice (chromosome 18) and that in human colon cancer cell lines (for example, chromosome 5q21 for HT-29) may be responsible for the discrepancy between the human colon cancer study and the *Min* mice experiments.⁴⁶ Also, the complicated environment *in vivo* including gut microbiota and other neighbor cells plays possible roles on the anticancer activities of compounds. Therefore, under the proposed study conditions, there was insufficient data to make a conclusion on the *in vivo* fate of nanoencapsulated apigenin. Further studies involving different dosages, other mouse models that better simulate human colon tumors as well as different cancer types are needed to better assess the potential *in vivo* efficacy of the studied nanodelivery system of apigenin.

3.5 Conclusions

Nanoencapsulation of apigenin with whey protein isolate enhanced the oral bioavailability of apigenin and absorption in colon mucosa. However, neither the free apigenin nor the encapsulated apigenin positively suppressed colon carcinogenesis *in vivo* in the current animal model, as analyzed based on polyps numbers, tissue apoptosis, tumor marker expressions, and inflammatory cytokines. The potential *in vivo* effectiveness of apigenin on cancer development must be further studied.

References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R. L.; Torre, L. A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians* **2018**, 68 (6), 394-424.
2. Lucas, C.; Barnich, N.; Nguyen, H. T. T. Microbiota, inflammation and colorectal cancer. *International journal of molecular sciences* **2017**, 18 (6), 1310.
3. Ginwala, R.; Bhavsar, R.; Chigbu, D. G. I.; Jain, P.; Khan, Z. K. Potential role of flavonoids in treating chronic inflammatory diseases with a special focus on the anti-inflammatory activity of apigenin. *Antioxidants* **2019**, 8 (2), 35.
4. Lucas, C.; Barnich, N.; Nguyen, H. Microbiota, inflammation and colorectal cancer. *International journal of molecular sciences* **2017**, 18 (6), 1310.
5. Martin, S. L.; Royston, K. J.; Tollefsbol, T. O. The Role of Non - Coding RNAs and Isothiocyanates in Cancer. *Molecular nutrition & food research* **2018**, 62 (18), 1700913.
6. Núñez - Sánchez, M. A.; González - Sarrías, A.; Romo - Vaquero, M.; García - Villalba, R.; Selma, M. V.; Tomás - Barberán, F. A.; García - Conesa, M. T.; Espín, J. C. Dietary phenolics against colorectal cancer—From promising preclinical results to poor translation into clinical trials: Pitfalls and future needs. *Molecular nutrition & food research* **2015**, 59 (7), 1274-1291.
7. Brenner, H.; Kloor, M. Pox cP. colorectal cancer. *Lancet* **2014**, 383 (9927), 1490-1502.
8. Madunić, J.; Madunić, I. V.; Gajski, G.; Popić, J.; Garaj-Vrhovac, V. Apigenin: A dietary flavonoid with diverse anticancer properties. *Cancer Letters* **2018**, 413, 11-22.
9. Dia, V. P.; de Mejia, E. G. Lunasin induces apoptosis and modifies the expression of genes associated with extracellular matrix and cell adhesion in human metastatic colon cancer cells. *Molecular nutrition & food research* **2011**, 55 (4), 623-634.
10. Davatgaran-Taghipour, Y.; Masoomzadeh, S.; Farzaei, M. H.; Bahramsoltani, R.; Karimi-Soureh, Z.; Rahimi, R.; Abdollahi, M. Polyphenol nanoformulations for cancer therapy: Experimental evidence and clinical perspective. *International Journal of Nanomedicine* **2017**, 12, 2689--2702 DOI: 10.2147/IJN.S131973.

11. Moskal, A.; Freisling, H.; Byrnes, G.; Assi, N.; Fahey, M. T.; Jenab, M.; Ferrari, P.; Tjønneland, A.; Petersen, K. E.; Dahm, C. C. Main nutrient patterns and colorectal cancer risk in the European Prospective Investigation into Cancer and Nutrition study. *British journal of cancer* **2016**, 115 (11), 1430.
12. Lambert, J. D.; Hong, J.; Yang, G.-y.; Liao, J.; Yang, C. S. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. *The American journal of clinical nutrition* **2005**, 81 (1), 284S-291S.
13. Rasouli, H.; Farzaei, M. H.; Khodarahmi, R. Polyphenols and their benefits: A review. *International Journal of Food Properties* **2017**, 20 (sup2), 1700-1741.
14. Liu, R.; Zhang, H.; Yuan, M.; Zhou, J.; Tu, Q.; Liu, J.-J.; Wang, J. Synthesis and biological evaluation of apigenin derivatives as antibacterial and antiproliferative agents. *Molecules* **2013**, 18 (9), 11496-11511.
15. Pápay, Z. E.; Balogh, E.; Zariwala, M. G.; Somavarapu, S.; Antal, I., Drug delivery approaches for apigenin: A review. Nova: 2015.
16. Lefort, É. C.; Blay, J. Apigenin and its impact on gastrointestinal cancers. *Molecular nutrition & food research* **2013**, 57 (1), 126-144.
17. Yan, X.; Qi, M.; Li, P.; Zhan, Y.; Shao, H. Apigenin in Cancer Therapy: Anti-cancer Effects and Mechanisms of Action. *Cell & bioscience* **2017**, 7 (1), 50.
18. Gupta, S.; Afaq, F.; Mukhtar, H. Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. *Oncogene* **2002**, 21 (23), 3727.
19. Brusselmans, K.; Vrolix, R.; Verhoeven, G.; Swinnen, J. V. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. *Journal of Biological Chemistry* **2005**, 280 (7), 5636--5645.
20. Lee, Y.; Sung, B.; Kang, Y. J.; Kim, D. H.; Jang, J.-Y.; Hwang, S. Y.; Kim, M.; Lim, H. S.; Yoon, J.-H.; Chung, H. Y. Apigenin-induced apoptosis is enhanced by inhibition of autophagy formation in HCT116 human colon cancer cells. *International journal of oncology* **2014**, 44 (5), 1599-1606.
21. Kuo, M.-L.; Lee, K.-C.; Lin, J.-K. Genotoxicities of nitropyrenes and their modulation by

- apigenin, tannic acid, ellagic acid and indole-3-carbinol in the Salmonella and CHO systems. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1992**, 270 (2), 87-95.
22. Van Dross, R. T.; Hong, X.; Pelling, J. C. Inhibition of TPA - induced cyclooxygenase - 2 (COX - 2) expression by apigenin through downregulation of Akt signal transduction in human keratinocytes. *Molecular Carcinogenesis: Published in cooperation with the University of Texas MD Anderson Cancer Center* **2005**, 44 (2), 83-91.
23. Shukla, S.; Gupta, S. Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft. *Molecular cancer therapeutics* **2006**, 5 (4), 843-852.
24. Zhong, Y.; Krisanapun, C.; Lee, S.-H.; Nualsanit, T.; Sams, C.; Peungvicha, P.; Baek, S. J. Molecular targets of apigenin in colorectal cancer cells: involvement of p21, NAG-1 and p53. *European Journal of Cancer* **2010**, 46 (18), 3365-3374.
25. Au, A.; Li, B.; Wang, W.; Roy, H.; Koehler, K.; Birt, D. Effect of dietary apigenin on colonic ornithine decarboxylase activity, aberrant crypt foci formation, and tumorigenesis in different experimental models. *Nutrition and cancer* **2006**, 54 (2), 243-251.
26. Jafari, S. M.; McClements, D. J., Nanotechnology approaches for increasing nutrient bioavailability. In *Advances in food and nutrition research*, Elsevier: 2017; Vol. 81, pp 1-30.
27. Pathak, K.; Raghuvanshi, S. Oral Bioavailability: Issues and Solutions via Nanoformulations. *Clinical Pharmacokinetics* **2015**, 54 (4), 325--357 DOI: 10.1007/s40262-015-0242-x.
28. Patel, D.; Shukla, S.; Gupta, S. Apigenin and cancer chemoprevention: progress, potential and promise. *International journal of oncology* **2007**, 30 (1), 233-245.
29. Luongo, C.; Moser, A. R.; Gledhill, S.; Dove, W. F. Loss of Apc⁺ in intestinal adenomas from Min mice. *Cancer Res.* **1994**, 54 (22), 5947-5952.
30. Jin, D.; Liu, T.; Dong, W.; Zhang, Y.; Wang, S.; Xie, R.; Wang, B.; Cao, H. Dietary feeding of freeze-dried whole cranberry inhibits intestinal tumor development in Apcmin/+ mice. *Oncotarget* **2017**, 8 (58), 97787.
31. Ziegler, C. C.; Rainwater, L.; Whelan, J.; McEntee, M. F. Dietary resveratrol does not affect intestinal tumorigenesis in Apc Min/+ mice. *The Journal of nutrition* **2004**, 134 (1), 5-

10.

32. Villegas, I.; Sánchez - Fidalgo, S.; de la Lastra, C. A. Chemopreventive effect of dietary curcumin on inflammation - induced colorectal carcinogenesis in mice. *Molecular nutrition & food research* **2011**, 55 (2), 259-267.

33. Tang, F. Y.; Pai, M. H.; Kuo, Y. H.; Wang, X. D. Concomitant consumption of lycopene and fish oil inhibits tumor growth and progression in a mouse xenograft model of colon cancer. *Molecular nutrition & food research* **2012**, 56 (10), 1520-1531.

34. Tang, F. Y.; Shih, C. J.; Cheng, L. H.; Ho, H. J.; Chen, H. J. Lycopene inhibits growth of human colon cancer cells via suppression of the Akt signaling pathway. *Molecular nutrition & food research* **2008**, 52 (6), 646-654.

35. Dia, V. P.; Krishnan, H. B. BG-4, a novel anticancer peptide from bitter gourd (*Momordica charantia*), promotes apoptosis in human colon cancer cells. *Scientific reports* **2016**, 6, 33532.

36. Wu, W.; Zu, Y.; Wang, L.; Wang, L.; Wang, H.; Li, Y.; Wu, M.; Zhao, X.; Fu, Y. Preparation, characterization and antitumor activity evaluation of apigenin nanoparticles by the liquid antisolvent precipitation technique. *Drug delivery* **2017**, 24 (1), 1713-1720.

37. Zhang, Z.; Cui, C.; Wei, F.; Lv, H. Improved solubility and oral bioavailability of apigenin via Soluplus/Pluronic F127 binary mixed micelles system. *Drug development and industrial pharmacy* **2017**, 43 (8), 1276-1282.

38. Hung, R. J.; Ulrich, C. M.; Goode, E. L.; Brhane, Y.; Muir, K.; Chan, A. T.; Marchand, L. L.; Schildkraut, J.; Witte, J. S.; Eeles, R. Cross cancer genomic investigation of inflammation pathway for five common cancers: lung, ovary, prostate, breast, and colorectal cancer. *Journal of the National Cancer Institute* **2015**, 107 (11), djv246.

39. Landskron, G.; De la Fuente, M.; Thuwajit, P.; Thuwajit, C.; Hermoso, M. A. Chronic inflammation and cytokines in the tumor microenvironment. *Journal of immunology research* **2014**, 2014.

40. Rajput, S.; Wilber, A. Roles of inflammation in cancer initiation, progression, and metastasis. *Front Biosci (Schol Ed)* **2010**, 2, 176-183.

41. Karmokar, A.; Marczyklo, T. H.; Cai, H.; Steward, W. P.; Gescher, A. J.; Brown, K. Dietary intake of rosmarinic acid by ApcMin mice, a model of colorectal carcinogenesis: levels of

parent agent in the target tissue and effect on adenoma development. *Molecular nutrition & food research* **2012**, 56 (5), 775-783.

42. Sale, S.; Tunstall, R. G.; Ruparelia, K. C.; Potter, G. A.; Steward, W. P.; Gescher, A. J. Comparison of the effects of the chemopreventive agent resveratrol and its synthetic analog trans 3, 4, 5, 4' - tetramethoxystilbene (DMU - 212) on adenoma development in the ApcMin⁺ mouse and cyclooxygenase - 2 in human - derived colon cancer cells. *International journal of cancer* **2005**, 115 (2), 194-201.

43. Klein, C. B.; King, A. A. Genistein genotoxicity: critical considerations of in vitro exposure dose. *Toxicology and applied pharmacology* **2007**, 224 (1), 1-11.

44. Xiao, J.; Kai, G. A review of dietary polyphenol-plasma protein interactions: characterization, influence on the bioactivity, and structure-affinity relationship. *Critical reviews in food science and nutrition* **2012**, 52 (1), 85-101.

45. Yokoyama, Y.; Wan, X.; Shinohara, A.; Takahashi, S.; Takahashi, Y.; Niwa, K.; Tamaya, T. Expression of PTEN and PTEN pseudogene in endometrial carcinoma. *International journal of molecular medicine* **2000**, 6 (1), 47-97.

46. Chung, C.; Jiang, Y.; Cheng, D.; Birt, D. Impact of adenomatous polyposis coli (APC) tumor supressor gene in human colon cancer cell lines on cell cycle arrest by apigenin. *Molecular Carcinogenesis: Published in cooperation with the University of Texas MD Anderson Cancer Center* **2007**, 46 (9), 773-782.

Appendix

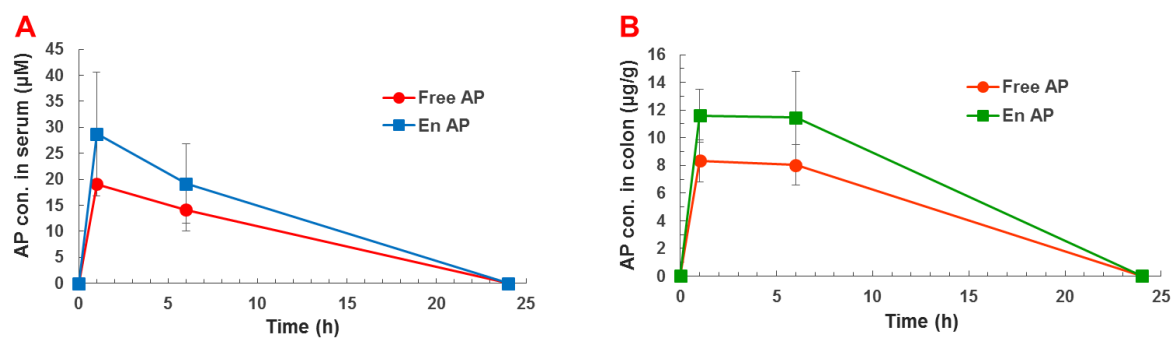


Figure 3-1. Apigenin concentration of mice after oral gavage with 50 mg/kg-body weight of free apigenin (AP) and encapsulated AP (En-AP) in the serum (A) and colon mucosa (B). Error bars are standard deviations ($n \geq 3$).

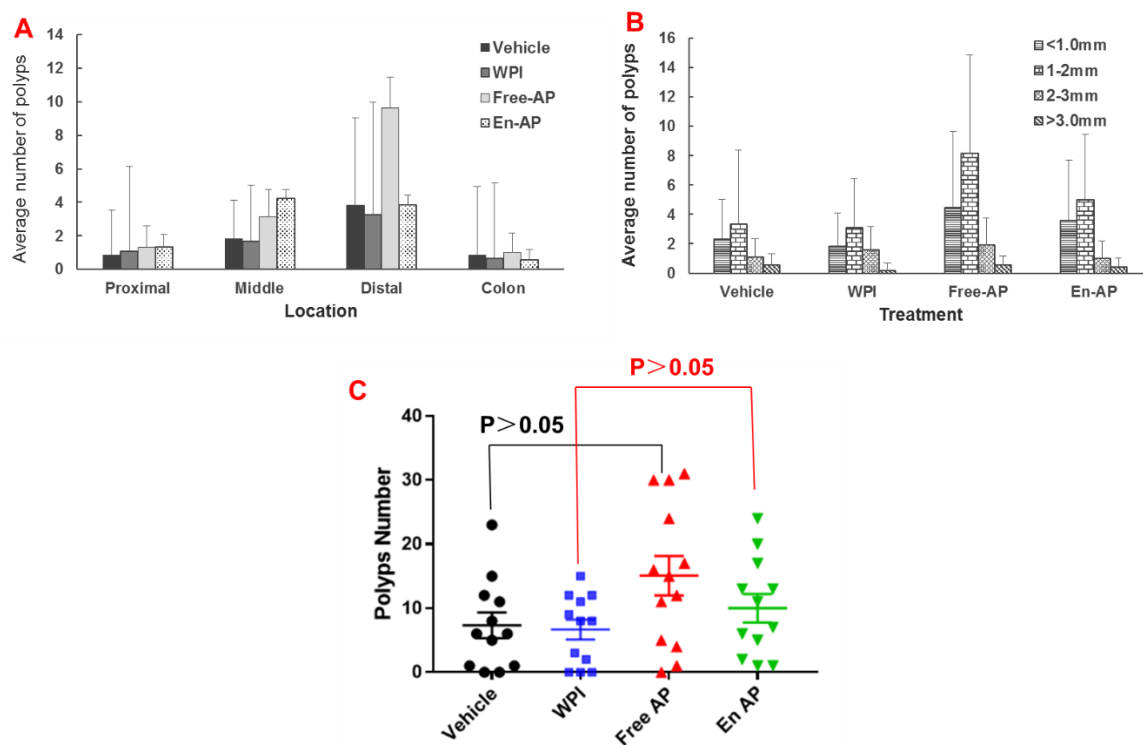


Figure 3-2. Tumorigenesis evaluation of mice treated with vehicle (0.5% w/v methylcellulose), pH-treated WPI, 50 mg/kg-mice-body-mass free apigenin (AP) or nanoencapsulated AP (En-AP): Location (A), size distribution (B) and total number (C) of polyps in the small intestine of mice.

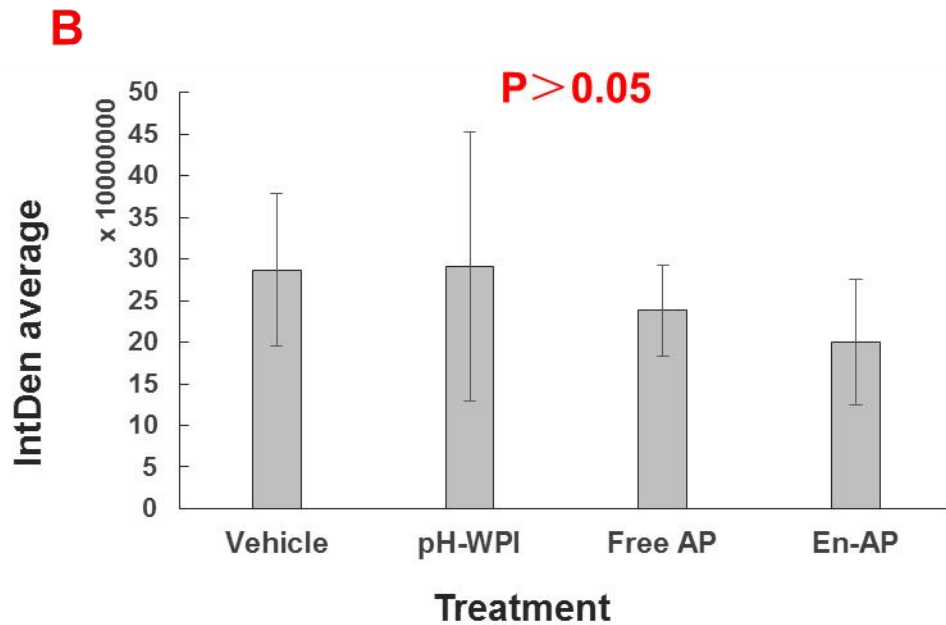
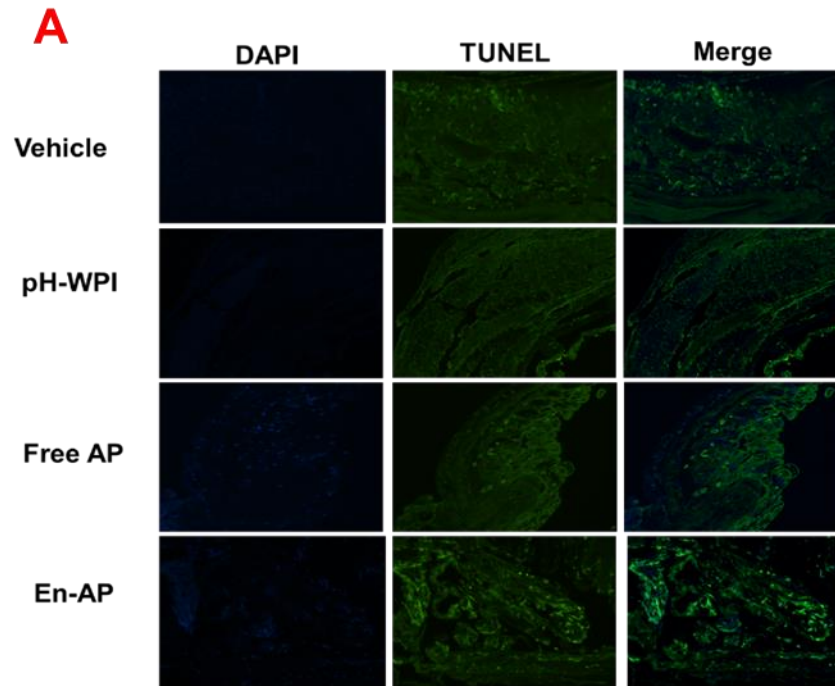


Figure 3-3. TUNEL assay of apoptotic cells in colon mucosa within different treatment groups: (A) Green staining represented apoptotic cells (10 $\times$ magnifications); (B) Green intensity calculated from 10 randomly selected fields with each section. Error bars represent standard deviations.

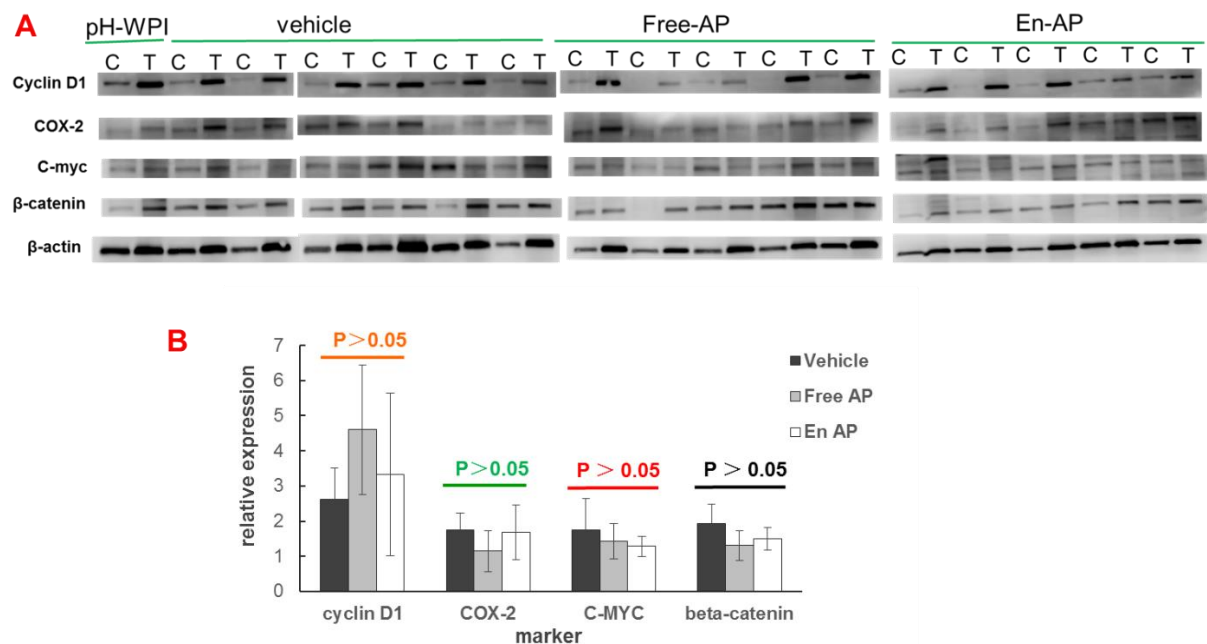


Figure 3-4. Western blot of tumor markers (COX-2, β-catenin, c-myc, cyclin D1). “T” and “C” represent tumor and normal colonic tissue, respectively, in (A). Relative expression is calculated based on standard marker of β-actin (B). Error bars are standard deviations ($n \geq 3$).

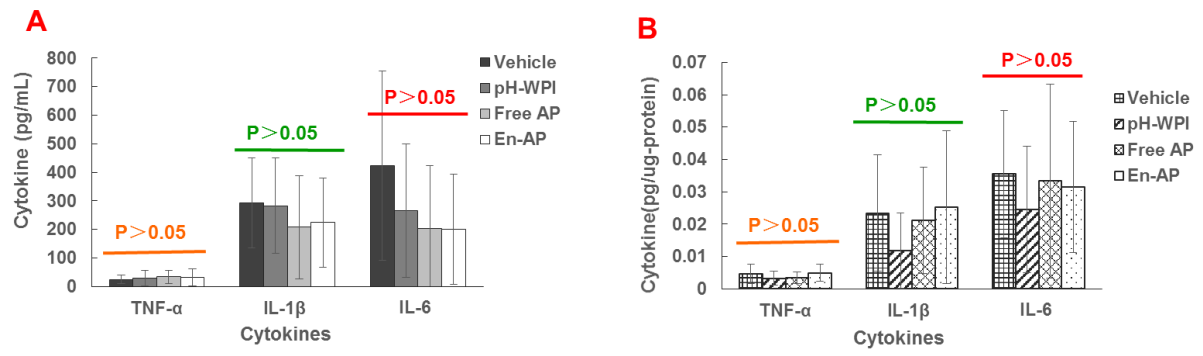


Figure 3-5. Effects of apigenin over inflammatory response *in vivo* as analyzed for cytokines (TNF- α , IL-1 β , IL-6) in mice serum (A) and colon mucosa (B). Error bars are standard deviations (n $\geq$ 3).

Chapter 4. Synergistic anti-inflammatory activity of free and co-encapsulated apigenin and curcumin in lipopolysaccharide-stimulated RAW 264.7 macrophages

4.1 Abstract

Inflammation in the chronic state is involved in initiation and promotion of many diseases including cancers. Dietary polyphenols have been widely accepted as potent anti-inflammatory agents. Combinations of two or more polyphenols potentially achieve better biological benefits with lower toxicity and side effects. The objective of this work was to investigate the potential synergistic anti-inflammatory activities of two important dietary compounds, apigenin and curcumin, in physical combinations, and the enhanced synergism by co-encapsulation. Non-toxic concentrations of apigenin, curcumin, and their physical mixtures as well as their coencapsulated dispersions were studied in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. For physical mixtures of unencapsulated polyphenols, a higher mass proportion of curcumin produced stronger inhibitory effects on nitric oxide (NO) production, and the synergistic interactions between apigenin and curcumin were enhanced by co-encapsulation based on the calculated combination index (CI). Western blot analysis revealed that the synergistic inhibition of NO was at least partially concomitant with a reduction in the protein expression of LPS-induced nitric oxide synthase (iNOS). To better understand the synergistic anti-inflammatory activities of apigenin and curcumin as well as their combinations, pro-inflammatory cytokines of IL-6 and TNF- α were further measured by ELISA. Since oxidative stress caused by the overproduction of intracellular reactive oxygen species (ROS) has been implicated in chronic inflammation and many diseases, ROS production was also analyzed by fluorescence microscopy and spectrophotometry. Similar synergistic activities of physical mixtures and coencapsulated dispersions were also found in mediating the production of

cytokines and ROS. Overall, these results demonstrated that apigenin and curcumin in specific combinations exerted synergistic effects in inhibiting LPS-induced inflammation in RAW 264.7 cells, and the co-encapsulation enhanced the synergistic activity.

Keywords: apigenin, curcumin, co-encapsulation, synergistic interaction, anti-inflammatory activity

4.2 Introduction

Inflammation is the defensive response of immune system to traumatic injuries or pathogenic infections, which cause disruptions of tissue homeostasis.¹ It is activated via the identification of exogenous pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by pathogen-recognition receptors (PRRs).² Acute inflammation is beneficial; however, the long-term chronic inflammation is always involved in progression of several chronic diseases, including cancers, diabetes, obesity and neurodegenerative disorders.^{3, 4} Chronic inflammation has been proposed to be oncogenic by inducing genomic instability, promoting cell growth, inhibiting cell apoptosis, and also stimulating angiogenesis.⁵ Therefore, controlling inflammation is an important strategy to prevent and treat cancers.

Inflammatory process is highly complicated, which is orchestrated by various immune cells through induction of a complex set of inflammatory mediators, such as cytokines, chemokines, pro-inflammatory transcription factors and pro-inflammatory enzymes.^{6, 7} Macrophages, as one of the primary immune cells, perform a critical role in initiating and developing inflammatory responses. Upon the stimulation by lipopolysaccharide (LPS) which is a major component of the outer membrane of Gram-negative bacteria,⁸ macrophages are activated and undergo a sequential release of diverse inflammatory factors including NO, IL-6 and TNF- α .⁴ Nitric oxide (NO), as a representative pro-inflammatory mediator, is regulated by inducible nitric oxide synthase (iNOS). Pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), on the other hand, can induce the expression of iNOS,

which in turn to increase the production of NO.⁹ Increasing evidences have demonstrated that the aberrant iNOS expression is closely associated with several malignant tumors and the excessive NO can cause DNA damage, mutagenesis and lead to cancer progression.^{10, 11} Likewise, cyclooxygenase-2 (COX-2) is another pro-inflammatory enzyme that is able to convert arachidonic acid into prostaglandins. Aberrant induction of COX-2 has been involved in several types of cancers, by upregulating generation of prostaglandins, which promote cell survival, angiogenesis, and metastasis.^{12, 13} In addition, oxidative stress occurs when the body's antioxidant defense system loses its capacity to response and neutralize the over generated reactive oxygen species (ROS), which are highly active radicals able to damage vital cellular components.¹⁴ Thus, suppression of these inflammatory molecules could be a potential strategy for alleviating chronic inflammation and preventing the downstream chronic diseases.

Recently, numerous studies have reported the anti-inflammatory roles of dietary polyphenols.^{15, 16} Dietary polyphenols are the most abundant antioxidants in our diets. The antioxidant activity of polyphenols results from their ability donating an electron or hydrogen atom to a wide range of reactive oxygen to neutralize free radicals, thereby attenuating oxidative stress caused inflammation and chronic disorders.¹⁷ Apigenin (4',5,7-trihydroxyflavone, AP) (Figure 1-1), as one of the most promising polyphenols, has been well documented as a potent anti-inflammatory agent. It was reported that apigenin significantly decreased various cytokines (IL-1 β , TNF- α , IL-6, and IL-12) in LPS-induced human periodontal ligament cells.¹⁸ Besides, *in vivo* studies reported the inhibitory action of apigenin on NO and prostaglandin E2 (PGE2) production, by inhibiting the expressions of iNOS and

COX-2, respectively.¹⁹ Curcumin (CUR, Figure 4-1), a constituent of turmeric ginger family, is another widely studied polyphenol due to its potential anti-inflammatory effects.²⁰ Pretreatment with CUR was able to suppress TNF- α induced COX-2 expression, and inhibit NF- κ B activation in human colonic epithelial cells.²¹ In human myeloid leukemia cells, CUR was found to inhibit the TNF- α induced nuclear translocation and DNA binding of NF- κ B, through suppression of I κ B α phosphorylation and subsequent degradation.²²

Single agents may not be able to inhibit the complex and multifactorial inflammation process. On the other hand, a growing body of evidence suggests that bioactive compounds can have different molecular targets, which may produce synergistic activities involving antioxidation, anti-inflammation, and anti-cancer.²³ Besides, polyphenols derived from fruits and vegetables are not under all circumstances beneficial, but in fact may cause unacceptable toxicity in a high-dose administration.⁷ Hence, anti-inflammatory agents with synergistic interactions may enhance anti-inflammatory efficacy, decrease the dosage of individual anti-inflammatory agents to achieve efficacy, and reduce toxicity or side effects.²³ With lower toxicity and non-mutagenesis, AP is a promising candidate to achieve significant synergism and simultaneously reduce the potential toxic high dosage of other agent when combined with other polyphenols.²⁴ In this sense, for the first time, the current study investigated the potential synergy of physical mixtures of AP and CUR as well as their coencapsulated dispersions of inhibiting pro-inflammatory responses in LPS-stimulated RAW 264.7 macrophage, with the hypothesis that co-encapsulation may enhance the synergistic effects.

4.3 Materials and methods

4.3.1 Materials

Apigenin (>98%) was purchased from Indofine Chemical Co. Inc (Hillsborough, NJ, USA). Curcumin (95% total curcuminoid content) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Casein sodium salt, Lipopolysaccharide (LPS; *Escherichia coli* O55:B5), and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Growth media DMEM 1X was purchased from Corning Inc. (Corning, NY, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA, USA). ELISA kits (mouse TNF- α and IL-6) were purchased from BioLegend (San Diego, CA, USA), Primary antibodies (iNOS 18985-1-AP, COX-2 66351-1-Ig and GAPDH 60004-1-Ig) were purchased from Proteintech (Rosemont, IL, USA). Secondary antibodies (goat anti-mouse and goat antirabbit) were purchased from Li-Cor Biosciences Inc. (Lincoln, NE, USA). All other chemicals were obtained from Thermo Fisher Scientific unless otherwise stated.

4.3.2 Co-encapsulation of apigenin and curcumin

A pH-cycle method was modified and applied to co-encapsulate AP and CUR based on a previous study.²⁵ Sodium caseinate (NaCas) solution (2% w/v) was prepared by dissolving desirable powder in deionized water until a complete dissolution, and stored overnight at 4 °C to ensure complete hydration of protein molecules. AP and CUR with various mass ratios (from 4:1 to 1:4) with a constant AP concentration of 0.2 mg/mL were dissolved in the above NaCas

solution, adjusted to pH 12.0 using 2M KOH. Mixtures were allowed to stir for 30 min at room temperature (21 °C) before neutralizing to pH 7.0 with 1 M HCl. Dispersions were centrifuged (Sorvall LYNX 6000 Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) at 10,000 *g* for 10 min to remove unencapsulated polyphenols, leaving the supernatant as dispersions with coencapsulated AP and CUR. The obtained precipitate was used to determine encapsulation efficiencies by the following equation based on the absorbance at 337 nm and 419 nm for respective apigenin and curcumin, and standard curves that were established previously.

$$EE \% = \frac{\text{total amount of polyphenol (mg)} - \text{amount of polyphenol in precipitate (mg)}}{\text{total amount of polyphenol (mg)}} \times 100$$

4.3.3 Physicochemical characterizations of dispersions

4.3.3.1 Dynamic light scattering and zeta-potential

Particle size distributions of dispersions with AP and CUR, NaCas and NaCas after the pH-cycle treatment under the same encapsulation conditions during 30-day ambient storage with the addition of 0.2% sodium azide to prevent microorganisms, as well as zeta-potential were measured using a Zetasizer Nano-ZS series instrument (Malvern Instruments Ltd., Worcestershire, UK).

4.3.3.2 Transmission electron microscopy (TEM)

Freshly prepared dispersions with AP and CUR at 1:1 mass ratio, NaCas, and NaCas after the pH-cycle treatment were diluted to 0.02% w/v protein and were imaged with TEM. One drop of each diluted sample was placed on a freshly glow-discharged carbon film on a 400-mesh copper grid, stained by 1% uranyl acetate and imaged with a Zeiss Libra micrope at 200

kV (Carl Zeiss Microscopy, Oberkochen, Germany).

4.3.4 Cytotoxicity assay

Cell viability of murine RAW 264.7 macrophages was tested to address the cytotoxicity of AP and CUR. Stock solutions of AP and CUR were prepared by dissolving a desired amount of compound in dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/mL. RAW 264.7 macrophages were cultured at 37 °C under a humidified atmosphere of 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) (Corning Inc., Corning, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Tech, Carlsbad, CA, USA) and 1% v/v penicillin/streptomycin (Life Tech, Carlsbad, CA, USA). A density of 2.5×10^4 cells/well was used for seeding in 96-well plates to allow cells to adhere to the plate. After overnight attachment, cells were treated with a series of concentrations of tested compounds or DMSO (as control) in 200 µL of serum complete medium, with the presence of 1 µg/mL LPS for 24 h. Subsequently, cells were studied with the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) to measure the viability.

4.3.5 Determination of NO production

Cells were seeded as described above, and were then treated with AP only, CUR only, physical mixtures of the two tested compounds with various mass ratios, and dispersions with various concentrations of coencapsulated AP and CUR. Compound concentrations were below toxicity levels, and cells were stimulated with LPS (1 µg/mL) for 24 h. Afterwards, the culture medium were collected to measure the nitrite accumulation as an indicator of NO production by the Griess reagent assay ²⁶. Briefly, supernatant of each treatment (100 µL) was plated in

microplates in triplicate, mixed with an equal volume of Griess reagent A (1% sulfanilamide in 5% phosphoric acid) and reagent B (0.1% naphthylethylenediamine dihydrochloride in water). After 5 min incubation at room temperature, absorbance at 550 nm was recorded (Synergy H1 Hybrid Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA) and the concentration of nitrite was calculated according to a standard curve constructed with sodium nitrite as a standard.

4.3.6 Preparation of whole cell lysates and western blot analysis

RAW 264.7 macrophage cells were seeded at a density of 2×10^5 in 6-well plates and exposed to treatments of a single compound (AP or CUR), a physical mixture of AP and CUR or a dispersion with encapsulated AP and CUR for 24 h in the presence of 1 $\mu\text{g/mL}$ LPS after overnight attachment. Whole cell lysates were collected after lysing with RIPA buffer followed by western blot analysis. According to a previous protocol.²⁷ Equal amounts of protein (20 μg) were loaded into wells of a 4-20% SurePAGE, Bis-Tris gel (GenScript Biotech, Piscataway, NJ, USA), and separated at 200V for 35 min in Tris-MOPS-SDS (GenScript Biotech, Piscataway, NJ, USA) running buffer at ambient temperature. The gel was then transferred to Trans-Blot Turbo PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blocking with 5% non-fat dry milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, membranes were washed three times for 5 min each with TBST. Then, membranes were incubated with primary antibodies against iNOS and COX-2 (ProteinTech, Chicago, IL, USA) at 1:1000 dilution in 1% non-fat dry milk of TBST, and

GAPDH at dilution of 1:5000 overnight at 4 °C. After washing three time for 10 min each with TBST, the membrane was then incubated with anti-rabbit or anti-mouse secondary antibody at dilution of 1:5000 for 1.5 h at room temperature. After which, the membrane was washed with TBST three time for 10 min each, immersed with Bio-Rad Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and visualized by a C-Digit blot scanner (Li-Cor Biosciences, Lincoln, NE, USA). The expression of proteins was calculated by quantifying the intensity of bands using ImageJ software (NIH, Bethesda, MD, USA).

4.3.7 Measurement of pro-inflammatory cytokines

Supernatants of macrophages under various treatments collected from section 4.3.5 were further subjected to respective enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (BioLegend, San Diego, CA, USA) to quantify levels of IL-6 and TNF- α in the culture medium. Absorbance at 450 nm was recorded in a Cambrex ELX808 Microplate Reader (bioMONTR Labs, Durham, NC, USA).

4.3.8 Determination of intracellular reactive oxygen species (ROS)

4.3.8.1 Fluorescent microscopy

RAW 264.7 cells were seeded as described earlier in the cell viability section in 96-well plates, and treated with AP or CUR individually, physical mixtures with various mass ratios of AP and CUR, or different concentrations of coencapsulated AP and CUR for 24 h. Cells were stimulated by 1 μ g/mL LPS for the last 18 h. Afterwards, supernatants were removed before cells were washed with 200 μ L ice-cold PBS. After washing, 200 μ L PBS containing 10 μ M 2',7'-dichlorofluorescein diacetate (DCFH-DA) was added into each well, followed by

incubation at 37 °C for 30 min in dark in a 5% CO₂ incubator. After removing the dye, cells were washed with ice-cold PBS twice. Pictures indicating ROS production were acquired immediately using a green fluorescent protein (GFP) channel with 20× magnification (model EVOS microscope, Thermo Fisher Scientific, Waltham MA, USA).

4.3.8.2 Fluorescence spectrophotometry

Cells were treated the same way as described for fluorescent microscopy, except being seeded in black 96-well plates. After treatments, washing with ice-cold PBS, and incubation with PBS containing 10 µM 2', 7'-dichlorofluorescein diacetate at 37 °C for 30 min, the fluorescence intensity of each treatment was recorded immediately using a Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT, USA) at excitation and emission wavelengths of 485 nm and 528 nm, respectively.

4.3.9 Determination of synergism

The synergistic effects of AP and CUR combinations were determined by the Chou-Talalay method,^{20, 28} by characterizing the interactions of drugs using the combination index (CI) given as:

$$CI = \frac{d_1}{D_{x,1}} + \frac{d_2}{D_{x,2}}$$

where d₁ and d₂ represent the doses of drug 1 (AP) and drug 2 (CUR) in combination; D_{x,1} and D_{x,2} are the corresponding doses of drug 1 (AP) and drug 2 (CUR) given individually to produce the same effect (x). A CI of <1, =1, or >1 suggests synergism, additive or antagonism, respectively. CI was obtained by using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA).

4.3.10 Statistical analysis

Data were presented as mean $\pm$ SD of at least three trials. Data were analyzed using one-way ANOVA with application of IBM SPSS Statistics version 25.0 software (SPSS Inc., Chicago, IL, USA) and means were considered to be significantly different at $p < 0.05$ as determined by Tukey's test for multiple comparisons.

4.4 Results and discussion

4.4.1 Physicochemical characteristics of dispersions

The optimized conditions resulted in high encapsulation efficiencies of both AP and CUR as shown in Table 4-1. Zeta potential indicates the surface charge properties of nanoparticles.²⁹ The magnitude of zeta-potential of native NaCas at pH 7.0 increased after the pH-cycle treatment at the encapsulation conditions (Table 4-1), which may be due to the reduced number of surface charges after reassembling during neutralization of acidity^{25, 30}. The zeta-potential of dispersions with various mass ratios of coencapsulated AP and CUR was similar to the pH-cycle treated NaCas. In addition, zeta-potential is commonly applied to indicate colloidal stability, with a magnitude higher than 30 mV always predicting a higher stability of nanodispersions by electrostatic repulsion.^{31, 32} Therefore, the more negatively charged nanodispersions with the elevated proportion of CUR may possess a higher stability (Table 4-1).

As shown in Figure 4-2A, the pH-cycle process resulted in a smaller hydrodynamic diameter (D_h) of NaCas, which is in agreement with some previous studies.^{33, 34} possibly due

to the supramolecular changes of casein particles. Encapsulating AP and CUR had insignificant influence on D_h when compared to the pH-cycle treated NaCas.

Morphologies of NaCas before and after the pH-cycle treatment as well as the dispersion prepared with AP and CUR at 1:1 mass ratio are presented in Figure 4-2B. Compared with the pH-cycle treated NaCas, the unprocessed NaCas had more irregular and heterogeneous structures. After the pH-cycle treatment, the structure became uniform and smaller, which was in consistent with the above D_h results. Besides, co-encapsulating AP and CUR further increased the uniformity and compactness of particles.

Co-encapsulating CUR together with AP resulted in the enhanced storage stability when comparing to the encapsulation treatment with AP only. As shown in Figure 4-3A, the treatment with AP only became turbid after overnight storage, while dispersions with both compounds maintained visually clear. The stability of co-encapsulation treatments with various mass ratios of AP and CUR was further investigated by measuring particle size distributions during 30-day storage at room temperature (21 °C). As shown in Figure 4-3B, increasing the proportion of CUR in the nanodispersion enhanced the storage stability of dispersions, increased from 15 days to 30 days ($p > 0.05$). Therefore, it is feasible to coencapsulate AP and CUR with NaCas and obtain a stable nanodelivery system.

4.4.2 Cell viability and toxicity of apigenin and curcumin

To test the toxicity of AP and CUR, and establish nontoxic dose ranges for these two compounds in RAW 264.7 cells, growth inhibitory activity of AP and CUR was initially investigated for cell viability using the MTS assay. As shown in Figure 4-4A and B, AP and

CUR at up to 12 and 6 $\mu\text{g/mL}$, respectively, did not cause a significant decrease on the viability of RAW 264.7 cells. The calculated IC_{50} of AP and CUR against RAW 264.7 was 19.22 $\mu\text{g/mL}$ and 9.81 $\mu\text{g/mL}$, respectively, which indicated a much higher toxicity of CUR than AP. Therefore, the combination of AP and CUR could decrease the dosage of CUR to potentially minimize the toxicity and side effects.

The combination treatments with different mass ratios (from 4:1 to 1:1) of AP and CUR at a total concentration of up to 6 $\mu\text{g/mL}$ were further confirmed for insignificant effect on the viability of RAW 264.7 cells (data not shown). Thus, nontoxic doses ranging from 0.5 $\mu\text{g/mL}$ to 6 $\mu\text{g/mL}$ were selected in the following studies, to determine the possible anti-inflammatory activities of AP and CUR, as well as their combinations in LPS-stimulated RAW 264.7 cells.

4.4.3 Synergistic inhibition of NO production

NO has been implicated as one of the key factors mediating tissue injury induced by inflammation. Excess NO has been reported to be genotoxic by inducing DNA damage as a reactive radical³⁵. Biologically produced NO is rather unstable and is rapidly oxidized to nitrite and nitrate in water.²⁰ As shown in Figure 4-5A, under the nontoxic dosage, AP inhibited LPS-induced NO production by 13.32, 30.23, 54.45, and 72.42% at 0.5, 1.5, 3 and 6 $\mu\text{g/mL}$, respectively. Similarly, CUR treatments also resulted in the inhibition of NO production by 12.46, 40.32, 76.38, and 97.91% at respective concentrations of 0.5, 1.5, 3, and 6 $\mu\text{g/mL}$ (Figure 4-5B).

To determine the combinational effects of AP and CUR, LPS-stimulated macrophages were treated with a series of combinations with different mass ratios of AP and CUR under the

established nontoxic concentrations. As shown in Figure 4-5C, combinations with an elevated proportion of CUR possessed a stronger inhibitory activity on NO production than individual treatments. The calculated CI (Table 4-2) decreased from 1.25 to 0.65 as the amount of CUR increased, suggesting the transition of additive to synergistic interactions between AP and CUR.

To overcome the low solubility and bioavailability, AP and CUR were coencapsulated and the inhibitory effects on NO production by nanodispersions with one or both compounds are shown in Figure 4-5B. The CI was smaller than 0.8 for all co-nanoencapsulation treatments (Table 4-2), suggesting enhanced synergistic interactions between AP and CUR at all mass ratios after co-encapsulation. When only one compound was nanoencapsulated with NaCas, the concentration needed to inhibit 50% NO production (IC_{50}) was higher than the corresponding free compound dissolved in DMSO (Table 4-2). This may result from the reduced direct interactions between polyphenols and cells, since, after encapsulation, polyphenol molecules bound to proteins through hydrophobic interactions and hydrogen bonds, lower the availability to cells. The synergistic activities after co-encapsulation of AP and CUR may result from different targets of the compounds. The enhanced synergistic interactions after nanoencapsulation may be ascribed to the improved stability, dispersibility, and proximity of polyphenols during cell incubation, which made it possible to better interact cells by two compounds simultaneously.

4.4.4 Decreased expression of iNOS and COX-2

The expression level of iNOS protein was subsequently analyzed by western blot, to obtain clear knowledge of the synergistic inhibitory mechanism of NO production by

combinations of AP and CUR. It was hypothesized that the synergism was due to the decreased production of the iNOS synthesis, since iNOS is responsible for sustained release of NO and is typically synthesized in response to pro-inflammatory stimuli, which is induced in a large variety of diseases.³⁶

As shown in Figure 4-6A, iNOS expression significantly increased following LPS stimulation. All treatments including individual polyphenols and physical combinations at doses of 3 and 6 $\mu\text{g/mL}$ resulted in a dose-dependent down-regulation of iNOS expression, although lower concentrations (0.5 and 1.5 $\mu\text{g/mL}$) did not show a considerable reduction. Treatments of 6 $\mu\text{g/mL}$ AP and CUR for 24 h induced 49.7 % and 40.6% inhibition of iNOS expression, respectively, as compared with the LPS-treated positive control cells (Figure 4-6B). However, administrations of physical mixtures of apigenin and curcumin at different mass ratios did not act in a synergism tendency as in the inhibition of NO production ($\text{CI} \geq 1$). On the other hand, compared to nanoencapsulation treatment with AP only or CUR only, co-encapsulation treatments with a higher proportion of CUR induced a synergistic inhibition of iNOS expression as shown in Figure 4-6C and Table 4-3, which agrees with the synergistic reduction of NO production (Figure 4-5). These results indicate that the synergistic inhibition of NO production by different treatments is at least partially through the suppression of iNOS expression.

COX-2 was also determined as another important pro-inflammatory enzyme.³⁷ As shown in Figure 4-7A, LPS stimulated a significant increase of COX-2 expression. Under the same treatment conditions, both free CUR and nanodispersion with CUR only at 6 $\mu\text{g/mL}$ inhibited

almost 100% expression of COX-2 (Figure 4-7B, C). The inhibitory activity of AP and encapsulated AP was not as strong as CUR, however, with respective reduction of 64% and 74.7% at 6 $\mu\text{g/mL}$ (Figure 4-7B, C). Due to the strong suppression of CUR, none of the combinations acted in a synergistic behavior, although all of them possessed a dose-dependent inhibitory manner and resulted in significant reduction of COX-2 level.

The exact cellular mechanisms of AP and CUR as well as their combinations for down-regulating iNOS and COX-2 in RAW 264.7 cells remain to be understood. They may have effects on upstream of signal transduction involving activation or translocation of transcription factors such as nuclear factor kappa B (NF- κ B), or they may affect the translational level of enzyme expression.³⁸ The differences of synergistic inhibitory activities noted in the iNOS and COX-2 expressions may be due to the degree of dependence of enzyme promoters or transcription factors on polyphenols and their combinations. Nevertheless, further studies are needed to clarify these points.

4.4.5 Synergistic suppression of IL-6 and TNF- α

IL-6 and TNF- α were quantified as they are two important pro-inflammatory cytokines. In negative control cells, both IL-6 and TNF- α were generated in vanishingly small quantities but produced in a large increasing amount when stimulated with LPS. As shown in Figure 4-8, all treatments showed a dose-dependent manner to inhibit the production of IL-6. AP significantly suppressed IL-6 production by 28.04%, 50.98%, and 81.32% at 1.5, 3, and 6 $\mu\text{g/mL}$, respectively. Compared to AP, free CUR induced a lower extent of inhibition at low concentrations but a larger degree of reduction in high concentration, with 13.88%, 56.07%

and 90.36% inhibition at respective concentrations of 1.5, 3, and 6 $\mu\text{g/mL}$ (Figure 4-8A). Similar to the NO production, combinations of free AP and CUR with increased concentrations of CUR resulted in synergistic inhibitory effects on IL-6 production, and co-encapsulation enhanced the synergism with CI values smaller than 1 (Table 4-4).

The studied treatments affected the production of TNF- α with a much lower inhibition extent. As shown in Figure 4-9A, at up to 6 $\mu\text{g/mL}$, AP and CUR induced up to 30.63% and 57.32% inhibition, respectively. These results indicate that separate mechanisms may possibly exist in the production of TNF- α and IL-6 in LPS-stimulated macrophages, which are differentially influenced by polyphenols. Several studies have demonstrated that polyphenols differentially modulate the expression of cytokines. It was reported that quercetin and galangin inhibited the production of IL-6, but not TNF- α .³⁹ It should be noted, however, that similar synergistic interactions were observed in the free polyphenol mixtures with a higher proportion of CUR (Table 4-5) and co-encapsulation enhanced synergism (Figure 4-9B), showing a significantly higher reduction of TNF- α production compared with nanodispersions with only one polyphenol at 6 $\mu\text{g/mL}$.

4.4.6 Synergistic inhibition of ROS production

In the current study, the levels of ROS were evaluated using fluorescence microscopy and spectrophotometry in the absence or presence of polyphenols and their combinations in RAW264.7 cells. As shown in Figure 4-10, without LPS treatment, ROS was not detectable but increased significantly following LPS treatment as strong fluorescence signals were observed. Compared with the LPS-treated control cells, the fluorescence intensity decreased dramatically

in the presence of 6 $\mu\text{g/mL}$ of polyphenols in all different treatments, suggesting a potent inhibitory activity of the studied compounds and the combinations.

The suppressant effects of these treatments were further confirmed by quantitative fluorescence spectrophotometry. As shown in Figure 4-11, all polyphenol treatments reduced the production of ROS triggered by LPS. Compared to the positive cells treated with LPS only, free AP at 1.5, 3, and 6 $\mu\text{g/mL}$ suppressed 13.55%, 24.41% and 43.18% of ROS production, respectively. CUR exerted a weaker inhibitory activity at lower concentrations as 1.5 and 3 $\mu\text{g/mL}$ administrations resulted in 8.69% and 18.84% reduction of ROS, respectively, while a stronger suppression of 75.96% was observed at 6 $\mu\text{g/mL}$. Besides, the calculated CI values (Table 4-6) confirmed the synergistic interactions between AP and CUR, with an enhanced synergism after co-encapsulation. Therefore, inhibiting ROS production is one of the possible mechanisms that contribute to the anti-inflammatory activities of AP and CUR as well as their combinations.

4.5 Conclusions

It was firstly to found that co-encapsulating curcumin together with apigenin using NaCas gave rise to the enhanced storage stability with respect to the nanodispersion encapsulated with apigenin only. With the increased proportion of curcumin, the nanodispersions coencapsulated with apigenin and curcumin resulted in a longer ambient storage period (21 °C). Besides, under non-toxic concentrations, combining apigenin and curcumin with higher mass ratios of curcumin in the simple mixtures synergistically diminished NO production, suppressed iNOS expression,

decreased pro-inflammatory cytokines (IL-6 and TNF- α), and reduced ROS production in LPS-induced RAW 264.7 macrophages (CI < 1). Co-encapsulation of apigenin and curcumin with NaCas resulted in the enhanced anti-inflammatory activities. These results provide new insights on synergistic interactions between dietary bioactive compounds. However, molecular mechanisms that are responsible for the enhanced synergistic interactions after co-encapsulation as well as those upstream signaling pathways that contribute to the above inflammatory mediators remains further elaboration.

References

1. Yoon, J.-H.; Baek, S. J. Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei medical journal* **2005**, 46 (5), 585-596.
2. Yi, Y. S. Regulatory roles of flavonoids on inflammasome activation during inflammatory responses. *Molecular nutrition & food research* **2018**, 62 (13), 1800147.
3. Gupta, S. C.; Kunnumakkara, A. B.; Aggarwal, S.; Aggarwal, B. B. Inflammation, a Double-Edge Sword for Cancer and Other Age-Related Diseases. *Frontiers in immunology* **2018**, 9.
4. Si, T. L.; Liu, Q.; Ren, Y. F.; Li, H.; Xu, X. Y.; Li, E. H.; Pan, S. Y.; Zhang, J. L.; Wang, K. X. Enhanced anti-inflammatory effects of DHA and quercetin in lipopolysaccharide-induced RAW264. 7 macrophages by inhibiting NF- κ B and MAPK activation. *Molecular medicine reports* **2016**, 14 (1), 499-508.
5. Schetter, A. J.; Heegaard, N. H.; Harris, C. C. Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* **2009**, 31 (1), 37-49.
6. Hougee, S.; Sanders, A.; Faber, J.; Graus, Y. M.; van den Berg, W. B.; Garssen, J.; Smit, H. F.; Hoijer, M. A. Decreased pro-inflammatory cytokine production by LPS-stimulated PBMC upon in vitro incubation with the flavonoids apigenin, luteolin or chrysin, due to selective elimination of monocytes/macrophages. *Biochemical pharmacology* **2005**, 69 (2), 241-248.
7. Guo, S.; Qiu, P.; Xu, G.; Wu, X.; Dong, P.; Yang, G.; Zheng, J.; McClements, D. J.; Xiao, H. Synergistic anti-inflammatory effects of nobiletin and sulforaphane in lipopolysaccharide-stimulated RAW 264.7 cells. *Journal of agricultural and food chemistry* **2012**, 60 (9), 2157--2164 DOI: 10.1021/jf300129t.
8. Kwon, D. H.; Cha, H.-J.; Choi, E. O.; Leem, S.-H.; Kim, G.-Y.; Moon, S.-K.; Chang, Y.-C.; Yun, S.-J.; Hwang, H. J.; Kim, B. W. Schisandrin A suppresses lipopolysaccharide-induced inflammation and oxidative stress in RAW 264.7 macrophages by suppressing the NF- κ B, MAPKs and PI3K/Akt pathways and activating Nrf2/HO-1 signaling. *International journal of molecular medicine* **2018**, 41 (1), 264-274.

9. Hibbs Jr, J. Synthesis of nitric oxide from L-arginine: a recently discovered pathway induced by cytokines with antitumour and antimicrobial activity. *Research in immunology* **1991**, 142 (7), 565-569.
10. Ohshima, H.; Bartsch, H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1994**, 305 (2), 253-264.
11. Arroyo, P. L.; Hatch-Pigott, V.; Mower, H.; Cooney, R. Mutagenicity of nitric oxide and its inhibition by antioxidants. *Mutation Research Letters* **1992**, 281 (3), 193-202.
12. Tsujii, M.; Kawano, S.; DuBois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proceedings of the National Academy of Sciences* **1997**, 94 (7), 3336--3340.
13. Liu, X.-H.; Kirschenbaum, A.; Yao, S.; Stearns, M. E.; Holland, J. F.; Claffey, K.; Levine, A. C. Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. *Clinical & experimental metastasis* **1999**, 17 (8), 687-694.
14. Hernández-Ledesma, B.; Hsieh, C.-C.; Ben, O. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochemical and biophysical research communications* **2009**, 390 (3), 803-808.
15. Ambriz-Pérez, D. L.; Leyva-López, N.; Gutierrez-Grijalva, E. P.; Heredia, J. B. Phenolic compounds: Natural alternative in inflammation treatment. A Review. *Cogent Food & Agriculture* **2016**, 2 (1), 1131412.
16. Guo, W.; Kong, E.; Meydani, M. Dietary polyphenols, inflammation, and cancer. *Nutrition and cancer* **2009**, 61 (6), 807-810.
17. Zhang, H.; Tsao, R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science* **2016**, 8, 33-42.
18. Jeong, G.-S.; Lee, S.-H.; Jeong, S.-N.; Kim, Y.-C.; Kim, E.-C. Anti-inflammatory effects of apigenin on nicotine-and lipopolysaccharide-stimulated human periodontal ligament cells via heme oxygenase-1. *International Immunopharmacology* **2009**, 9 (12), 1374-1380.
19. Leyva-López, N.; Gutierrez-Grijalva, E. P.; Ambriz-Perez, D. L.; Heredia, J. B. Flavonoids

as cytokine modulators: a possible therapy for inflammation-related diseases. *International journal of molecular sciences* **2016**, 17 (6), 921.

20. Saw, C. L. L.; Huang, Y.; Kong, A.-N. Synergistic anti-inflammatory effects of low doses of curcumin in combination with polyunsaturated fatty acids: docosahexaenoic acid or eicosapentaenoic acid. *Biochemical pharmacology* **2010**, 79 (3), 421-430.

21. Plummer, S. M.; Holloway, K. A.; Manson, M. M.; Munks, R. J.; Kaptein, A.; Farrow, S.; Howells, L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF- κ B activation via the NIK/IKK signalling complex. *Oncogene* **1999**, 18 (44), 6013.

22. Singh, S.; Aggarwal, B. B. Activation of transcription factor NF- κ B is suppressed by curcumin (diferuloylmethane). *Journal of Biological Chemistry* **1995**, 270 (42), 24995-25000.

23. Phan, M. A. T.; Paterson, J.; Bucknall, M.; Arcot, J. Interactions between phytochemicals from fruits and vegetables: Effects on bioactivities and bioavailability. *Critical Reviews in Food Science and Nutrition* **2018**, 58 (8), 1310--1329 DOI: 10.1080/10408398.2016.1254595.

24. Xu, Y.; Xin, Y.; Diao, Y.; Lu, C.; Fu, J.; Luo, L.; Yin, Z. Synergistic effects of apigenin and paclitaxel on apoptosis of cancer cells. *PLoS ONE* **2011**, 6 (12), DOI: 10.1371/journal.pone.0029169.

25. Pan, K.; Luo, Y.; Gan, Y.; Baek, S. J.; Zhong, Q. pH-driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity. *Soft Matter* **2014**, 10 (35), 6820-6830.

26. Lee, J.-A.; Ha, S.; Cho, E.; Choi, I. Resveratrol as a bioenhancer to improve anti-inflammatory activities of apigenin. *Nutrients* **2015**, 7 (11), 9650-9661.

27. Dia, V. P.; Krishnan, H. B. BG-4, a novel anticancer peptide from bitter melon (*Momordica charantia*), promotes apoptosis in human colon cancer cells. *Scientific reports* **2016**, 6, 33532.

28. Xu, Y.; Xin, Y.; Diao, Y.; Lu, C.; Fu, J.; Luo, L.; Yin, Z. Synergistic effects of apigenin and paclitaxel on apoptosis of cancer cells. *PloS one* **2011**, 6 (12), e29169.

29. Wang, S.; Su, R.; Nie, S.; Sun, M.; Zhang, J.; Wu, D.; Moustaid-Moussa, N. Application of nanotechnology in improving bioavailability and bioactivity of diet-derived phytochemicals. *The Journal of nutritional biochemistry* **2014**, 25 (4), 363-376.

30. Pan, K.; Chen, H.; Davidson, P. M.; Zhong, Q. Thymol nanoencapsulated by sodium caseinate: physical and antilisterial properties. *Journal of agricultural and food chemistry* **2014**, 62 (7), 1649-1657.
31. Hunter, R. J., Zeta potential in colloid science: principles and applications. Academic press: 2013; Vol. 2.
32. Webb, P. A.; Orr, C., *Analytical methods in fine particle technology*. Micromeritics Instrument Corp: 1997.
33. Liu, Y.; Guo, R. pH-dependent structures and properties of casein micelles. *Biophysical Chemistry* **2008**, 136 (2-3), 67-73.
34. Zhang, B.; Luo, Y.; Wang, Q. Effect of acid and base treatments on structural, rheological, and antioxidant properties of α -zein. *Food chemistry* **2011**, 124 (1), 210-220.
35. Choi, J. S.; Islam, M. N.; Ali, M. Y.; Kim, E. J.; Kim, Y. M.; Jung, H. A. Effects of C-glycosylation on anti-diabetic, anti-Alzheimer's disease and anti-inflammatory potential of apigenin. *Food and Chemical Toxicology* **2014**, 64, 27-33.
36. Ben, P.; Liu, J.; Lu, C.; Xu, Y.; Xin, Y.; Fu, J.; Huang, H.; Zhang, Z.; Gao, Y.; Luo, L.; Yin, Z. Curcumin promotes degradation of inducible nitric oxide synthase and suppresses its enzyme activity in RAW 264.7 cells. *International Immunopharmacology* **2011**, 11 (2), 179--186 DOI: 10.1016/j.intimp.2010.11.013.
37. Villegas, I.; Sánchez - Fidalgo, S.; de la Lastra, C. A. Chemopreventive effect of dietary curcumin on inflammation - induced colorectal carcinogenesis in mice. *Molecular nutrition & food research* **2011**, 55 (2), 259-267.
38. Kim, H. K.; Cheon, B. S.; Kim, Y. H.; Kim, S. Y.; Kim, H. P. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochemical pharmacology* **1999**, 58 (5), 759-765.
39. Lee, H. N.; Shin, S. A.; Choo, G. S.; Kim, H. J.; Park, Y. S.; Kim, B. S.; Kim, S. K.; Cho, S. D.; Nam, J. S.; Choi, C. S. Anti-inflammatory effect of quercetin and galangin in LPS-stimulated RAW264. 7 macrophages and DNCB-induced atopic dermatitis animal models. *International journal of molecular medicine* **2018**, 41 (2), 888-898.

Appendix

Table 4-1. Encapsulation efficiency and zeta-potential of dispersions after the pH-cycle treatment of 2% w/v sodium caseinate (NaCas) and 0.2 mg/mL polyphenols or with different mass ratios of apigenin (AP) : curcumin (CUR) at a constant AP concentration of 0.2 mg/mL.

Encapsulation Efficiency			Zeta-potential	
AP:CUR	EE% of AP	EE% of CUR	Sample	Zeta-potential (mV)
1:0	96.17% ± 0.008 ^{b,c}	-	NaCas	-21.10 ± 1.44 ^c
0:1	-	96.70% ± 0.004 ^{a,b}	pH-NaCas	-31.40 ± 1.73 ^b
4:1	96.63% ± 0.001 ^{a,b,c}	93.26% ± 0.010 ^d	AP:C=1:1	-33.43 ± 1.00 ^{a, b}
3:1	95.99% ± 0.001 ^c	94.11% ± 0.008 ^{c, d}	AP:C=1:2	-33.10 ± 0.70 ^{a, b}
2:1	96.41% ± 0.002 ^{b,c}	95.70% ± 0.002 ^{b, c}	AP:C=1:3	-32.03 ± 0.84 ^b
1:1	96.26% ± 0.003 ^{b,c}	95.81% ± 0.019 ^{b, c}	AP:C=1:4	-35.17 ± 0.42 ^a
1:2	97.46% ± 0.002 ^a	98.47% ± 0.001 ^a	Numbers are mean ± standard deviations (n = 3). Different superscript letters indicate significant differences ($p < 0.05$).	
1:3	97.09% ± 0.001 ^{a,b}	98.73% ± 0.001 ^a		
1:4	97.00% ± 0.004 ^{a,b}	98.32% ± 0.008 ^a		

Table 4-2. Concentrations of free or encapsulated (En) apigenin (AP) and curcumin (CUR) to inhibit 50% of NO production (IC₅₀) and combination index (CI) calculated for combinations with various mass ratios of free or co-encapsulated (CoEn) AP and CUR on NO production in LPS-stimulated RAW 264.7 cells.

	IC ₅₀ (µg/mL)		IC ₅₀ (µg/mL)
Free-AP	2.41	En-AP	4.40
Free-CUR	1.43	En-CUR	3.51
	CI		CI
Free-AP:CUR=4:1	1.246	CoEn-AP:CUR=4:1	0.629
Free-AP:CUR=3:1	1.099	CoEn-AP:CUR=3:1	0.703
Free-AP:CUR=2:1	1.125	CoEn-AP:CUR=2:1	0.474
Free-AP:CUR=1:1	1.128	CoEn-AP:CUR=1:1	0.582
Free-AP:CUR=1:2	0.957	CoEn-AP:CUR=1:2	0.543
Free-AP:CUR=1:3	0.652	CoEn-AP:CUR=1:3	0.683
Free-AP:CUR=1:4	0.789	CoEn-AP:CUR=1:4	0.553

Table 4-3. Combination index (CI) calculated for combination effects of coencapsulated (CoEn) apigenin (AP) and curcumin (CUR) at different mass ratios on expression of iNOS triggered by LPS activation.

	CI		CI
CoEn-AP:CUR=4:1	1.405	CoEn-AP:CUR=1:2	0.777
CoEn-AP:CUR=3:1	1.472	CoEn-AP:CUR=1:3	0.609
CoEn-AP:CUR=2:1	0.870	CoEn-AP:CUR=1:4	0.457
CoEn-AP:CUR=1:1	0.932		

Table 4-4. Concentrations of free or encapsulated (En) apigenin (AP) and curcumin (CUR) to inhibit 50% of IL-6 level (IC₅₀) and combination index (CI) calculated for combinations with various mass ratios of free or co-encapsulated (CoEn) AP and CUR on IL-6 production induced by LPS stimulation.

	IC ₅₀ (µg/mL)		IC ₅₀ (µg/mL)
Free-AP	2.69	En-AP	5.36
Free-CUR	2.78	En-CUR	5.83
	CI		CI
Free-AP:CUR=4:1	1.349	CoEn-AP:CUR=4:1	0.519
Free-AP:CUR=3:1	1.310	CoEn-AP:CUR=3:1	0.721
Free-AP:CUR=2:1	1.244	CoEn-AP:CUR=2:1	0.664
Free-AP:CUR=1:1	1.243	CoEn-AP:CUR=1:1	0.675
Free-AP:CUR=1:2	0.860	CoEn-AP:CUR=1:2	0.647
Free-AP:CUR=1:3	0.699	CoEn-AP:CUR=1:3	0.866
Free-AP:CUR=1:4	0.725	CoEn-AP:CUR=1:4	0.568

Table 4-5. Concentrations of free apigenin (AP) and curcumin (CUR) to inhibit 50% of TNF- α level (IC₅₀) and combination index (CI) calculated for combinations with various mass ratios of free AP and CUR on TNF- α level induced by LPS stimulation.

	IC ₅₀ (μ g/mL)		IC ₅₀ (μ g/mL)
Free-AP	12.32	Free-CUR	5.45
	CI		CI
Free-AP:CUR=4:1	1.500	Free-AP:CUR=1:2	0.978
Free-AP:CUR=3:1	1.486	Free-AP:CUR=1:3	0.563
Free-AP:CUR=2:1	1.162	Free-AP:CUR=1:4	0.732
Free-AP:CUR=1:1	1.381		

Table 4-6. Concentrations of free or encapsulated (En) apigenin (AP) and curcumin (CUR) to inhibit 50% of ROS production (IC₅₀) and combination index (CI) calculated for combinations with various mass ratios of free or co-encapsulated (CoEn) AP and CUR on ROS production in LPS-induced RAW 264.7 cells.

	IC ₅₀ (µg/mL)		IC ₅₀ (µg/mL)
Free-AP	7.79	En-AP	11.48
Free-CUR	4.26	En-CUR	4.22
	CI		CI
Free-AP:CUR=4:1	0.878	CoEn-AP:CUR=4:1	0.620
Free-AP:CUR=3:1	0.816	CoEn-AP:CUR=3:1	0.665
Free-AP:CUR=2:1	0.832	CoEn-AP:CUR=2:1	0.653
Free-AP:CUR=1:1	0.875	CoEn-AP:CUR=1:1	0.720
Free-AP:CUR=1:2	0.794	CoEn-AP:CUR=1:2	0.652
Free-AP:CUR=1:3	0.744	CoEn-AP:CUR=1:3	0.750
Free-AP:CUR=1:4	0.727	CoEn-AP:CUR=1:4	0.672

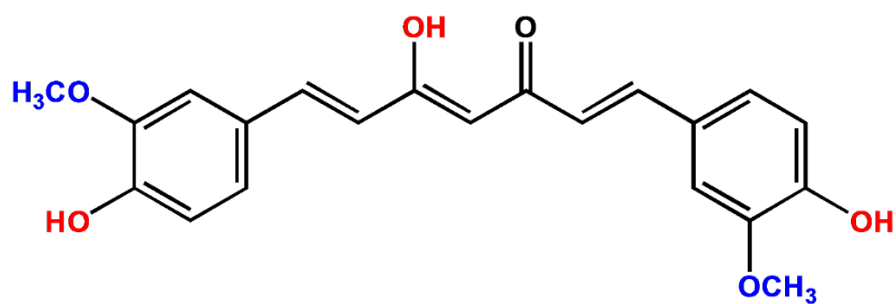


Figure 4-1. Molecular structure of curcumin, (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione.

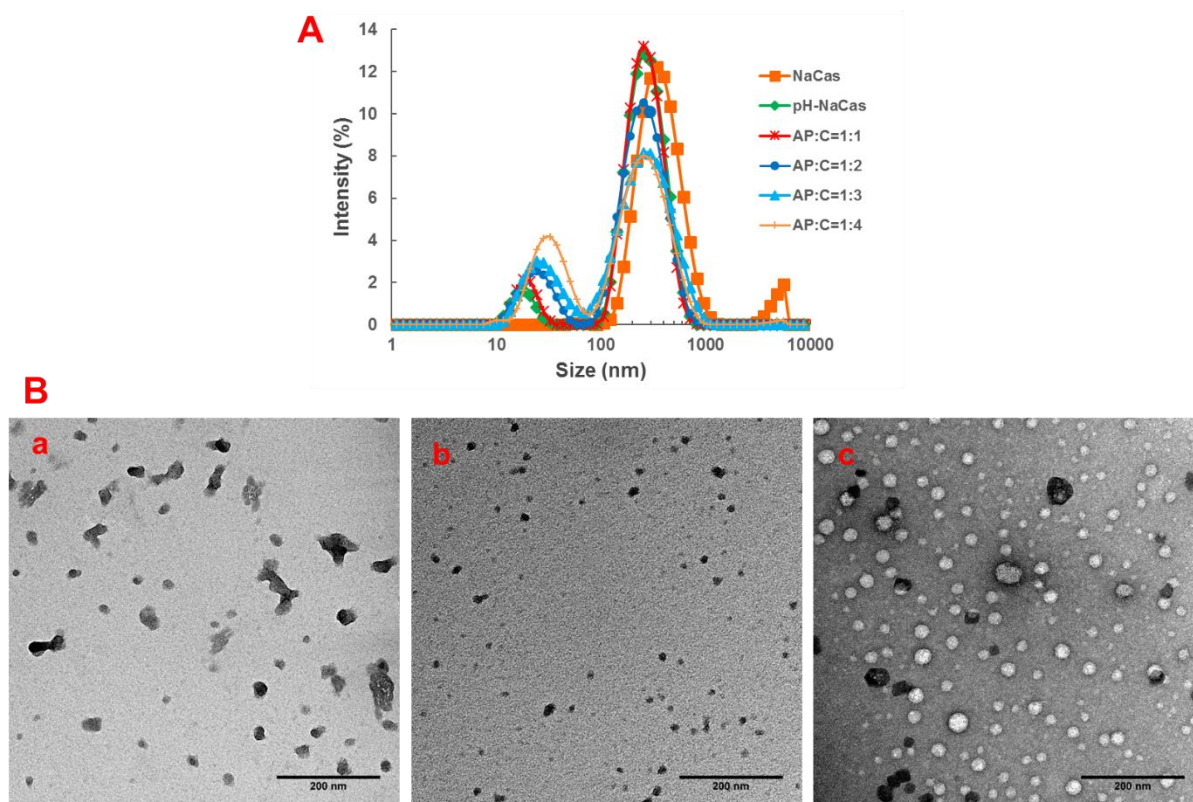


Figure 4-2. (A) Hydrodynamic diameter distributions of pH 7.0 dispersions of sodium caseinate (NaCas), pH-cycle treated NaCas (pH-NaCas) and coencapsulated apigenin (AP) and curcumin (CUR) at different mass ratios; (B) TEM images of (a) NaCas; (b) pH-NaCas, and (c) dispersion with AP and CUR at 1:1 mass ratio.

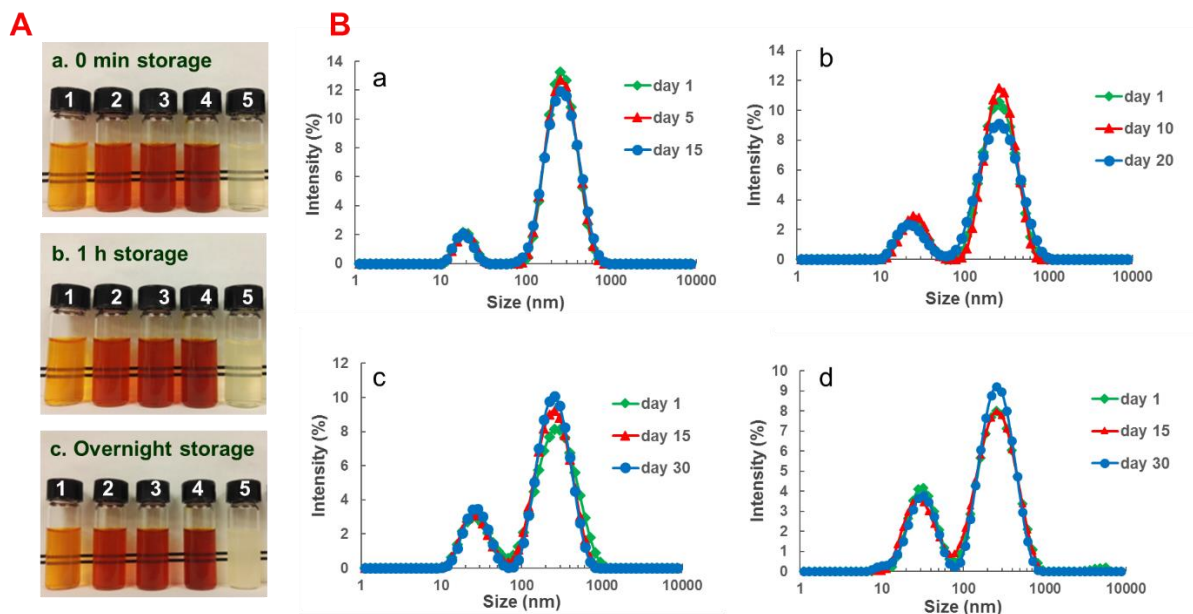


Figure 4-3. (A) Visual appearance of nanodispersions prepared in 2% w/v NaCas and constant 0.2 mg/mL apigenin (AP) at mass ratio of AP:curcumin (CUR) (1) 1:1, (2) 1:2, (3) 1:3, (4) 1:4, and (5) 1:0; (B) Particle size distributions of the dispersions prepared with AP and CUR at mass ratios of (a) 1:1, (b) 1:2, (c) 1:3, and (d) 1:4 during ambient storage (21 °C) for up to 30 days.

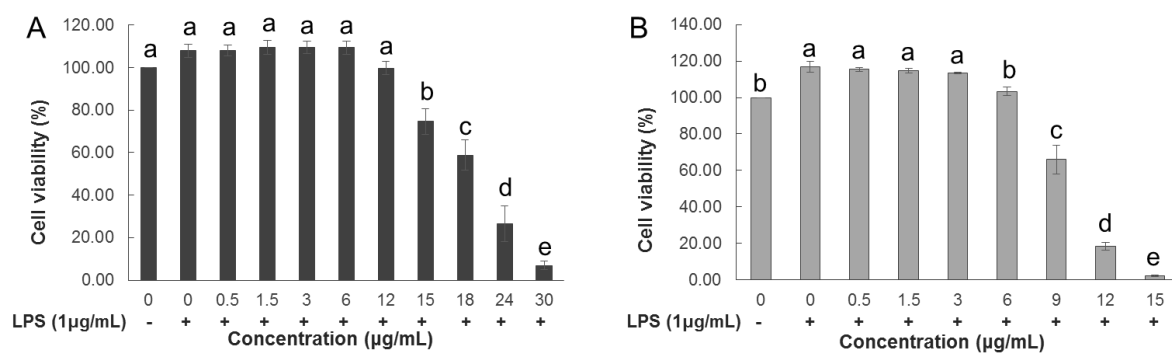


Figure 4-4. Effects of 0-30 μg/mL apigenin (A) and 0-15 μg/mL curcumin (B) on viability of lipopolysaccharide (LPS+) activated macrophages. Error bars are standard deviations (n ≥ 3). Different letters above columns indicate significant differences ($p < 0.05$).

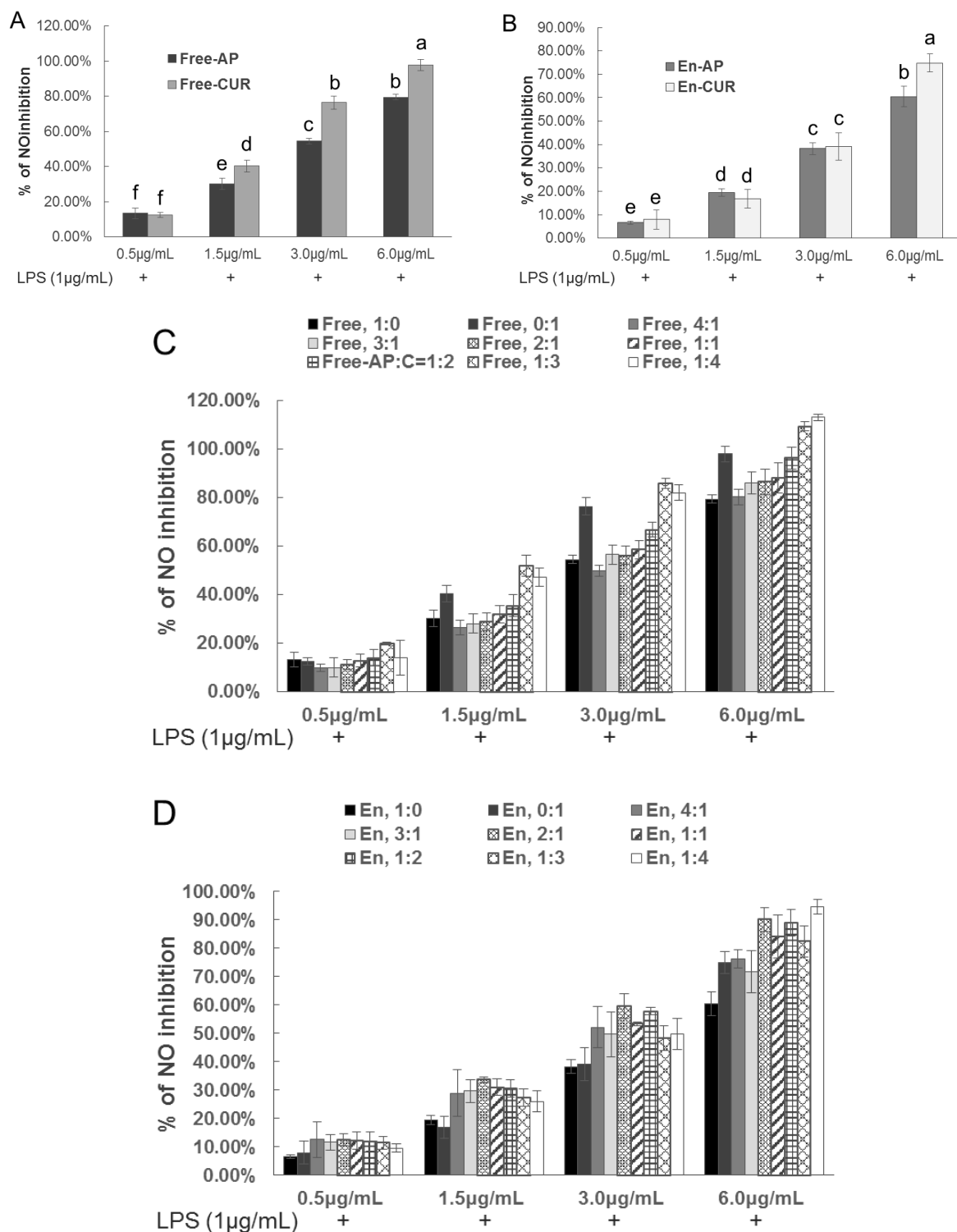


Figure 4-5. Percentage of inhibition on NO production in LPS-induced RAW 264.7 cells by free (A) or encapsulated (B) apigenin (AP) or curcumin (CUR); free AP and CUR at different mass ratios (C); and AP and CUR co-encapsulated at different mass ratios (D). Error bars are standard deviations ($n \geq 3$). Different letters above columns in A and B indicate significant differences ($p < 0.05$).

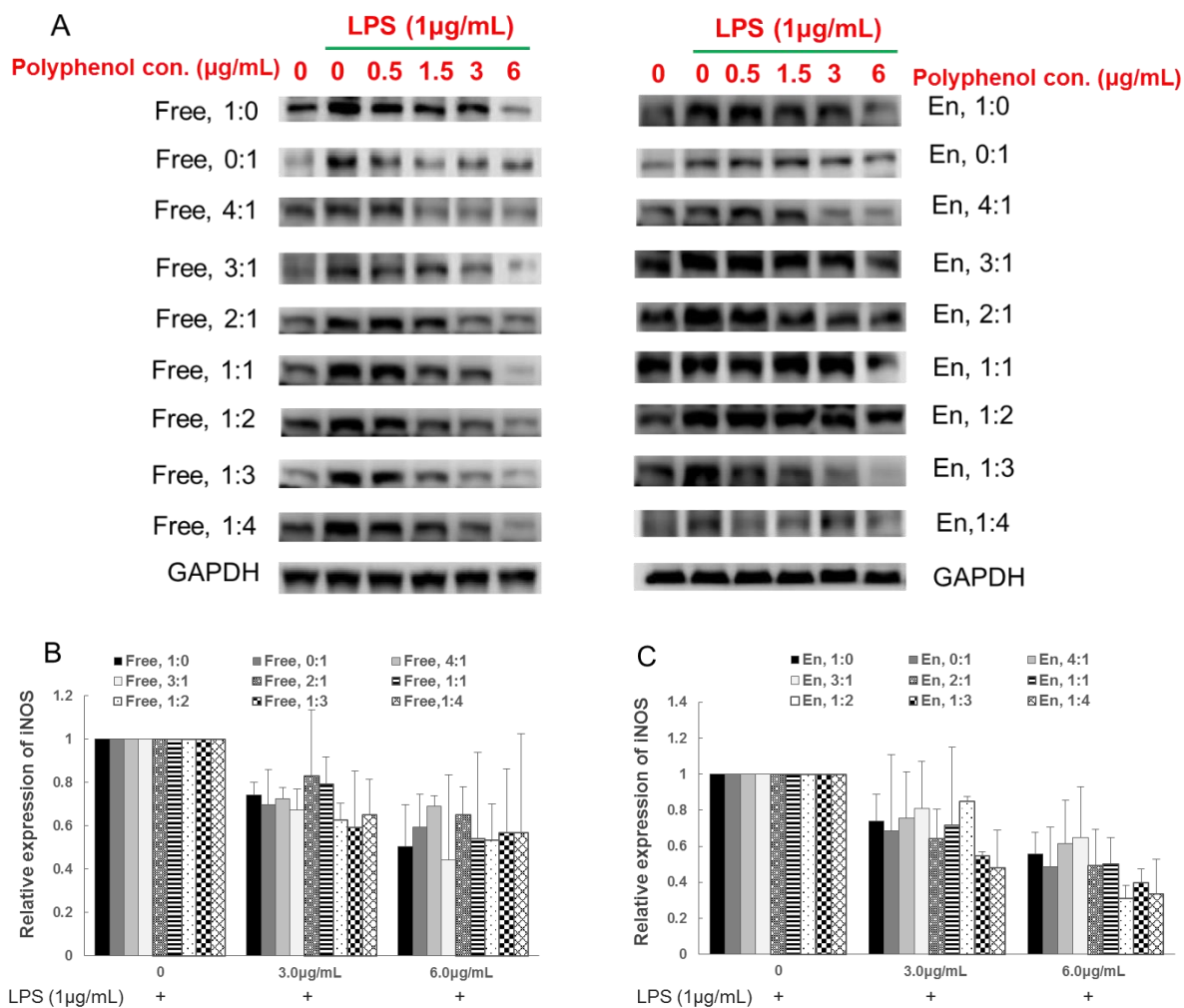


Figure 4-6. Western blot analysis of iNOS expression in LPS-induced RAW 264.7 cells as inhibited by 0-6 μg/mL free or encapsulated (En) apigenin (AP) and curcumin (CUR) at mass ratios from 1:0 to 0:1: images (A) and relative expression (B, free; C, En). GAPDH represents the housekeeping protein. The first “0 μg/mL” represents the negative control without LPS and polyphenols. The second “0 μg/mL” represents the positive control with LPS but without polyphenols. Relative expression of iNOS protein was calculated compared to the positive control cells. Error bars are standard deviations (n = 3).

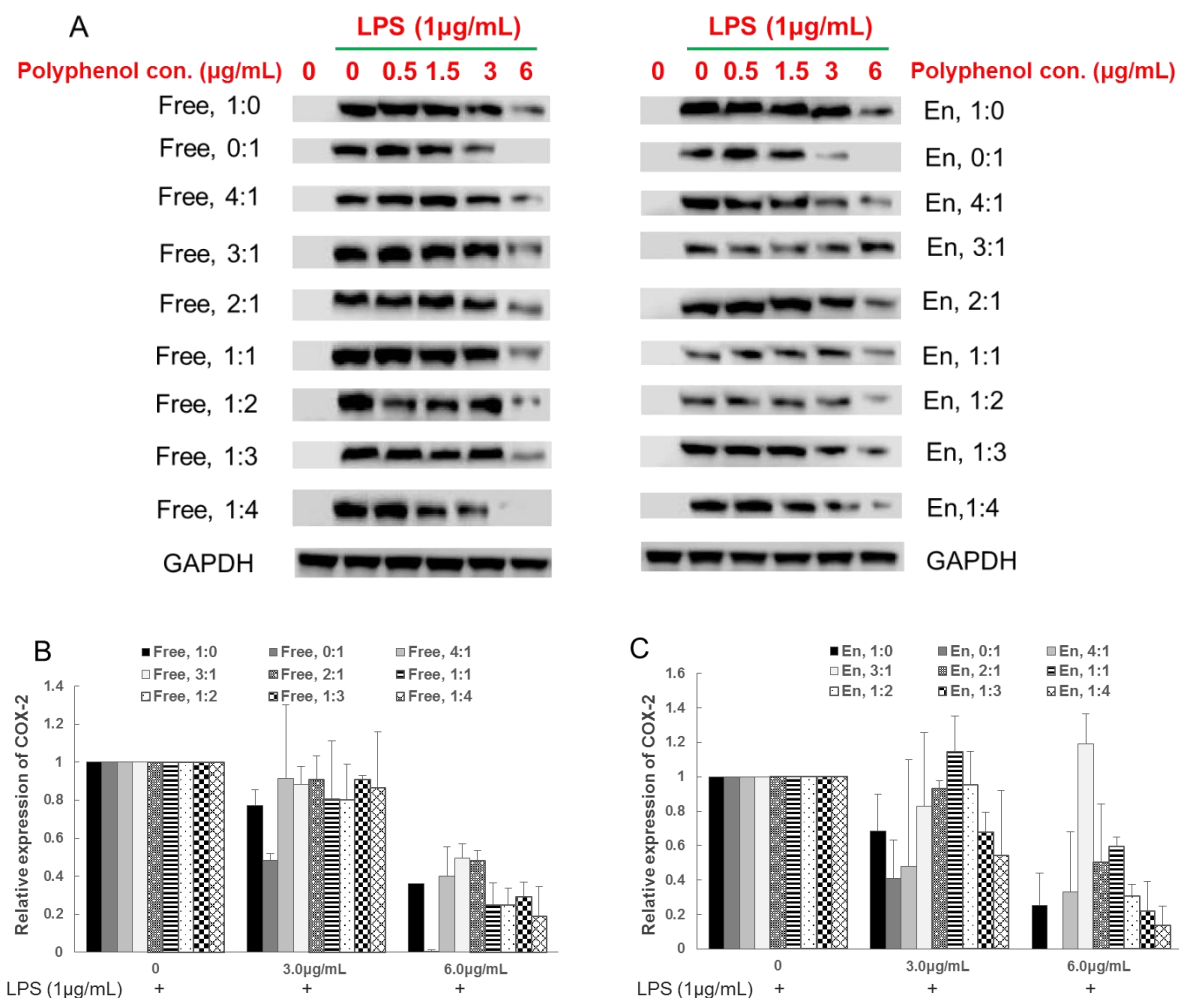


Figure 4-7. Western blot analysis of COX-2 expression in LPS-induced RAW 264.7 cells as inhibited by 0-6 μg/mL free or encapsulated (En) apigenin (AP) and curcumin (CUR) at mass ratios from 1:0 to 0:1: images (A) and relative expression (B, free; C, En). GAPDH represents the housekeeping protein. The first “0 μg/mL” represents the negative control without LPS and polyphenols. The second “0 μg/mL” represents the positive control with LPS but without polyphenols. Relative expression of COX-2 protein was calculated compared to the positive control cells. Error bars are standard deviations (n = 3).

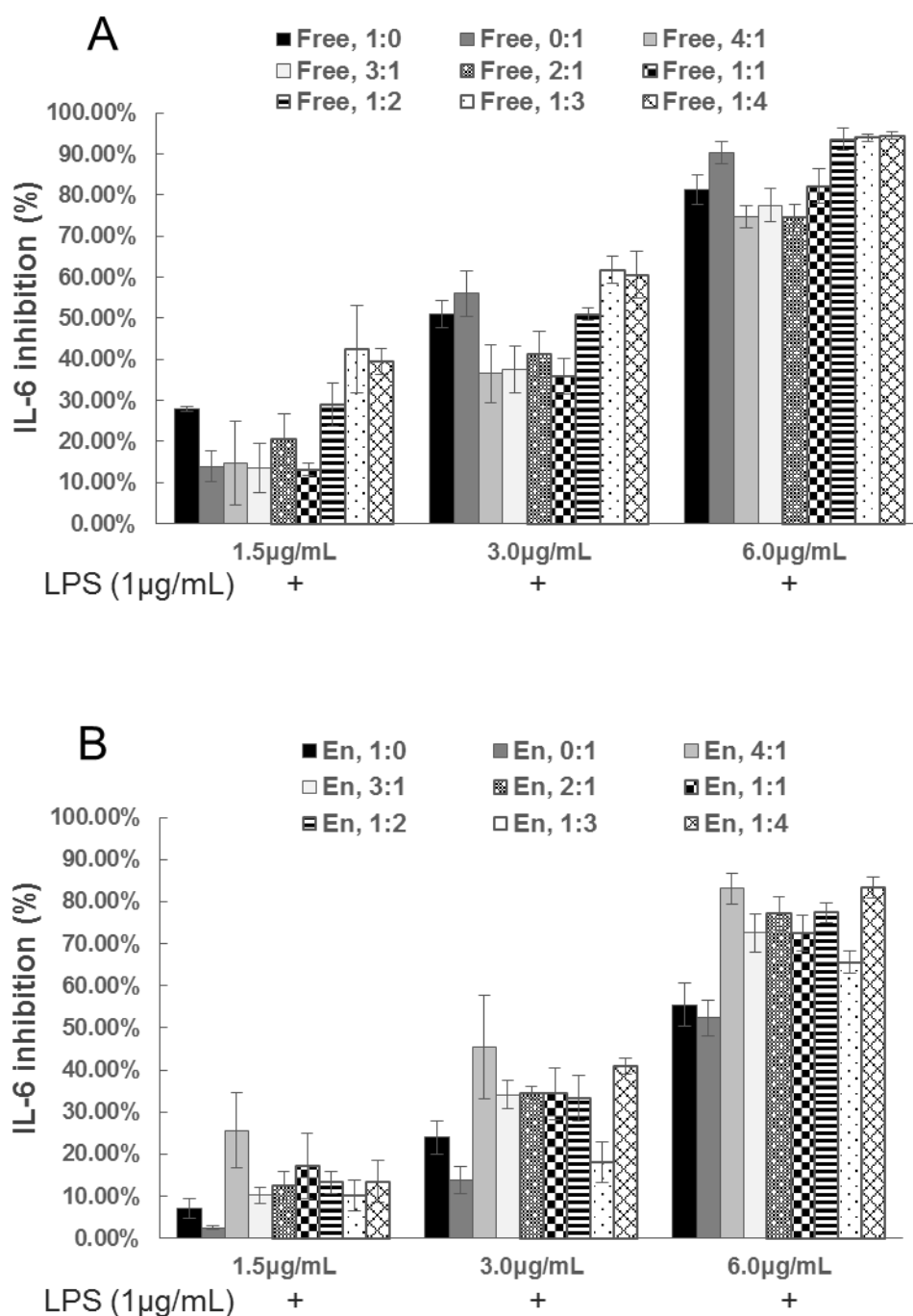


Figure 4-8. IL-6 production in RAW 264.7 cells stimulated with 1µg/mL LPS for 24 h as inhibited by 1.5-6.0 µg/mL free (A) or encapsulated (B) apigenin (AP) and curcumin (CUR) at different mass ratios. The inhibition percentage was calculated with respect to the LPS-treated positive control. Error bars are standard deviations (n = 3).

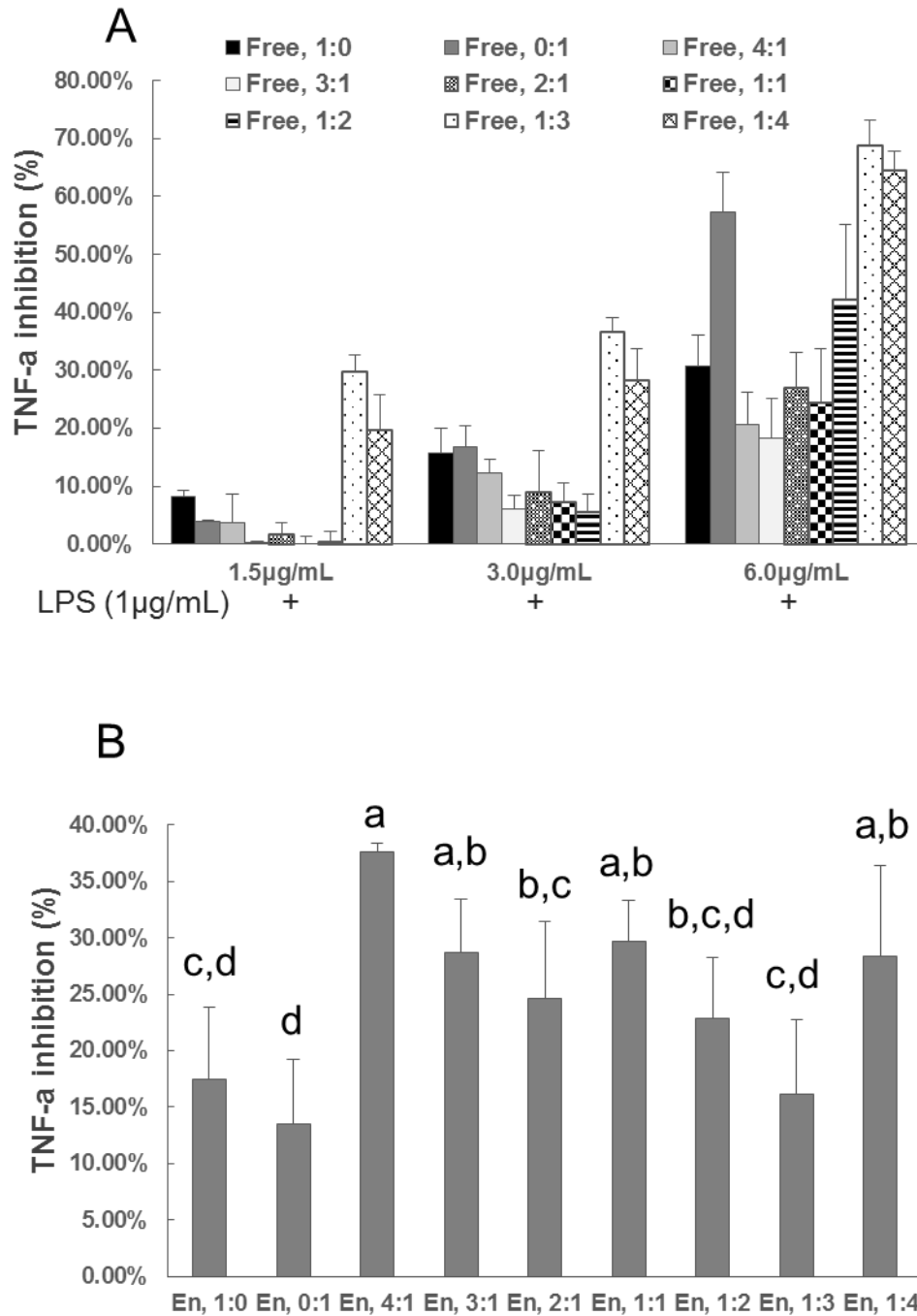


Figure 4-9. TNF- α production in 1 μ g/mL LPS-induced RAW 264.7 cells for 24 h as inhibited by 1.5-6.0 μ g/mL free (A) or 6.0 μ g/mL encapsulated (B) apigenin (AP) and curcumin (CUR) at different mass ratios. The inhibition percentage was calculated with respect to the LPS-treated positive control. Error bars are standard deviations ($n = 3$). Different letters above columns in B indicate significant difference ($p < 0.05$).

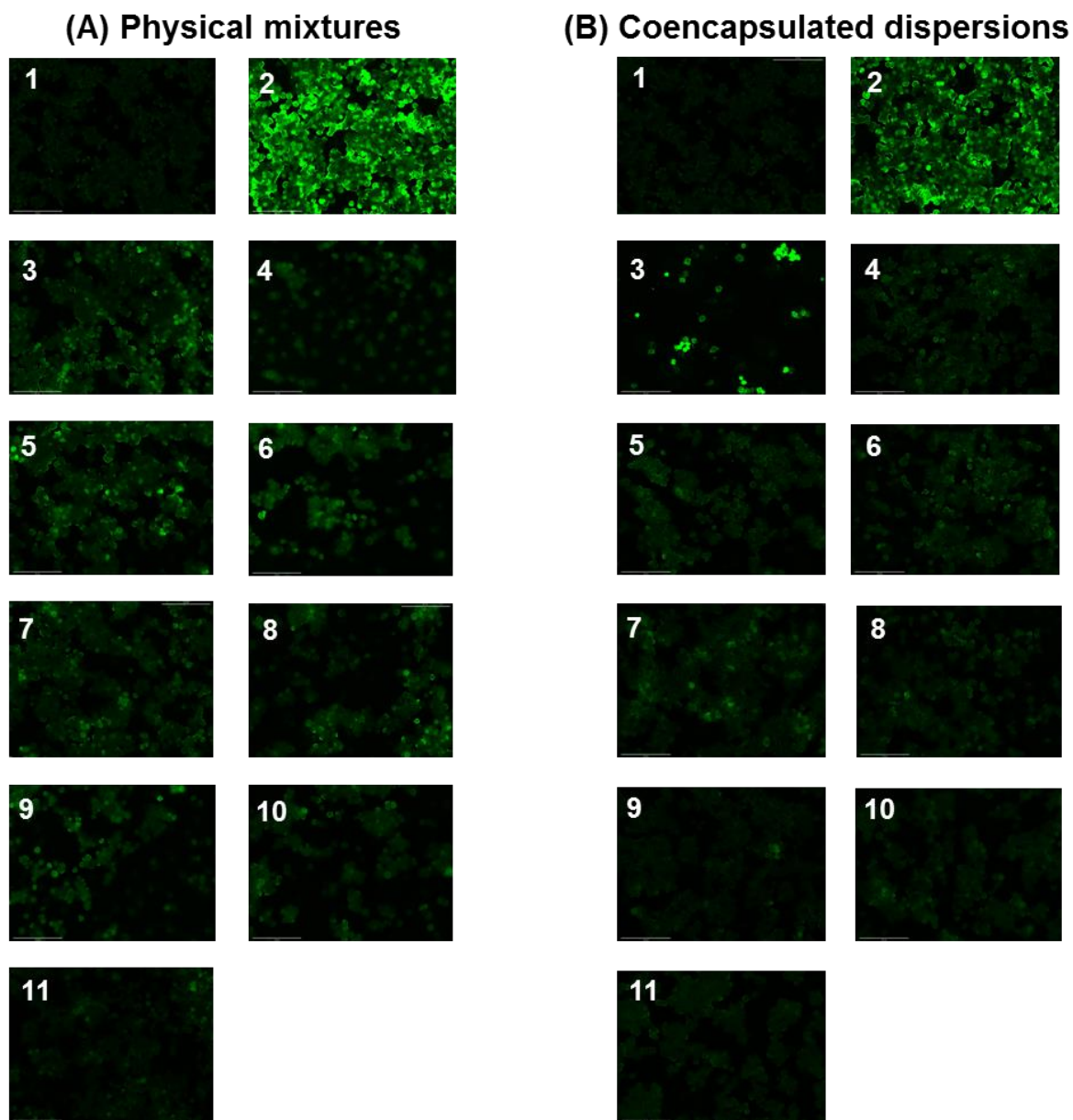


Figure 4-10. Fluorescence microscopy analysis of intracellular reactive oxygen species (ROS) production as inhibited by 6 $\mu\text{g/mL}$ free (A) and encapsulated (B) apigenin and curcumin at mass ratios of (3)1:0, (4) 0:1, (5) 4:1, (6) 3:1, (7) 2:1, (8) 1:1, (9) 1:2, (10) 1:3, and (11) 1:4. Cells were treated for 24 h and were activated by 1 $\mu\text{g/mL}$ LPS in the last 18 h. Figure 1 shows the negative control without LPS and polyphenols. Figure 2 shows the positive control with LPS but without polyphenols. Pictures were taken at 20 $\times$ magnification after incubation with 10 μM of 2',7'-dichlorofluorescein diacetate.

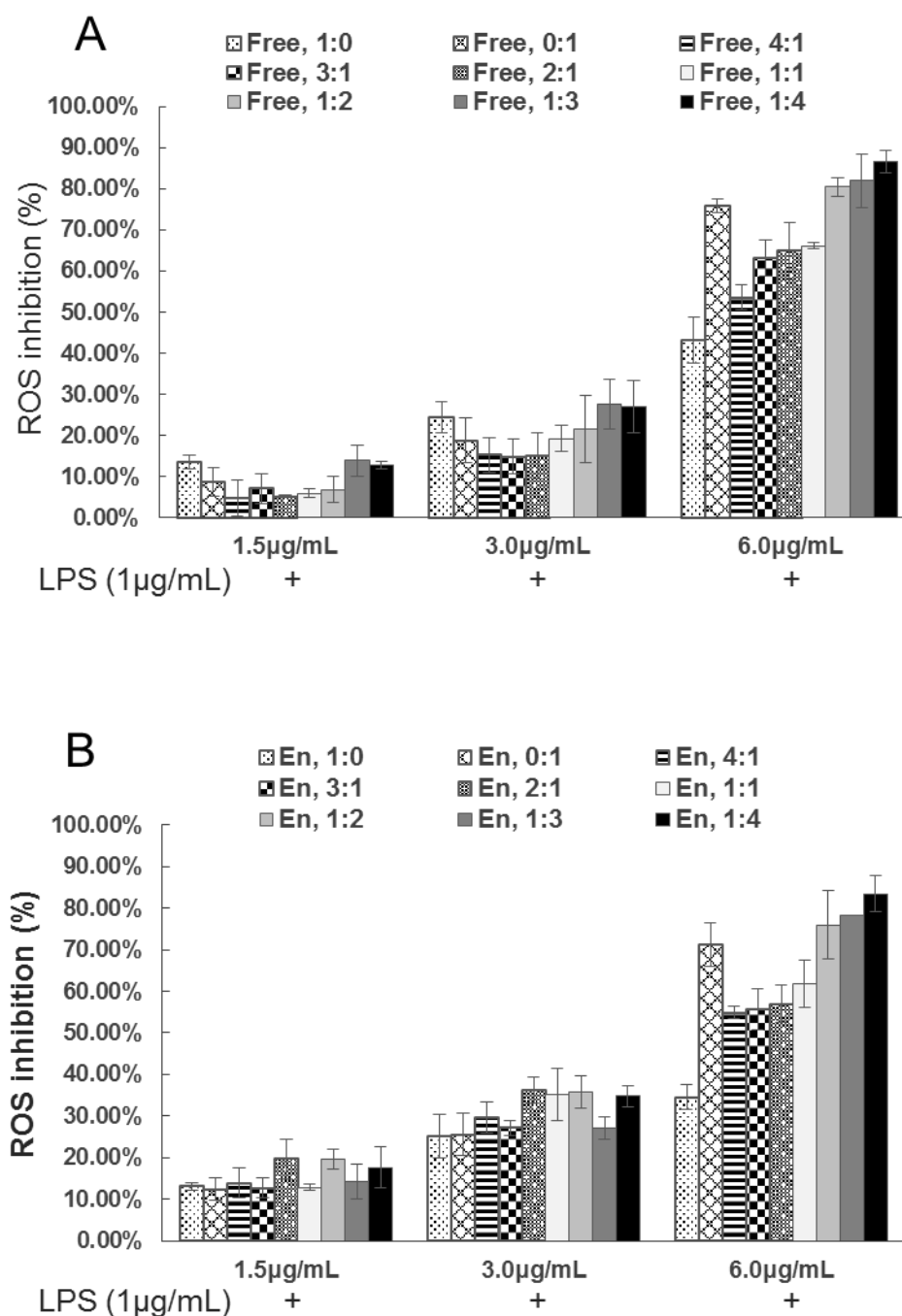


Figure 4-11. Production of reactive oxygen species (ROS) as inhibited by 1.5-6.0 µg/mL free (A) or encapsulated (B) apigenin (AP) and curcumin (CUR) at different mass ratios investigated by fluorescence spectrophotometry at 485 nm/528 nm. Cells were treated for 24 h and were activated by 1 µg/mL LPS in the last 18 h. Inhibition percentage was calculated based on fluorescence intensity at 485 nm/528 nm with respect to the positive control. Error bars are standard deviations (n = 3).

Chapter 5. Concluding remarks and future work

5.1 Conclusions

Colorectal cancer (CRC) continues to be a major health concern worldwide, accounting for the second leading cause of cancer-related death. The poor lifestyle and unhealthy diets are largely responsible for the increased incidence. Chronic inflammation is another great threat to public health since it has long been recognized as a risk factor on the progression of various human cancers. Due to the side effects of current treatments, many efforts have been focused on natural polyphenols as potential alternatives to intervene the above threatening health problems.

Apigenin is one of the most promising polyphenols with low toxicity. However, the poor water solubility of apigenin is an important obstacle to achieve higher efficacy as a therapeutic agent. The aim of this dissertation was to fabricate a low-cost, organic solvent-free and food biopolymer-based nanodelivery system, and evaluate the anti-colorectal cancer fate of the nanodelivered apigenin both *in vitro* and *in vivo*, as well as the anti-inflammatory activities in the LPS-activated macrophages, with a focus to enhance synergistic effects of co-encapsulated apigenin and curcumin.

In chapter 2, a pH-cycle method was effectively applied to encapsulate apigenin in whey protein isolate, to achieve high encapsulation efficiency, loading capacity and good storage stability. The improved solubility, stability and dispersibility of apigenin after nanoencapsulation significantly enhanced the cellular uptake of apigenin in human colon cancer cells (HCT-116 and HT-29). Both free and nanoencapsulated apigenin possessed promising anti-proliferative activity against HCT-116 and HT-29, which was correlated to the

induction of cell apoptosis. These results suggest the potential of the nanodelivered apigenin in the development of functional beverages for disease prevention.

In Chapter 3, the *in vivo* efficacy of apigenin encapsulated in WPI was evaluated. Oral bioavailability of apigenin in the mice serum and colon mucosa was significantly enhanced by nanoencapsulation to 1.39 times and 1.41 folds that of free apigenin, respectively. *In vivo* anti-cancer activity investigated in Apc^{Min/+} mice, by evaluating tumorigenesis, tissue apoptosis, tumor markers expression and levels of pro-inflammatory cytokines, however, was not significantly different among different treatment groups. Both free and encapsulated apigenin did not positively suppress colon carcinogenesis *in vivo* in the current animal model. More comprehensive and extensive studies need to be done to address the potential *in vivo* anticancer activity of nanoencapsulated apigenin.

In Chapter 4, the synergistic anti-inflammatory activity in LPS-stimulated RAW 264.7 cells was investigated for apigenin and curcumin, in simple mixture and coencapsulated forms. Results showed that, under non-toxic concentrations, the simple mixtures with a higher proportion of curcumin showed a greater extent of synergism inhibiting NO production, suppressing iNOS enzyme expression, decreasing pro-inflammatory cytokines (IL-6 and TNF- α), and reducing ROS level. These synergistic anti-inflammatory effects of apigenin and curcumin were found to be enhanced after co-encapsulation.

Overall, this dissertation research resulted in a novel, scalable and food grade nanodelivery system for apigenin. The nanodelivered apigenin showed the anticancer and anti-inflammatory activities. The understanding of the *in vivo* efficacy of nanoencapsulated

apigenin and the enhanced synergistic interactions of coencapsulated apigenin and curcumin may provide multi-perspectives to develop functional foods incorporated with lipophilic phytochemicals for disease intervention.

5.2 Future work

Results in the current dissertation have demonstrated that nanoencapsulation using dairy proteins enhanced bioavailability of apigenin in the colon mucosa of apigenin. However, the nanoencapsulated apigenin did not significantly affect colon carcinogenesis *in vivo*. In addition to further studies that involve different dosages and mouse models to assess the potential *in vivo* anticancer efficacy of nanodelivered apigenin, nanoparticles can be further modified to improve their stability and enhance the anticancer effects of encapsulated compounds *in vivo*. As found in chapter 2, WPI was easily hydrolyzed by enzymes in gastrointestinal digestions, and covering another layer of indigestible polymer can be studied to result in a slower release of apigenin and thereby may lower the elimination rate and enhance the bioavailability *in vivo*. For instance, as a positive charged biocompatible polysaccharide, chitosan not only can improve the stability of nanoparticles, but also has been used as an absorption enhancer as the outside layer due to its mucous adhesiveness, and thus enhance the bioavailability of polyphenols.^{1, 2} Besides, since apigenin can accumulate in the colon mucosa, it would be of great importance to incorporate target ligands such as antibodies, small peptides and receptor binding agents on the surface of nanoparticles to increase the target specificity for colon cancer delivery meanwhile decrease the potential toxicity and adverse effects to normal cells.³ Other

studies such as analyzing the metabolites of apigenin, fluorescent labeling⁴ of nanoparticles to investigate the bio-distribution, absorption, and metabolism of apigenin, may help to explain the mechanisms and *in vivo* anticancer efficacy of apigenin.

On the other hand, it has been reported that combinations of two or more phytochemicals bring about changes in the ultimate biological effects, such as synergistic anti-oxidant, anti-cancer and anti-inflammatory activities. Compared with free polyphenols, the enhanced synergistic anti-inflammatory activities were observed after co-encapsulating apigenin and curcumin. Interestingly, the storage stability of apigenin in dispersion was also enhanced by co-encapsulating with curcumin. Therefore, apart from the biological activities, further studies such as molecular modelling approaches that investigate the molecular and atomic level mechanisms of such interactions may help to provide valuable insights to develop coencapsulated systems to achieve synergistic biological activities.

Reference

1. Werle, M.; Takeuchi, H. Chitosan–aprotinin coated liposomes for oral peptide delivery: development, characterisation and in vivo evaluation. *International journal of pharmaceutics* **2009**, 370 (1-2), 26-32.
2. Werle, M.; Takeuchi, H.; Bernkop-Schnürch, A. Modified chitosans for oral drug delivery. *Journal of pharmaceutical sciences* **2009**, 98 (5), 1643-1656.
3. Urbinati, G.; Marsaud, V.; Renoir, J.-M. Anticancer drugs in liposomal nanodevices: a target delivery for a targeted therapy. *Current topics in medicinal chemistry* **2012**, 12 (15), 1693-1712.
4. Laroui, H.; Dalmaso, G.; Nguyen, H. T. T.; Yan, Y.; Sitaraman, S. V.; Merlin, D. Drug-loaded nanoparticles targeted to the colon with polysaccharide hydrogel reduce colitis in a mouse model. *Gastroenterology* **2010**, 138 (3), 843-853. e2.

Vita

Shan Hong was born in Zhejiang, China. She received her Bachelor of Science degree in Food Science and Engineering from Sichuan University (Chengdu, China) and continued her master's education at Zhejiang University (Hangzhou, China), majoring in Food Science. In August 2016, she started her PhD program in Food Science at the University of Tennessee, Knoxville, working under the guidance of Dr. Qixin Zhong.