APPLICATIONS OF ZEIN-BASED MATERIAL IN NANO-ENCAPSULATION, PICKERING EMULSIONS, MICROSPHERES AND AMORPHOUS SOLID DISPERSIONS

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ABSTRACT OF THE DISSERTATION

Applications Of Zein-based Material In Nano-encapsulation, Pickering Emulsions, Microspheres And Amorphous Solid Dispersions

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With the increasing environmental concerns about the synthetic plastic materials, prolamine originated from grains have come back to people’s sight due to their unique characteristics: GRAS (Generally Recognized as Safe) nature with good biocompatibility and biodegradability, highly hydrophobic but soluble in 60%-80% aqueous alcohol, easy fabrication of particulate carrier at micro even nano level, and relatively slower digestibility for oral route-controlled release design, etc. Among them, zein is the first commercial prolamine originated from maize byproduct, which is usually produced as a white or light yellowish powder. As one of the historically manufactured plant proteins, zein has various applications in the industry like coatings, binders, fibers, etc. In the decade, zein’s advantages make it one potential biomaterial with intensive explorations in nutraceutical and medical delivery use, as well as an environmentally friendly structural material for the soft matter. However, there is still the specific limitation or scarce study for zein based materials to fulfill its application potentials in each trending field, including nano-encapsulation, Pickering emulsions, microspheres and amorphous solid
My Ph.D. thesis exploits the solutions to break through the instability limitations of zein-based material in nano-encapsulation and Pickering emulsions via chemical modification, and also fills its research gap for scaling up bioactive compound encapsulation using microsphere system and amorphous solid dispersions. Firstly, hydrophilic modification was successfully performed on zein material, in order to improve its colloidal stability by intrinsically enhancing its amphiphilic nature. The zein was conjugated with hydrophilic carboxymethyl dextran (CMD) through covalent linking, and a novel amphiphilic zein-based material, zein-carboxymethyl dextran (Zein-CMD), was successfully synthesized to self-assemble nano-micelles for delivery potential application. The results suggested that about 2 CMD molecules were conjugated per zein chain, which resulted in the transformation from secondary α-helix to β-sheet and random coil structure. After modification, Zein-CMD still took a rod-like conformation but more elongated than pristine zein in aqueous alcohol solution. The modified zein-based material could self-assemble spherical nano-micelles by conventional anti-solvent method, with a good control on colloidal stability and particle size within a physiological pH environment, regardless of zein’s precipitation tendency due to its isoelectric point of ~ 6.2.

Secondly, hydrophilic modified zein-based material was self-assembled into nano-sized micelles to enhance the encapsulation efficiency of lipophilic phytochemicals. Dihydromyricetin (DMY) loaded Zein-CMD nano-micelles, DMY/Zein-CMD, was prepared through anti-solvent method. The Dihydromyricetin load was up to 30 wt.%, and a better colloidal stability was achieved overwhelming solely zein carrier or zein with CMD as the outer. In addition, DMY encapsulated in the Zein-CMD carrier was confirmed as amorphous status, with hydrogen bonding found to synergistically prevent
recrystallization of DMY with hydrophobic interactions. In comparison, dissolution profile of DMY/Zein-CMD was significantly improved as compared to pristine zein in bio-relevant media.

In the following section, hydrophobic modification on zein material was also conducted to resolve the unstable issues of Pickering emulsions (PE) based on pristine zein particles. The typical hydrophobic lauryl chains were successfully grafted onto zein to adjust its hydrophile-lipophile balance (HLB) to better stabilize PE. It is indicated that the HLB of lauryl-zein conjugate could be tuned by 1-8 lauryl chains per zein chain, which was visually quantified by water contact angle as well. Through anti-solvent and ultrasonic treatments, modified zein conjugate particles were successfully prepared with narrowly size distribution. These particles at low concentration of 1 wt% could better stabilize 70% oil internal phase than that at 50% oil phase, and overwhelmed the breakage of pristine zein based Pickering emulsions after 1 week’s storage at the ambient environment. The potential mechanism was discussed that the increase of hydrophobicity through the lauryl grafting correspondingly enhanced the surface tension. A new insight was introduced on PE stabilization containing high oil internal phase, using hydrophobic modified zein conjugate particles without other additives.

Besides chemical modification on zein material, encapsulation of poor soluble bioactive compounds at scale-up level was also discussed on zein-based microsphere delivery system and amorphous solid dispersions. A novel zein-based microsphere delivery system containing amorphous resveratrol was prepared by anti-solvent method and following freeze drying technology. The results showed that up to 20 wt% of amorphous resveratrol was stabilized in zein microspheres possibly via hydrogen bonding, and the amorphous formulation could maintain stable for 3 months. A better dissolution performance with enhanced solubility from amorphous resveratrol was achieved from
zein microspheres as compared to equivalent crystalline resveratrol. Free drying technology can be a way to manufacture hydrophobic phytochemical loaded microspheres based on zein material, but a limit of the initial load could be a concern regarding on the high crystallization tendency.

Lastly, amorphous solid dispersions (ASD) containing felodipine and polymeric carrier zein were produced by spray drying technology. The solid state characterization results demonstrated that amorphous origin of ASD was maintained under 3 months’ accelerated stability study, with spherical particles of about 1 um were observed without any birefringence in the micro condition. Only one single glass transition ($T_g$) was detected around 128.6 °C without exotherms or endotherms, indicating the good miscibility of felodipine in polymeric zein through spray drying. Based on cumulative bioaccessibility of felodipine through TIM-1 in vitro digestion model, a 6-8 times increased bioaccessibility from ASD was achieved as compared to equivalent crystalline felodipine mixed with zein. The spray dried amorphous solid dispersions using zein as polymeric excipient was proved to maintain saturation status of felodipine and enhance its bioaccessibility in simulated upper intestinal tract.
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TABLE OF CONTENTS

ABSTRACT OF THE DISSERTATION .......................................................................................... ii
ACKNOWLEDGEMENT ...........................................................................................................vi
TABLE OF CONTENTS .......................................................................................................... viii
LIST OF TABLES .................................................................................................................. xii
LIST OF FIGURES ................................................................................................................. xiii

CHAPTER I. INTRODUCTION. ............................................................................................... 1
1. Rationale and Hypothesis. ................................................................................................. 1
2. Specific Research Objectives ............................................................................................. 3
   3.1. Zein-based Particulate Delivery System. ................................................................. 6
   3.2. Zein Stabilized Pickering Emulsions ......................................................................... 11
   3.3. Solid Dispersions ....................................................................................................... 12
   3.4. Phytochemicals ........................................................................................................... 15
   3.5. Solid-state Characterizations ...................................................................................... 21

CHAPTER II. PREPARATION AND CHARACTERIZATION OF ZEIN-CARBOXYMETHYL DEXTRAN AS SELF-ASSEMBLED NANO-MICELLES .................................................................................................................. 25
1. Introduction ....................................................................................................................... 25
2. Materials and Methods ..................................................................................................... 27
   2.1. Materials .................................................................................................................... 27
   2.2. Preparation of Zein-Carboxymethyl Dextran (Zein-CMD) ......................................... 28
   2.3. Characterizations of Zein-CMD .................................................................................. 29
   2.4. Small-angle X-ray scattering (SAXS) ....................................................................... 30
   2.5. Preparation of Zein-CMD micellar dispersion ............................................................ 30
   2.6. Critical micellar concentration (CMC) ...................................................................... 30
   2.7. Particle size .................................................................................................................. 31
   2.8. Micellar morphology observation .............................................................................. 31
3. Results and Discussions .................................................................................................... 31
   3.1. Carboxymethylation of dextran ................................................................................. 31
   3.2. Conjugation of CMD onto zein .................................................................................. 33
3.3. Conjugation degree estimated by SEC and XPS.................................34
3.4. Secondary structural difference between Zein-CMD and pristine zein ........35
3.5. Molecular conformation and dimensional difference between Zein-CMD and pristine zein.................................................................36
3.6. Critical Micellar Concentration (CMC) of Zein-CMD ........................39
3.7. Particle Size and Stability of Zein-CMD Micellar Dispersions.............39
3.8. Morphology study of Zein-CMD Nano-Micelles............................43

4. Conclusions.....................................................................................45

CHAPTER III. PREPARATION OF AMORPHOUS DIHYDROMYRICETIN WITH ZEIN-CARBOXYMETHYL DEXTRAN AS THE NANO-CARRIER........46
1. Introduction....................................................................................46
2. Materials and Methods.................................................................49
   2.1. Materials.................................................................................49
   2.2. Preparation of DMY/Zein-CMD.............................................50
   2.3. Loading and encapsulation efficiency....................................50
   2.4. Particle size and zeta potential. .............................................51
   2.5. Solid-state characterizations..................................................51
   2.6. Thermal analysis......................................................................52
   2.7. Morphology study.................................................................53
   2.8. Dissolution study in bio-relevant media.................................53
3. Results and Discussions.................................................................54
   3.1. DMY/Zein-CMD dispersion....................................................54
   3.2. Interactions between DMY and Zein-CMD..............................57
   3.3. Accelerated stability test.........................................................58
   3.4. Thermal analysis......................................................................60
   3.5. Morphology study.................................................................62
   3.6. Dissolution study in bio-relevant media....................................64
4. Conclusions.....................................................................................66

CHAPTER IV. OIL-IN-WATER PICKERING EMULSIONS STABILIZED BY LAURYL MODIFIED ZEIN PARTICLES...........................................68
1. Introduction....................................................................................68
2. Materials and Methods.................................................................71
   2.1. Materials................................................................................71
CHAPTER VI. ENHANCING FELODIPINE DISSOLUTION AND BIOACCESSIBILITY THROUGH AMORPHOUS SOLID DISPERSIONS CONTAINING ZEIN. .................................................................109

1. Introduction........................................................................................................109

2. Materials and Methods....................................................................................111
   2.1. Materials......................................................................................................111
   2.2. Preparation and Stability of ASD................................................................112
   2.3. Solid State Characterizations......................................................................112
   2.4. Dissolution Study ......................................................................................114
   2.5. Suspension of ASD Felodipine/Zein.........................................................115
   2.6. TNO gastro-intestinal model-1 (TIM-1)..................................................116

3. Results and Discussions.....................................................................................117
   3.1. Basic Characterizations of Felodipine and Zein......................................117
   3.2. Solid State Characterizations of ASD Felodipine/Zein...........................118
   3.3. Dissolution Study in FeSSIF: ....................................................................121
   3.4. ASD Suspension..........................................................................................123
   3.5. In Vitro Digestion of ASD Felodipine/Zein..............................................124

4. Conclusions........................................................................................................127

CONCLUSIONS .......................................................................................................129

REFERENCES .........................................................................................................131
LIST OF TABLES.

Table I-1. Representative zein-based particulate delivery system.................................8
Table II-1. Average particle size of Zein-CMD nano-micelles.................................................39
Table III-1. Loading and encapsulation efficiency by DMY load ..............................................54
Table III-2. Kinetic solubility of DMY released from DMY/Zein-CMD or crystalline DMY in bio-relevant media .............................................................................................................64
Table IV-1. Stability characteristics of Pickering emulsions stabilized by zein conjugate or zein particles .................................................................................................................................................84
Table V-1. Zein microspheres with different initial resveratrol loading..................................97
Table VI-1. Felodipine Solubility in the simulated intestinal (SIF, pH 6.5) with or without added polymers at 37 °C .................................................................................................................................121
LIST OF FIGURES.

Figure I-1. Typical proportions of amino acids in a commercial zein (reprint from ref. [19]). ............................................................... 10
Figure I-2. Schematic diagram of two types of Pickering emulsion. ...................... 11
Figure I-3. Diagram of solid dispersions classified at four generations (Reprinted from ref. [33]) ................................................................. 15
Figure I-4. Illustration of dietary phytochemicals classification (Reprinted from Ref. [58]) ............................................................................. 16
Figure I-5. Sub-groups of dietary flavonoids (Reprinted from Ref. [58]) ............... 17
Figure I-6. Molecular structure of Dihydromyricetin (DHM)........................................... 19
Figure I-7. Trans and cis structure of Resveratrol (Reprinted from Ref. [75]) ....... 20
Figure I-8. Typical XRPD pattern of crystalline material, amorphous material and mixture of crystalline and amorphous material ........................................... 22
Figure II-1. Schematic preparation of Zein-CMD............................................................ 29
Figure II-2. (a) FTIR spectra and (b) 1H NMR spectra of CMD and pristine dextran. 1H NMR (500 MHz, D2O): δ 5.10-4.85 (anomeric, 1, CH1 of CMD), δ 4.10 (m, 2, CH2COO-), δ 3.90-3.30 (m, 6, CH2,3,4,5,6 of CMD). ................................................................. 32
Figure II-3. (a) FTIR spectra and (b) 1H NMR spectra of Zein-CMD and pristine zein. 1H NMR (500 MHz, DMSO-d6): δ 3.30-2.80 (m, 6, CH2,3,4,5,6 from CMD backbone)... 33
Figure II-4. Size-exclusion chromatography (SEC) of Zein-CMD and zein. ............ 34
Figure II-5. XPS of Zein-CMD and zein......................................................................... 35
Figure II-6. CD spectra of Zein-CMD and zein............................................................... 36
Figure II-7. (a) SAXS profiles of Zein-CMD in 70% ethanol solution fitted by power law. (b) SAXS profiles of Zein-CMD in 70% ethanol solution fitted by the ellipsoid model. The concentration gradients included 5 mg/mL (red squares), 10 mg/mL (yellow diamonds), 20 mg/mL (blue triangles), and 40 mg/mL (green circles). ................................................................. 37
Figure II-8. Sizes of Zein-CMD or zein in 70% ethanol solution plotted against concentration. Squares and triangles represented the gyration radius (Rg) and cross-sectional radius (Rc), respectively. Solid line and dash line were used to distinguish Zein-CMD and zein. ...................................................................................... 38
Figure II-9. Critical micelle concentration (CMC) of Zein-CMD................................. 39
Figure II-10. The effect of concentration and pH on particle size of Zein-CMD nanomicelles. .................................................................................................................................................................................. 42

Figure II-11. (a) 2D AFM topography image and (b) 3D AFM image of Zein-CMD micelles at 0.1 mg/mL with scale at 1um. SEM images of Zein-CMD micelles at (c) 0.1 mg/mL and (d) 2 mg/mL (scale bar is 2um and magnification is x16000). ...................... 44

Figure II-12. TEM images of (a) a single Zein-CMD micelle and (b) aggregated Zein-CMD micelles prepared from 0.1 mg/mL micellar dispersion. Accelerating voltage is 100 kV and magnification is x75000. .................................................................................................................................................................................. 45

Figure III-1. Visual images of three formula dispersion: (a) DMY/Zein-CMD in water, (b) DMY/zein in water and (c) DMY/zein in CMD solution. The initial load of DMY for all three formula is 30 wt.%. .................................................................................................................................................................................. 56

Figure III-2. FTIR spectra of carrier Zein-CMD and DMY/Zein-CMD....................... 58

Figure III-3. (a) XRPD overlay of DMY/Zein-CMD under exposure to 25°C, 40%RH after 1 week, 1 month and 3 months. (b) XRPD overlay of DMY/Zein-CMD after 1 month’s exposure to three different storage conditions (40°C, 75%RH), (25°C, 75%RH) or (25°C, 40%RH). XRPD pattern of carrier Zein-CMD and the mixture of crystalline DMY and Zein-CMD (w/w, 3:7) are shown as the reference. (c) PLM image of mixture of crystalline DMY and Zein-CMD (w/w, 3:7). (d) PLM image of DMY/Zein-CMD after 3 month’s exposure to 25°C, 40%RH. (e) PLM image of DMY/Zein-CMD after 1 month’s exposure to 25°C, 75%RH. All PLM images were taken under magnification x200. ..... 59

Figure III-4. (a) DSC overlay of crystalline DMY and amorphous DMY/Zein-CMD. (b) mDSC curve of amorphous DMY/Zein-CMD. .................................................................................................................................................................................. 61

Figure III-5. (a) Height and (b) 3D AFM image of DMY/Zein-CMD. The Z range was 50 nm. .................................................................................................................................................................................. 63

Figure III-6. SEM image of DMY/Zein-CMD.............................................................................. 63

Figure III-7. (a) Image of turbid solution of crystalline DMY incubated in FaSSIF after filtration through 0.25 PTFE membrane. (b) Image of filtered clear supernatant of DMY/Zein-CMD incubated in FaSSIF. Time points from left to right were 15 min, 30 min and 60 min. .................................................................................................................................................................................. 65

Figure III-8. Dissolution curves of DMY/Zein-CMD or crystalline DMY alone in FaSSGF or FeSSIF. Each data point is a mean of three measurements ± SD. ..................... 66

Figure IV-1. FTIR spectra of lauryl-modified and pristine zein. Characteristic bands for protein and lauryl chains are labeled. .................................................................................................................................................................................. 76
Figure IV-2. $^1$H NMR spectra of overlay of zein and zein conjugate. $^1$H NMR spectra of both zein and zein-C0.5 around 0.7-1.3 ppm were zoomed in. .............................. 78

Figure IV-3. Intensity profile and ellipsoid model fit of zein-C1 (a) and zein-C4 (a’) zein conjugates in acetic acid. Variation trends of perpendicular semiaxis (solid point) and parallel semiaxis (hollow point) are shown in (b). In (c), solid triangles and squares are measurements of modified zein in acetic acid, the empty circles are measurements in acetic acid by Li et al \[186\], the empty diamond is the measurement in aqueous methanol solution by Tatham et al.\[187\], whereas the empty squares are from zein in aqueous ethanol solutions collected by Matsushima et al.\[188, 189\] ................................................................. 80

Figure IV-4. Intensity profile and ellipsoid model fit of zein (a), 1:1 zein conjugate (a’) and 4:1 zein conjugate (a'”) in 80% ethanol. Pair distance distribution function (PDDF) of zein (b), 1:1 modified zein (b’) and 4:1 modified zein (b”’). Variation trend of perpendicular semiaxis (c), parallel semiaxis (d) and radius of gyration $R_g$ calculated using ellipsoid model (solid point) and GNOM (hollow point) (e). ............................................. 81

Figure IV-5. Visual image of freshly prepared 1 wt% of (a) zein-C0.5 particle and (b) zein particle dispersions in water ........................................................................................................ 82

Figure IV-6. Water-in-air contact angle ($\theta_{wa}$) image (one droplet on the film): (a) zein, (b) zein-C0.5, (c) zein-C1, (d) zein-C2 and (e) zein-C4. Each data is an average of six measurements ± SD. ........................................................................................................ 83

Figure IV-7. Visual image of Pickering emulsions stabilized by 1 wt% of zein-C0.5 or zein particles at $\varphi = 0.5$ after storage period of (a) 1 day and (c) 7 days, or at $\varphi = 0.7$ after storage period of (c) 1 day and (d) 7 days. ........................................................................................................................................ 85

Figure IV-8. PE microscopic images for 1 wt% zein-C0.5 at (a) $\varphi = 0.5$ or (b) $\varphi = 0.7$. (c) PE microscopic image for 1 wt% zein at $\varphi = 0.5$. The scale bar of microscopic images was 100 μm in length. (d) Frequency sweep curves with frequency ranging at 0.1-100 rad/s. Storage modulus ($G'$) - filled, loss modulus ($G''$) - opened. (e) Apparent viscosity vs. shear rate (0.1-100 s$^{-1}$) fitted by power-law model ................................................................. 87

Figure IV-9. Relationship of degree of lauryl grafting and HLB. The red triangle is the measurement of water contact angle of kafirin by Xiao et al.\[164\]. ..................................................... 89

Figure V-1. Trans and cis structure of resveratrol .............................................................. 91

Figure V-2. TGA/DSC curve of Resveratrol ..................................................................... 98

Figure V-3. (a) XRPD overlay of four batches of freshly prepared microsphere samples (R10, R20, R30 and R40), compared with the crystalline pattern of equivalent mixture of
resveratrol/zein (wt./wt. = 9/1). (b) XRPD overlay of R10 under exposure to 25 °C, 40% RH after 0 day, 1 month and 3 months. (c) XRPD overlay of R20 under exposure to 25 °C, 40% RH after 0 day, 1 month and 3 months.

Figure V-4. PLM image of four batch samples (R40, R30, R20, and R10). Magnification was ×100.

Figure V-5. PLM image of microsphere suspension of R40 and R20 during solvent evaporation at different time points.

Figure V-6. FTIR spectra of resveratrol, R20, R40 and zein protein.

Figure V-7. Particle size distribution (PSD) of R20 and R40.

Figure V-8. SEM image of R20. Scale bar was 200 µm.

Figure V-9. (a) DSC overlay of resveratrol, R20 and R10. (b) mDSC of R20.

Figure V-10. Dissolution curves of D20 or resveratrol alone in bio-relevant media.

Figure VI-1. DSC and TGA thermograms of felodipine.

Figure VI-2. PXRD patterns of felodipine and zein.

Figure VI-3. (a) PXRD patterns of ASD Felodipine/Zein under condition of 40 ºC and 75% RH for 0 M and 3 M. (b) PLM image of ASD Felodipine/Zein. Magnification ×500.

Figure VI-4. (a) DSC and TGA thermograms of ASD Felodipine/Zein. (b) Modulated DSC thermograms of ASD Felodipine/Zein.

Figure VI-5. Dissolution of felodipine from solid dispersions containing three different polymeric excipients: zein, HPMC-AS and PVP-VA.

Figure VI-6. (a) PXRD patterns and (b) PLM images (magnification ×500) of suspension of ASD Felodipine/Zein in 0.5% w/v methylcellulose at the concentration of 3 mg/mL and 16 mg/mL.

Figure VI-7. Bioaccessible felodipine accumulated in every 30 min or 60 min interval. (a) Bioaccessible felodipine in jejunum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. (b) Bioaccessible felodipine in ileum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. (c) Total bioaccessible felodipine in both jejunum and ileum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation.

Figure VI-8. Cumulative bioaccessibility profile of felodipine (% of input) accumulated in every 30 min or 60 min. (a) Cumulative bioaccessibility of felodipine in jejunum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. (b)
Cumulative bioaccessibility of felodipine in ileum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. (c) Total cumulative bioaccessibility of felodipine in both jejunum and ileum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. .......................................................... 127
CHAPTER I. INTRODUCTION.

1. Rationale and Hypothesis.

In food science field, GRAS (Generally Recognized as Safe) protein-based materials originated from natural products are favored as alternatives to synthetic or semi-synthetic ones within a broad range of applications. Among them prolamines from grains have attracted massive of interests due to their productive and economic characters. Especially zein protein, as the representative commercialized prolamine extracted from corn process, is a very promising candidate with various unique properties for a wide spectrum of applications. Firstly, the alcohol soluble but hydrophobic nature of zein protein renders the fabrication of particulate delivery system, combined with its good biodegradability and biocompatibility. Besides, the soluble facts in neither water phase nor oil phase make it a Pickering emulsion stabilizer, to maintain a proper wettability between two phases. What’s more, the ability to withstand in gastric fluid provides more insights for zein protein as an novel and natural extended-released excipient in nutraceutical or drug product, which opens the gate to investigate its pre-formulation values as a carrier, e.g. microspheres, amorphous solid dispersions. Nevertheless, up to now though tons of research work have been carried out on the applications of zein-based materials, turning inward is a big trend by mixing other additives or polymers with respect to zein’s own limitations, e.g. hydrophobic nature usually fails encapsulation of highly lipophilic bioactive compounds, wettability preference over water phase increases instability index of Pickering emulsions, etc. Quite few effort has been tried in fulfilling its own demands in different application fields through chemical modification, typically by conjugating hydrophilic or hydrophobic functional molecules and breaking the barriers. In addition, zein being used as an biomaterial carrier for encapsulation of amorphous bioactive ingredients is still a gap, waiting to be filled for the evaluation on its future applications.
in oral solid dosage drug or nutraceutical product development.

Based on the above rational and our primary study results, I hypothesize that:

1) The chemical modification of zein towards a hydrophilic direction may compensate for adverse effects of zein’s hydrophobicity on its colloidal stability, and by enhancing the amphiphilic nature, self-assembly behaviors of modified zein-based material would be expected with good stability and particle size control within a physiological pH range.

2) As compared to pristine zein material or the physical mixture containing additives, our hydrophilic modified zein-based material would serve as an more effective nano-carrier for encapsulating highly lipophilic bioactive compounds under the same dosage, and a more stable colloidal delivery system with enhanced dissolution merits would be expected.

3) The hydrophobic modification might be a novel solution to achieving a better hydrophile-lipophile balance for the stability of zein-based Pickering emulsions, especially to accommodate high oil internal phase with low particle concentration.

4) The alcohol soluble but water insoluble character of zein material would drive the one-step microsphere formation via anti-solvent method, and the self-assembled microspheres may function as an encapsulation vehicle of amorphous bioactive compounds for the increase of solubility and enhancement of dissolution rate.

5) Being resistant to gastric environment, zein-based material may serve as a potential excipient for amorphous solid dispersions development of bioactive compound, thus serving as an extended-release carrier for the enhancement of bioaccessibility.

We expect that the exploratory work of modified zein-based materials towards either hydrophilic or hydrophobic path would pave a new way for breaking its own limitations and expanding its applications in various aspects. In addition, encapsulation of
amorphous bioactive compounds using zein-based vehicles would explore its applications in pre-formulation region and even in future nutraceutical or drug product development. The abovementioned academic research work would surely endow the extending applications of zein-based material covering aspects from nano-size or micro-size delivery system to surfactant-free Pickering emulsions, which should be of great importance for this commercialized prolamine product.

2. Specific Research Objectives.

This thesis presents five objectives with the aim to expand application potentials of zein-based material with respect to nano-scale delivery system, Pickering emulsions, microsphere delivery system and amorphous solid dispersions.


1) Carboxymethyl dextran (CMD) will be synthesized with dextran as raw hydrophilic polysaccharide.

2) Novel biomaterial zein-carboxymethyl dextran (Zein-CMD) will be synthesized by conjugating CMD with zein material, and the conjugation reaction will be confirmed by Fourier-transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (¹H-NMR).

3) The conjugation degree of CMD will be determined by gel permeation chromatography (GPC) and X-ray photoelectron spectroscopy (XPS).

4) The structural change post modification will be investigated by circular dichroism (CD) and small angle X-ray scattering (SAXS).

5) The Zein-CMD nano-micelles will be self-assembled via anti-solvent method, and the stability and particle size will be monitored within a physiological pH range.

6) The morphology of Zein-CMD nano-micelles will be studied by atomic force
microscopy (AFM) and scanning electron microscopy (SEM), and micro-scale structure of the nano-micelles will be imaged by transmission electron microscopy (TEM).

2.2. Preparation of Amorphous Dihydromyricetin with Zein-Carboxymethyl Dextran as the Nano-carrier.

1) Zein-CMD nano-micelles containing amorphous Dihydromyricetin (DMY) will be prepared by anti-solvent method, and under the same DMY input, pristine zein nanoparticles or zein particles coated by CMD will also be prepared as the comparative references.

2) The highest input of DMY will be determined by calculating loading efficiency and encapsulation efficiency based on UV-Vis spectrometry, and particle size with zeta-potential will be measured as the optimal formula determined.

3) The interactions between DMY and nano-carrier Zein-CMD will be studied by FTIR.

4) Amorphous origin of DMY will be confirmed by powder X-ray diffraction (PXRD) and polarized light microscopy (PLM), and accelerated stability study will be conducted to determine the predominant impact factors.

5) The glass transition ($T_g$) of Zein-CMD nano-micelles containing amorphous DMY will be detected by modulated differential scanning calorimetry (mDSC).

6) The dissolution study in bio-relevant media will be performed to compare dissolution behaviors of amorphous DMY encapsulated using Zein-CMD nano-micelles with the one of pristine crystalline DMY.

2.3. Oil-in-water (O/W) Pickering Emulsions Stabilized by Lauryl Modified Zein conjugates.

1) Lauryl modified zein material will be synthesized by grafting lauryl chains onto zein material.

2) The novel structure will be confirmed by $^1$H-NMR and FTIR, and the modification
degree will be sorted by peak area ratio of $^1$H-NMR and water-in-air contact angle.

3) The structural change of lauryl modified zein material will be investigated by SAXS.

4) The lauryl zein conjugates will be prepared by anti-solvent method, and the particle size will be monitored as well.

5) Pickering emulsions based on lauryl zein conjugates will be produced at high oil internal phase (e.g. 70%), and stability will be evaluated as compared to pristine zein-based PE.

6) The rheology study will be performed using strain-controlled rheometer.

2.4. Zein Microsphere Delivery System of Amorphous Resveratrol.

1) Zein microspheres of amorphous Resveratrol will be developed based on the strategy of solid dispersions by combining anti-solvent method and freeze-drying technology.

2) A variety of Resveratrol input will be tried with the loading efficiency and encapsulation efficiency determined based on UV-Vis spectrometry.

3) The molecular interactions between Resveratrol and zein microsphere will be studied by FTIR.

4) The particle size distribution and morphology of zein microsphere containing Resveratrol will be studied by laser diffraction and SEM respectively.

5) Amorphous identity of Resveratrol within 3 months’ stability test will be confirmed by PXRD, PLM and DSC.

6) The dissolution behaviors of zein microsphere containing amorphous Resveratrol will be studied in bio-relevant media with pristine crystalline Resveratrol as the blank.

2.5. Enhancing Felodipine Dissolution and Bioaccessibility through Amorphous Solid Dispersions Containing Zein.

1) Amorphous solid dispersions (ASD) of felodipine using zein as the excipient (Felodipine/Zein) will be prepared by spray drying technology, and another two ASD
batches of felodipine containing commercialized excipient hydroxypropyl methylcellulose acetate succinate (Felodipine/HPMC-AS) or polyvinylpyrrolidone-vinyl acetate (Felodipine/PVP-VA) will also be prepared as the references.

2) Amorphous identity of felodipine in ASD formulation will be confirmed by a series of solid-sate characterizations within 3 months.

3) The single glass transition ($T_g$) of ASD will be detected by reversible heat flow of mDSC.

4) The dissolution behaviors of Felodipine/Zein will be compared with Felodipine/HPMC-AS and Felodipine/PVP-VA in bio-relevant media.

5) Solid suspension of Felodipine/Zein will be developed, with its physical stability for pre-formulation purpose will be evaluated by PXRD and PLM.

6) The TNO dynamic gastro-intestinal model-1 (TIM-1) study will be performed to assess the bioaccessibility input of ASD formulation with pure crystalline felodipine suspension as the blank.


3.1. Zein-based Particulate Delivery System.

Zein, as the major storage protein extracted from the endosperm of the corn, is regarded as the typical commercial prolamine with a large production in the market. The unique characteristic of hydrophobicity is credited to its more than one half of nonpolar amino acids. Though the nutritional deficiency due to lack of basic and acidic amino acids, the unique physicochemical property has made zein protein as one promising GRAS and biodegradable [1] vehicle in delivery system of both nutraceutical [2] and drugs [3]. The alcohol-soluble but aqueous-insoluble character renders the fabrication of zein-based particulates with multiple advantages, such as controlled release in gastrointestinal
tract due to the relatively slow digestibility of Zein protein \[^4\], easy localization with enhanced retention in GI, biodegradability and related less regulatory concerns \[^5\], etc.

Through anti-solvent method, zein particulates with different size (from nano-size to micro-size) can be fabricated. Generally, zein in 60%-90% alcohol solution was added into anti-solvent, typically water, to abruptly change the polarity around zein molecules leading to the formation of zein particles. As the alcohol composition is decreased significantly, zein becomes insoluble in the solution to induce the phase separation. The core mechanism is simultaneous particle aggregation and solidification due to the solubility decrease. Based on the mechanism above, both bioactives and zein material are dissolved in alcohol binary solution, and then pre-mixed homogenous solution can be added into water to co-precipitate zein particles containing bioactive candidates.

Typically, zein particles are classified into nano scale (nano-particles) or micro scale (microspheres) based on the particle size distribution, which can be effected by multiple fabrication parameters, including alcohol concentration of zein stock solution, stirring rate or shearing rate, pH condition, etc. Normally, smaller particle size can be achieved by increasing stirring/shearing rate and alcohol concentration \[^6\]. Moreover, ethanol has been reported to be prior to methanol and isopropanol for fabricating zein particles with relatively smaller size and particle size distribution index (PDI) \[^7\].

**Table I-1** shows some representative zein-based particulate delivery system at nano-scale or micro-scale. A variety of zein-based nanoparticles or microspheres have been studied for encapsulation of different bioactives, \(e.g\). curcumin-loaded nanoparticles with particle size at 175-250 nm. It is noted that curcumin-loaded nano-micelles based on mPEG-zein had smaller particle size at 124 with lower PDI, indicating that hydrophilic part of mPEG could help fabricate core-shell micelles with smaller size, to enhance
passive permeation through the intestinal epithelial cell and improve stability in the mimicking serum [8]. In addition, physical modification of zein nanoparticles by coating hydrophilic polysaccharide, such as carboxymethyl chitosan, also reduced the particle size and increased surface charge to enhance colloidal stability in the solution [9]. For the micro-scale, Ivermectin-loaded zein microspheres are studied as one model and zero-order release of Ivermectin was achieved after tablet formation [10].

Table I-1. Representative zein-based particulate delivery system

<table>
<thead>
<tr>
<th>Fabrication method</th>
<th>Bioactive</th>
<th>Characters</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| Nanoparticles by phase separation | 5-Fluorouracil | Size: 114.9 nm  
Surface potential: $-45.75 \pm 0.3$ mV  
Good drug loading stability at 4 °C for 6 months | [11] |
| Nanoparticles by pH induced nano-precipitation | Coumarin | Size: 365 nm with 0.36 PDI  
Surface potential: $-11.3 \pm 1.8$ mV | [12] |
| Nanoparticles by electro-hydrodynamic atomization | Curcumin | Size: 180 - 250 nm  
No big change at 23 °C/43% RH for 3 months | [13] |
| Nano-micelles based on mPEG-zein conjugation | Curcumin | Size: 124 nm with 0.25 PDI  
Surface potential: $-7 \pm 1.6$ mV | [8] |
<table>
<thead>
<tr>
<th>Fabrication method</th>
<th>Bioactive</th>
<th>Characters</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoparticle using spray dried</td>
<td>N/A</td>
<td>To reduce ethanol content in zein nanoparticle fabrication</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Size: 120–140 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surface charge: $-44 \pm 3$ mV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controlled release and enhanced stability</td>
<td></td>
</tr>
<tr>
<td>Nanoparticles based on zein/</td>
<td>Indole-3-carbinol and 3,3’-diindolylmethane</td>
<td>Size: 90-115 nm by coating compared to 250 nm without coating</td>
<td>[9]</td>
</tr>
<tr>
<td>carboxymethyl chitosan</td>
<td></td>
<td>Surface charge: $-20.0$ mV by coating compared to $-11.0$ mV without coating</td>
<td></td>
</tr>
<tr>
<td>Microsphere by phase separation</td>
<td>Ivermectin</td>
<td>Size: 0.3–1.2 um</td>
<td>[10]</td>
</tr>
<tr>
<td>method</td>
<td></td>
<td>Zero-order release as tablet</td>
<td></td>
</tr>
</tbody>
</table>

Based on the previous results, it is very promising to use zein-based particulate system for the delivery use. However, isoelectric point (~6.2) of zein has been a limitation, triggering the aggregates in water solution around neutral pH. In order to better stabilize the zein-based particulate system, surfactants or polysaccharides are often physically mixed with the particles as an outer coater. Among small-molecule surfactants, natural and safe sodium caseinate (SC) is widely acceptable to stabilize particulate suspension at neutral pH. In addition, Luo et al. developed
zein/carboxymethyl chitosan (zein/CMC) nanoparticle system to improve stability and control release \(^9, 17\).

Though many efforts have been taken with respect to physical modification on zein particulate system, there is few work about the chemical modification for intrinsic change of zein molecular structure and the corresponding demanded property. Based on the amino acid proportion of zein protein (as displayed in Figure I-1), there are about 30% of amine side group containing potential reaction sites \(^1\), where acylation reaction with hydrophilic carboxyl methylated polymers has been reported to be performed by using bio-conjugate reagent EDC/NHS \(^18\). It opens a novel path considering the instability issue of zein particulate system due to the nearly neutral isoelectric point, that zein material can be conjugated with other hydrophilic biomaterial to improve the amphiphilic property and subsequent colloidal stability during the preparation process.

![Figure I-1. Typical proportions of amino acids in a commercial zein (reprint from ref. \(^19\)).](image-url)
3.2. Zein Stabilized Pickering Emulsions

Emulsions have been used widely in a variety of industries including cosmetic, pharmaceuticals, food, etc. Different from common emulsions stabilized by either common surfactants or amphiphilic macromolecules, Edible particles based PE have received lots of attention due to its outstanding resilience to coalescence in the recent decades. The Pickering emulsions is named by the scientific contribution from S.U. Pickering [20], and the solid particles are stabilizers which should be only partially wetted but still insoluble in both of phases (one is considered as continuous phase and another one is dispersed phase), so that sufficient interface absorption can be achieved. Moreover, the particle size is supposed to be smaller than emulsion droplet size. Regarding the PE types and its storage stability, the contact angle of three phases $\theta$ (continuous phase, dispersed phase and solid particles) plays the key role. If particles are hydrophilic with the $\theta < 90^\circ$, colloidal particles tend to be immersed in oil phase to fabricate Oil in water (O/W) type; otherwise the Water in Oil (W/O) type would be formed by hydrophobic particles with the $\theta > 90^\circ$ (Figure I-2).

![Figure I-2. Schematic diagram of two types of Pickering emulsion.](image)

For both two types of Pickering emulsions, the value of $\theta$ should fall between 30°
and 150 ° in order to obtain the irreversible attachment of particles towards the interface, resulting in the strong stabilization against coalescence [21]. Up to now, massive PE studies are based on inorganic particles excluding biodegradability and biocompatibility, such as silica particles, which has limited the practical application in food and nutraceautical industries. Thus, various edible particles have been studied to fabricate Pickering emulsions, such as starch [22], cellulose [23] and prolamine particles [24]. As the commercialized representative of prolamine, zein stabilized Pickering emulsions has been first reported in 2012 [25], since when extensive studies are conducted on this system. However, PE instability based on pristine zein particles was also discussed because of the wettability preference of zein particles over the water phase [26]. The current solution is to fabricate zein-polymer or zein-surfactant complexes to stabilize PE as oil phase fraction (φ) is increased [27, 28], but numerous conditionality had to be considered including formation of complex at specific pH, amount of added surfactant, etc.[29] To our best knowledge, there is no work about chemical modification instead of conditional physical blending allows an alternative exploration to intrinsically tune surface wettability of zein-based colloidal particles, with a better hydrophile-lipophile balance (HLB) of zein particles achieved at the interface between water phase and oil phase.

3.3. Solid Dispersions

As one of popular strategies on dissolution enhancement of low aqueous soluble bioactives by reducing crystallinity, and it is also very useful to help improve the bioavailability of bioactive ingredients [30, 31]. Especially for amorphous solid dispersion, the key content is that crystalline bioactives (like crystalline drugs or nutraceautical) are dispersed as amorphous state in a polymeric matrix, and the selected polymer as the excipient contributes to inhibiting the recrystallization under long-term storage. The core
value is the amorphous status of encapsulated APIs or nutraceutical shows less physically and chemically stable than crystalline counterparts. The latter are at the lowest energy level, thus higher energy is required to break the lattice energy to disperse the molecule into the water-based solvent. However, amorphous materials only possess the short range order structure without any crystalline lattice energy, resulting in the improvement of release behavior, including apparent solubility and dissolution rate, when compared with the crystalline counterparts [31, 32].

As shown in Figure 1-3, solid dispersions technology has been developed through four generations [33, 34]. The definition of solid dispersions was first time brought up by Sekiguchi and Obi in the 1960s, who found eutectic mixtures could increase release of poorly water soluble drugs and improve the related bioavailability [35]. Later, solid dispersion systems with mannitol as the vehicle were developed by Levy and Kaning using molecular dispersions instead of eutectic mixtures [36, 37]. Faster dissolution than conventional same drugs were achieved due to smaller particle size and more wettability of produced drug solids through dispersion. However, in the first generation only microcrystals or small crystalline particles of drug were produced because crystalline carriers were used in the solid dispersions, which actually leaded to slower release of drugs due to the more thermodynamically stable state of crystalline solids [37, 38].

In the second generation, a milestone occurred credited to the introduction of amorphous excipients instead of crystalline ones. The amorphous state is less thermodynamically stable resulting in more efficient release of drugs than the first generation of crystalline carriers [39]. Thus the symbol of second generation of solid dispersions is that the drugs are molecularly dispersed in amorphous excipients [40]. The major of amorphous carriers are synthetic and semi-synthetic polymers, including
Povidone (PVP) and its derivative \(^{[41-43]}\), cellulose and its derivatives \(^{[44-46]}\), starch derivatives \(^{[47]}\), etc. Compared to first generation, the second generation solid dispersions makes drug as a super-saturated state dispersed in amorphous polymeric carrier. In this way particle size could be minimized to molecular level, resulting in better wettability and faster dissolution of the drug coordinated by the water soluble polymers \(^{[40,48]}\).

Since dissolution profile of drugs can be improved via further augment of carrier surface, additives like surfactants and self-emulsifiers are added into the amorphous carriers, which generate the third generation solid dispersions. In essence, third-generation solid dispersions are still amorphous dispersions as same as second generation. The difference is drawbacks of previous generation is greatly prevented credited to the addition of third additive, through which re-crystallization of drug in super-saturated state will be stopped or inhibited \(^{[34]}\). In addition, enhanced surface activity can help prevent drug agglomeration and subsequently improve physical stability in vitro \(^{[49]}\). The major of additives are surfactants, including poloxamers \(^{[50,51]}\), lauroyl macrogol-32 (glycerides gelucire 44/14) \(^{[52,53]}\), glyceryl behenate (compritol 888 ATO) \(^{[54,55]}\), etc. The complexation of amorphous excipient and additive can prevent the drug agglomeration in solid dispersion and precipitation when exposed to the aqueous media, subsequently resulting in the improvement of drug dissolution \(^{[56,57]}\). However, the complexation sometimes also can bring the potential heterogeneity issue when excipient (like polymers) and the third additive are not very miscible.

Zein-based solid dispersion have not been widely studied up to now, and it showed potentials as the novel delivery system to encapsulate and maintain the amorphous bioactive candidates of high recrystallization tendency, so that dissolution enhancement would be achieved leading to bioaccessibility improvement for the oral solid dosage
products. For the lab scale, spray drying is normally used to produce solid dispersions.

![Diagram of solid dispersions classified at four generations](image)

**Figure I-3.** Diagram of solid dispersions classified at four generations (Reprinted from ref. [33])

### 3.4. Phytochemicals

Phytochemicals derived from plants might have an essential role in improving our health condition as one part of diet or in medical way [58]. Originated from Greek word *phyto* which means plant, phytochemicals refer to bioactive chemical substances extracted from plant such as fruits, vegetables, grains, etc. They are classified as nonnutrients but show the potential health benefits, like anti-oxidant and anti-inflammatory bioactivities associated with prevention from some major chronic diseases like cancer or cardiovascular disorders [59, 60]. According to different biosynthetic origins and molecular structures, classification of phytochemicals is shown in Figure I-4. Most of the recent research candidates focus on the phenolics, which typically possesses $\geq 1$ aromatic ring with multiple hydroxyls, including phenolic acids, flavonoids, stilbenes, etc.
Among them more than half of the phenolics are flavonoids. Flavonoids are a sub group of phenolics typically consisting of 2 aromatic rings, which are connected via three-carbons oxygenated heterocyclic ring, and the structural variation further subdivides them as displayed in Figure I-5.

**Figure I-4.** Illustration of dietary phytochemicals classification (Reprinted from Ref. [58])

- Flavonoids
- Flavones
- Flavanols (Catechins)
- Flavanones
- Anthocyanidins
- Isoflavonoids
Even though the controversy of potential treatment or prevention against some intractable diseases like cancers and angiocardiopathy, it is undeniable that plant foods in our diary diet can provide great source of phytochemicals for humans in terms of health benefits. Furthermore, natural occurring phytochemicals originated from plants make people feel safer than synthetic medicines considering any treatment of disease. Thus nowadays the connection between phytochemicals extracted from diet plants and their positive effects on human health has urged exponentially increasing number of research investigations in both nutraceuticals and pharmaceuticals.

However, administration enhancement of bioactive phytochemicals are facing to the same low bioaccessibility issues as most of drugs especially from BCS II or IV regions, where the exposure in GI system is very low $^{[61,62]}$. Multiple strategies using prolamin as vehicle system can be proposed to enhance bioaccessibility of various phytochemicals. In the proposal, one flavonoid representative Dihydromyricetin from BCS IV and one natural phenol model Resveratrol of BCS II are chosen as the research objectives in preliminary experiments. They all show the very poor aqueous solubility leading to their limited exposure in the simulated GI fluid. Below are the brief introduction of each model phytochemical used in our thesis work.

3.4.1. **Dihydromyricetin.**

Dihydromyricetin (molecular structure as shown in Figure I-6) is extracted in a large scale from traditional Chinese herb, *Ampelopsis grossedentata*. It is a promising active flavanonol in pharmaceutical and nutraceutical industry, e.g. as main extraction from traditional medicine, *Hovenia dulcis*, in Asian area to treat fever and parasitic
infection, as a treatment of liver diseases or as commercial health-care product for hangover \[^{63-65}\], especially as one promising candidate for resolving alcohol use disorders (AUD) issues in American \[^{66}\]. As one typical flavonoid, however, Dihydromyricetin shows the typical poor solubility in aqueous solution (0.2 mg/mL at ambient condition) leading to dissolution limited absorption \[^{67}\]. Moreover, the high temperature melting point of Dihydromyricetin indicates its high recrystallization tendency and good stability, which makes its solid dosage form superior to the liquid formulation. In recent study, little literature systematically reported the solution to enhance bioaccessibility of Dihydromyricetin through either increasing solubility (maximum dose) or improving permeability. Chenguang Wang, et al. used cocrystallization method to bind Dihydromyricetin with two high soluble conformers, caffeine and urea, to enhance the apparent solubility. However, both of the cocrystals DHM-caffeine and DHM-urea undergo rapid precipitation when exposing to aqueous solution, leading to the formation of poorly soluble Dihydromyricetin dihydrate. Thus extra crystallization inhibitors must be added to maintain supersaturation of Dihydromyricetin solution over a certain period \[^{67}\]. More importantly, though stable incubated in gastric fluids at pH 1.2, the degradation of Dihydromyricetin has been found in both simulated intestinal fluids and pH 6.8 buffer solutions without pancreatin, which indicates administration of Dihydromyricetin alone would result in degradation in GI fluid very limiting its bioaccessibility. However, no other more effective Dihydromyricetin delivery systems have been developed up to now. In this proposal, zein-based nanoencapsulation was introduced using novel hydrophilic modified Zein derivative, Zein-CMD, as polymeric carrier to resolve dissolution limited bioaccessibility issue by enhancing dissolution of Dihydromyricetin and improving stability in GI media.
3.4.2. Resveratrol.

Resveratrol, as one polyphenol phytoalexin naturally produced by plants in response to exogenous stress, has attracted lots of attention because of its wide distribution in the common dietary sources and promising properties at the aspect of therapy and healthcare [68-71]. Though a number of in vitro and in vivo trials above have indicated the therapeutic potential of Resveratrol, which is mainly related to possible treatment or prevention against cancer and cardiac diseases, the high crystallinity and high melting point (above 250 °C) of Resveratrol lead to its very low solubility (0.03 mg/mL at ambient condition) limiting the oral bioaccessibility [72, 73].

Essentially in terms of solubility enhancement, changing crystalline form of Resveratrol into amorphous through amorphous solid dispersion is a rational strategy [74]. Wegiel et al. has studied re-crystallization inhibition of amorphous formulations of Resveratrol using different polymers and found that strength of interactions between Resveratrol and polymers played a key role. In this proposal, we introduced Zein, one commercial food-grade prolamin, as the polymer excipient. Because it contains high
percentage of non-polar amino acids with amine residues [11], a strong hydrogen bonding interaction between Resveratrol and Zein polymer would be expected.

As show in Figure I-7, on the other hand, Resveratrol is very photo sensitive and isomerization from trans- (E) to cis- (Z) would occur at the exposure to light [75]. Trans isomer is the primary biologically active component in nature and cis isomer is not commercialized due to its relative chemical instability [75, 76]. Thus how to preserve Resveratrol’s integrity as the trans form is another essential task for formulation development. Nam et al. used porous polymeric microspheres to stabilize Resveratrol but the adopted polymer material is not FDA accepted, let alone toxic organic solvent toluene, heptane, etc. [77]. Zein protein, as the GRAS and biodegradable material, again shows the potential as the carrier of Resveratrol. Liu et al. has prepared ivermectin-loaded Zein microspheres, in which Zein protected drug ivermectin from photo-degradation and in vitro sustained-release was also achieved [10].

To enhance Resveratrol’s accessibility through amorphous solid dispersions, multiple specific requirements have to be met, like to increase its solubility in aqueous solution and stabilize its integrity in gastric fluid, etc. Thus in the present proposal a novel microsphere delivery system of Resveratrol with Zein as the polymeric carrier was introduced by using the strategy of amorphous solid dispersions.

![Figure I-7. Trans and cis structure of Resveratrol (Reprinted from Ref. [75])](image-url)
3.5. Solid-state Characterizations

Though solubilization is introduced to dissolve crystalline phytochemicals and then amorphous material can be expected in the latter formula preparation, solid-state samples as the final solid dosage form should be obtained via freeze drying or spray drying to remove any liquid, so that the consequent characterization can be performed for origin (amorphous or crystalline) and physical stability evaluation before dissolution study in the simulated GI tract. Solid dosage forms of phytochemicals-loaded products have multiple advantages over other dosage forms, like longer shelf life than liquid form, easy handling and shipping, less storage space, control release and accurate dosage, etc. The common characterization methods will be briefly introduced below.

3.5.1. X-Ray Powder Diffraction (XRPD)

X-Ray diffraction is a powerful technique to detect any crystalline presence in the samples \(^{[78, 79]}\). As shown in Figure 1-8, no diffraction peaks related to crystalline component are observed for typical amorphous material while significant diffraction peaks are found for crystalline material. As for mixture of crystalline and amorphous material, it shows crystalline peaks with amorphous halo. The typical pattern of the mixture is similar to the failed formula, in which re-crystallization of drug occurs. Based on this method, XRPD is also used to exam physical stability at specific time point when the solid samples are stored in different conditions of temperature and relative humidity (RH). In addition, the scan time of each step also affects the resolution of detected XRPD pattern. For amorphous material, the ratio of signal to noise may be below the limit of detection (LOD) if the scan time is too short, and longer test time is needed to enhance the ratio of signal to noise (S/N) to give a sharper image, based on which small crystalline peaks can be detected ignorant of noise.
3.5.2. Differential scanning calorimetry (DSC) and Thermogravimetric analysis (TGA)

DSC and TGA are two thermo analytical techniques to detect the thermal event of solid state material.\textsuperscript{80, 81} DSC is very effective to determine the difference of energy absorbance between the sample and the reference as temperature increases linearly vs. time, and heat capacity of sample over a range of temperatures can be scanned and recorded. TGA is used to determine the mass loss as the temperature increases, giving the information of physical and chemical phenomena, like heat decomposition, phase transitions, desorption of solvent, sublimation, etc. The data information obtained from these two techniques are often complementary, and the combination of data analysis can provide thermal events of the analyzed samples, like endotherms related to solvent loss or melting, exotherms corresponding to the re-crystallization of amorphous material, glass transition and enthalpy relaxation due to the phase transition.
TGA/DSC analysis is very essential to validate the amorphous formulation products and support the XRPD data \[82\]. Especially for amorphous bioactives in the solid samples, TGA data can provide weight loss (%) of the sample up to 100 °C so that we can estimate the moisture of sample after vacuum dry process. The residue solvent can negatively decrease the glass transition and increase the mobility of drug molecules, leading to the unexpected recrystallization. DSC can quickly provide the thermal events over the range of RT to high temperature (like 300 °) and identify any endothermic peak related to crystalline component.

For amorphous material, however, glass transition can be difficult to be detected in some cases of fast heating rate, e.g. 10 °C/min. Modulated DSC is very useful to determine the characteristics of amorphous solids dispersions \[83\]. As per the equation \( \frac{dH}{dt} = C_p \frac{dT}{dt} + f(T,t) \), where \( \frac{dH}{dt} \) represents the total heat flow of all thermal transitions of the sample; \( C_p \frac{dT}{dt} \), the reversing heat flow, contains all information of heat capacity, glass transition and most of the melting; and \( f(T,t) \) describes the non-reversible heat flow of material including enthalpy recovery, evaporation, crystallization, decomposition, etc. Compared to conventional DSC, modulated DSC can allow the separation of reversing heat flow (glass transition) from non-reversing heat flow (accompanying relaxation), and glass transition of amorphous drug in the solid dispersions can be detected from the reversible heat flow \[84\].

### 3.5.3. Polarized light optical microscopy (PLM)

PLM is one very straight method to observe birefringent specimens under polarized light, which represents the crystalline material \[85\]. This method is very simple and quick to provide the feedback about whether designed amorphous formula is successfully produced. And it is more sensitive to detect crystalline particles by vision. Another
advantage is that a little amount of material is qualified for the PLM detection than other common testing methods, and particle size of the dispersed solid particles can be estimated by PLM as well.

Hot-stage can be combined with the PLM to determine the physical stability of solid samples upon heating [86]. Polymeric carriers are often used to protect the amorphous bioactive compounds from re-crystallization, but increasing temperature will enhance the mobility of bioactive molecule and induce the crystal growth. Thus hot-stage PLM is often used to observe whether any birefringent plates corresponding to the crystalline material occur under the specific temperature or to see the phase transition point where there will be the significant morphological change.
CHAPTER II. PREPARATION AND CHARACTERIZATION OF 
ZEIN-CARBOXYMETHYL DEXTRAN AS SELF-ASSEMBLED 
NANO-MICELLES

1. Introduction

Zein is a class of prolamin protein originated from the maize (corn). Due to ‘GRAS’ food-grade identity and self-assembling behavior induced by the unique hydrophobicity in aqueous system, zein-based particulate systems have been extensively studied as promising carriers for hydrophobic bioactive ingredients.\[87, 88\] Mass of literature have reported the nanoencapsulation of nutrients by pristine zein particles through anti-solvent method, such as coumarin,\[12\] 5-fluorouracil,\[89\] thymol and carvacrol,\[90\] etc. However, zein nano-particulates tend to aggregate in aqueous solution around neutral pH due to the isoelectric point of 6.2, inevitably limiting its future for delivery use.

To improve the zein colloidal stability, additional stabilizers including surfactants or polysaccharides are often introduced to physically modify the outer coating of zein particulates. Regarding on safety concern, food grade surfactants are preferred for stabilization of zein colloidal system around neutral pH, e.g. sodium caseinate (SC),\[16\] lecithin,\[91\] Other commercial surfactants such as Tween 80, poloxamer 188, and sodium deoxycholate have been studied as well, but significant toxicity was found as concentration up to 50 μg/mL post 1 day incubation.\[92\]

Alternatively, zein–polysaccharide complex systems through physical blending have received increasing interests in decades. For instance, zein/carboxymethyl chitosan complex as one widely investigated category has been proposed to improve the particulate stability and redispersibility in aqueous solution, as compared to zein
nanoparticles without coating of carboxymethyl chitosan.\textsuperscript{[9, 17]} Sodium carboxymethyl cellulose has also been reported to constitute with zein nanoparticle acting as an effective drug carrier and transporter, with stable performance within a broad pH region in aqueous solutions.\textsuperscript{[93]} Other polysaccharides, such as soluble soybean polysaccharide,\textsuperscript{[94]} hyaluronic acid,\textsuperscript{[95]} are shown to be potential stabilizers for fabrication and stabilization of zein-based nanoparticles as well. Moreover, a zein/polysaccharide/surfactant ternary complex has been recently developed to improve stability and bio-accessibility of curcumin.\textsuperscript{[96]} Ternary complex appears to take a better stabilizing effect under bio-relevant conditions than binary complex, however, more steps are required increasing the complexity of preparation procedure.\textsuperscript{[96, 97]}

Though lots of efforts have been put into physical blending approaches for stabilization of zein colloidal dispersion, scant research relying on the chemical way has been reported so far. There is still adequate space for researchers seeking novel zein-based particulate delivery systems with target functionalities through chemical conjugations. Not like physical blending with another biopolymers, chemical modification can intrinsically improve the amphiphilic nature of zein-based material by linking the hydrophilic biopolymer with zein protein, resulting in a polymeric micelle with a better stability in aqueous environment and higher encapsulation and loading efficiency. Podaralla et al. covalently conjugated a hydrophilic synthetic polymer, polyethylene glycol (PEG), to zein protein, which enabled the self-assembly of micellar carriers enhancing the bioavailability of insoluble curcumin.\textsuperscript{[18]} To the best of our knowledge, however, little literature has reported conjugation of polysaccharide carboxymethyl dextran (CMD) with zein protein. As a highly hydrophilic and flexible negative polyelectrolyte, CMD has arisen an increasing interest for functional modification of nano-carriers in biomedical engineering and healthcare,\textsuperscript{[98]} such as
tailoring the surface of a model gene delivery nanoparticle by using a library of CMD with different molecular weights and charge densities,\cite{99} enhancing colloidal stability and cellular uptake of magnetic nanoparticles,\cite{100} etc. On the other hand, zein protein could provide reactive side groups of lysine amino acid,\cite{11} providing targeted sites on which CMD can be conjugated by using mild linking reagents 1-[3-dimethylaminopropyl]-3-ethyl-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS).\cite{18,101}

Thus in this chapter work, bearing the purpose of promoting the functionalities of zein–polysaccharide systems, we conjugated CMD with zein through covalent linking, and successfully synthesized a novel amphiphilic food-originated modified zein, which could self-assemble nano-micelles for potential delivery use. The structural properties were investigated before and after chemical modification. The Zein-CMD micellar dispersion can be prepared through anti-solvent, with micellar morphology investigated as well.


2.1. Materials.

The α-zein (abbreviated as zein) was purchased from Wako Chemical Industries, Ltd. (Tokyo, Japan). Dextran (Mₙ ~5,000 g·mol⁻¹) was given as a gift from pK Chemicals, Ltd. (Køge, Denmark). Sodium chloroacetate and sodium hydroxide were purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Bio-conjugating reagents 1-[3-dimethylaminopropyl]-3-ethyl-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), pyrene, deuterated dimethyl sulfoxide (DMSO-\textit{d}₆) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Milli-Q water and pure ethanol were obtained from Food Science Department, Rutgers. (New Brunswick, NJ). All organic
solvents such as dimethyl sulfoxide (DMSO) were purchased from VWR, Ltd. (Bridgeport, NJ).

2.2. Preparation of Zein-Carboxymethyl Dextran (Zein-CMD)

As illustrated in Figure II-1, the chemical modification contains two steps. Firstly, carboxymethylation of dextran was conducted according to the literature.\textsuperscript{[102]} Typically 10 g of dextran were dissolved in a 40 mL of 3.8 M NaOH solution while stirring for one hour at room temperature. Then sodium chloroacetate was added into reaction mixture at a feed molar ratio of 1:1 ([Chloride group of sodium chloroacetate] : [Hydroxyl group of dextran monomer]) while continuously stirring to obtain homogeneity. The reaction mixture was then incubated in an oil bath at 60°C under stirring of 300 rpm for 2 h. Finally the crude product was precipitated into methanol three times to remove any small molecule reagents and solvent, and then vacuum dried at 40°C overnight. The refined CMD was obtained as white fine powder in a yield above 95%.

Secondly, zein was conjugated with CMD by amidation based on previous literature about using zero-length and efficient bioconjugate reagents, EDC/NHS,\textsuperscript{[103]} to covalently link zein proteins with other bio-polymers.\textsuperscript{[104–106]} CMD, EDC and NHS (feed molar ratio was 1:3:3) were dissolved in DMSO/water solution (v/v = 1:4) while stirring for 2.5 h to activate carboxyl groups of CMD. The reaction mixture was added dropwisely into DMSO solution of zein (molar ratio of zein and CMD was 1:1) until the final volume ratio of DMSO/water reached to be 4:1. The reaction system was stirred overnight at ambient condition and dialyzed (MWCO 8~10 kDa) against pure water for 1 day, then against 60% ethanol solution for 1 day and against pure water for 1 day, to remove any small molecule reagents, unreacted dissolved CMD and organic solvent. Final powder sample of Zein-CMD was obtained by freeze drying.
2.3. Characterizations of Zein-CMD

The solution proton nuclear magnetic resonance (\(^1\text{H NMR}\)) spectra of CMD and pristine dextran in D\(_2\)O, Zein-CMD and pristine zein in Dimethyl sulfoxide-\(d6\) (DMSO-\(d6\)) were recorded at room temperature using a Varian VNMRS 500 MHz spectrometer.

Fourier-transform Infrared Spectroscopy (FTIR) spectra was acquired using a Nicolet Nexus 670 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Each sample powder was flattened for close contact with ZnSe crystal. All spectrums were averaged by 128 scans with 16 cm\(^{-1}\) resolutions within range of 4000-500 cm\(^{-1}\).

Size-exclusion Chromatography (SEC) for Zein-CMD and pristine zein were obtained as followed.\(^{[107]}\) The 9.4*250 mm ZORBAX GF-250 column (Agilent, USA) was equilibrated with starting buffer (mobile phase A: water; B: 40% ACN with 0.1% TFA), and ratio of B was increased at 1.6 %/min for 5 min and then 0.1 %/min for 70 min. Flow rate was 1 mL/min and elute was monitored at 280 nm.

By X-ray Photoelectron Spectroscopy (XPS), the elemental compositions of Zein-CMD and pristine zein were measured by a Thermo ESCALAB 250 X-ray photoelectron spectrometer with Al X-ray source.

Circular Dichroism (CD) spectra for each sample was measured at far-UV region of 190-260 nm with a path length of 0.1 cm. Recorded speed was 100 nm/min with a 1.0 nm
of bandwidth and 0.2 nm resolution at ambient environment. The composition ratio of \(\alpha\)-helix, \(\beta\)-sheet and random coil of Zein-CMD and pristine zein were calculated using an online K2d program.\(^{[108]}\)

2.4. Small-angle X-ray scattering (SAXS).

The configuration of chemically modified zein-CMD was investigated in solution as compared to pristine zein by SAXS. The measurements were performed using Bio-CAT, 18-ID beam-line section of Advanced Photon Source (Argonne National Laboratory). Single exposure period was 1 s with X-Ray wavelength fixed at 1.033 Å. Each sample solution was loaded to a cylindrical flow cell (\(\Phi = 1.5\) mm) at 25°C, and load rate was constant at 10 \(\mu\)L/s. All SAXS profiles were collected from the averaged curve of 15 measurements post proper solvent background subtraction. Software Igor Pro was used to fit the scattering intensity profiles and sequential Guinier plots.

2.5. Preparation of Zein-CMD micellar dispersion.

Typically, Zein-CMD in 70% alcohol solution was loaded into water under stirring at 500 rpm. Then ethanol of the resulting dispersion was removed using nitrogen flow through the solution while continuous stirring for 1 h. Additional water was compensated for ethanol lose with pH adjusted within the range of 6.8-2.0, and Zein-CMD micellar dispersion was prepared within 0.1-5 mg/mL.

2.6. Critical micellar concentration (CMC)

CMC of Zein-CMD was measured by pyrene 1:3 ratio approach.\(^{[109]}\) Firstly, equivalent acetone solution of pyrene was added to each sample vial. After drying, isometric Zein-CMD dispersion was added to ensure that the final concentration of pyrene was 1.2 \(\times 10^{-7}\) M. All prepared sample solutions with pyrene were placed at room temperature overnight. The excitation wavelength was set as 335 nm. Excitation and
emission slit width were both set to 3 nm. The emission intensity was recorded between 350 nm and 410 nm. Each data point obtained is an average of three fluorescence scan experiments.

2.7. Particle size.

The colloidal particle size was measured by dynamic light scattering by a Zetasizer Nano instrument (Nano S90, Malvern) at room temperature. A solid state laser at 633 nm was operated at a fixed vertical angle with the refraction index as 1.330. Measurements were performed in triplicate.

2.8. Micellar morphology observation.

Air-dried Zein-CMD micelles were obtained by casting dispersion onto a silicon chip, and the morphology was observed by Atomic Force Microscopy (AFM, Seiko SPA-300HV, Japan), Field Emission Scanning Electron Microscope (FESEM, XL-30, Japan) and Transmission Electron Microscope (TEM, JEM-1011, Japan). Tapping mode was used in AFM test, and the spring constant of silicon tip was 2 N·m⁻¹. Before SEM observation, each sample was gold coated (300 A) to improve the surface electrical conductivity. Nano-scale structure of the micelles was further investigated by TEM, with the accelerating voltage of field emission gun at 100 kV and magnification × 75000.

3. Results and Discussions.

3.1. Carboxymethylation of dextran.

Carboxymethylation was affirmed via FTIR and ¹H NMR (Figure II-2), with chloride groups of sodium chloroacetate substituted by activated hydroxyl groups of dextran in the presence of 3.8 M NaOH. As displayed in Figure II-2a, FTIR spectra of CMD revealed the typical absorption peak at 1592 cm⁻¹ credited to asymmetrical stretching vibration of carboxyl, and the absorption band at about 1420 cm⁻¹ which was
the carboxyl characteristic peaks of symmetrical stretching vibration in sodium salt form.\textsuperscript{[110, 111]} The substitution degree was then calculated by integration of corresponding \textsuperscript{1}H NMR signals of the CMD \textbf{Figure II-2b}. Hydroxyl groups (-OH) in D\textsubscript{2}O are undetectable due to the proton-deuterium exchange. The overall integral of the CH(1) proton of CMD backbone including sites from CH(1) α-1,3, CH(1') α-1,6, and CH(1) α-1,6 was set to be 1.0 as reference.\textsuperscript{[112]} The proton signal of CH\textsubscript{2}COO\textsuperscript{-} (δ 4.10) were integrated to be 4.6,\textsuperscript{[113]} which was divided by 2 to determine the substitution degree as 2.3.\textsuperscript{[114]}

\textbf{Figure II-2}. (a) FTIR spectra and (b) \textsuperscript{1}H NMR spectra of CMD and pristine dextran. \textsuperscript{1}H NMR (500 MHz, D\textsubscript{2}O): δ 5.10-4.85 (anomeric, 1, CH\textsubscript{1} of CMD), δ 4.10 (m, 2, CH\textsubscript{2}COO\textsuperscript{-}), δ 3.90-3.30 (m, 6, CH\textsubscript{2,3,4,5,6} of CMD).
3.2. Conjugation of CMD onto zein.

Figure II-3 shows FTIR and $^1$H NMR spectra of Zein-CMD and pristine zein. Zein protein contains lysine with side amine group -NH$_2$, where amide bond (CO-NH) can be formed as the bridge to conjugate zein with CMD. As displayed in Figure II-3a, IR band of 2350 cm$^{-1}$ was due to stretching vibration of newly formed amide bond (CMD-CO-NH-Zein). In addition, the typical absorption bands in fingerprint region belonging to 1100 cm$^{-1}$ and 1018 cm$^{-1}$ were credited to stretching vibration of C-O bond from CMD backbone. The conjugation was also confirmed by $^1$H NMR (Figure II-3b). The intensive signals at $\delta$ 3.30-2.80 were attributed to 6 protons located at CH$_{2,3,4,5,6}$ positions of CMD backbone.

Figure II-3. (a) FTIR spectra and (b) $^1$H NMR spectra of Zein-CMD and pristine zein. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 3.30-2.80 (m, 6, CH$_{2,3,4,5,6}$ from CMD backbone).
3.3. Conjugation degree estimated by SEC and XPS.

Figure II-4 displays two peaks in the SEC race of pristine zein, corresponding to two bands of molecular weight (Mw.) from commercial α-zein resource.\[107, 115]\] After chemical modification, the peak area of higher Mw. band increased from 11.7% to 37.2%, which suggested that about 1/4 of lower Mw. Band zein chains were mainly involved in the conjugation of CMDs, resulting in this proportional increase of higher Mw. Band and decrease of lower Mw. Band. The SEC result indicated that not every single band of zein was conjugated with CMD, but from an average respect the conjugation degree of CMDs per zein can be estimated based on the change of elemental content.

![SEC chromatogram of Zein-CMD and zein](image)

**Figure II-4.** Size-exclusion chromatography (SEC) of Zein-CMD and zein.

As shown in Figure II-5, the elemental nitrogen (N) content decreased from 15.0% to 8.0%, while the ratio of elemental oxygen (O) increased from 16.5% to 23.3% after conjugation. Assume that the mass of zein was 1.0, which contributed 15.0% of N and 16.5% of O, and the conjugated CMD should provide the rest of O. Because the ratio of
N/O for zein and CMD together was the same as that for Zein-CMD, the CMD would contribute 27.2% of O. The content of O accounted for about 54% in CMD, thus the mass of conjugated CMD was calculated to be 0.5. For Zein-CMD$_n$ with n representing the number of conjugated CMD per zein, n was estimated as 2.0 when the mass of each component was divided by its molar mass. Thus in average, about 2 CMD molecules were conjugated per zein.

![XPS of Zein-CMD and zein](image)

**Figure II-5.** XPS of Zein-CMD and zein.

### 3.4. Secondary structural difference between Zein-CMD and pristine zein.

**Figure II-6** shows the difference in ellipticity of spectra curves at 210 and 222 nm, suggesting a significant reduction of $\alpha$-helix structure after conjugation.\textsuperscript{[108]} The content of $\alpha$-helix decreased from 84.2% to 67.4% in 70% aqueous ethanol solution after chemical modification, while $\beta$-sheet ratio slightly went up to 3.3% and random coil content also increased from 14.5% to 29.3% by calculation using K2d program.\textsuperscript{[108]} The transformation from $\alpha$-helix to $\beta$-sheet and random coil might be attributed to the incorporation of CMD into zein chain, which is in agreement with other reports that interaction between zein and other components could impede the ordered $\alpha$-helix
3.5. Molecular conformation and dimensional difference between Zein-CMD and pristine zein.

The effects of grafted CMDs on the molecular conformation and dimensions of zein was studied using solution SAXS (Figure II-7), complementing the picture of the structure-function relationship sketched from single molecule to the formation of zein colloidal system. The power-fit SAX scattering intensity profiles of Zein-CMD in 70% alcohol solution within 5 - 40 mg/mL were presented in logarithmic scale in Figure II-7a. The solid lines showed the fractal region at intermediate q range, which can provide the contour shapes in solutions. The values of fractal dimension varied from 1.58 to 0.94, suggesting that Zein-CMD in 70% aqueous ethanol was more likely to take an elongated conformation with the concentration increase. It agrees with previous observation by Uzun et al. that rod-like growth of zein proteins can result in the fractal dimension approaching to 1.\textsuperscript{[117]} Furthermore, isometric ellipsoid model was found to best match the scattering profiles as displayed in Figure II-7b. The dimension of Zein-CMD at 5 mg/mL was 14.3×3.4×3.4 nm\textsuperscript{3} using software of Igor Pro, which was almost 3 times
smaller than that of pristine zein in 70% aqueous ethanol solution (33.8×4.4×4.4 nm³) as mentioned in the previous work by Li et al. It can be understood that aqueous ethanol is more of a $\theta$ solvent to zein, in which zein protein tends to form dimer or even multimer resulting in a larger size. However, the CMD decoration may facilitate dissolving of Zein-CMD in the same solvent, and suppressing dimers or aggregates as well. In addition, the axial ratio of Zein-CMD was found to increase from 4.2 to 7.2 as concentration increased from 5 to 40 mg/mL. It suggests no change for the rod-like conformation of zein proteins after conjugation with CMDs, but increasing concentration may promote intermolecular helix packing with a more elongated conformation as well proposed by Shi et al.

![Figure II-7](image)

**Figure II-7.** (a) SAXS profiles of Zein-CMD in 70% ethanol solution fitted by power law. (b) SAXS profiles of Zein-CMD in 70% ethanol solution fitted by the ellipsoid model. The concentration gradients included 5 mg/mL (red squares), 10 mg/mL (yellow diamonds), 20 mg/mL (blue triangles), and 40 mg/mL (green circles).
Figure II-8 indicates gyration radius \( (R_g, \text{ standard compact object}) \) and cross-sectional radius \( (R_c, \text{ rodlike form}) \) of both zein and Zein-CMD in 70% ethanol solution by Guinier fittings at a region safe with \( R_g \times q < 1.3 \) at the very beginning of the \( q \).\[120\] The \( R_g \) and \( R_c \) of zein protein had no obvious dependence on the concentration between 10 mg/mL and 40 mg/mL, however for Zein-CMD, \( R_c \) decreased from 15.5 Å to 11.8 Å while \( R_g \) remained around 60 Å, which may result in the longer elongated conformation with concentration increase as well suggested by Xiao et al.\[121\] Above all, one may conclude that Zein-CMD in 70% aqueous ethanol solution was more likely to behave as zein in acetic acid (polyelectrolyte solution), rather than the behaviors of zein in \( \theta \) solvents.\[122\] The consequence is brought up that Zein-CMD is partially unfolded with hydration layer (CMD outer) suppressed when increasing the concentration, leading to a more elongated rod-like conformation of Zein-CMD with a smaller volume than zein protein in 70% aqueous ethanol solution.

![Figure II-8](image)

**Figure II-8.** Sizes of Zein-CMD or zein in 70% ethanol solution plotted against concentration. Squares and triangles represented the gyration radius \( (R_g) \) and cross-sectional radius \( (R_c) \), respectively. Solid line and dash line were used to distinguish Zein-CMD and zein.
3.6. Critical Micellar Concentration (CMC) of Zein-CMD.

**Figure II-9** shows CMC determination of Zein-CMD by using fluorescence spectrometer with pyrene as the fluorescence probe. When Zein-CMD micellar dispersion was prepared, the self-assembly of Zein-CMD micelles above CMC can cause polarity change of solvent medium, which was detected by sharp variation of the I₁/I₃ value (ratio of fluorescence emission intensity corresponding to the 1st and 3rd vibronic bands of pyrene molecule at the wavelengths of 372 nm and 383 nm). As can be seen in **Figure II-9**, the pyrene I₁/I₃ ratio plot matches the decreasing sigmoid of the Boltzmann type,[109] and the CMC of Zein-CMD was determined as around 0.08 mg/ml from the intersection point of the tangent line (y₁) to the sigmoid and the bottom straight line (y₂), suggesting a relatively good thermodynamic stability of the self-assembly micelles on dilution as described by previous work of Podaralla et al.[18]

![Figure II-9. Critical micelle concentration (CMC) of Zein-CMD.](image)

3.7. Particle Size and Stability of Zein-CMD Micellar Dispersions.

**Table II-1.** Average particle size of Zein-CMD nano-micelles
As displayed in Table II-1 and Figure II-10, the Zein-CMD nano-micelles were self-assembled under different conditions, and effects of concentration, pH or storage time on dispersion stability were investigated. Firstly, a concentration-dependent relationship was found that the average micellar size decreased by half as Zein-CMD was diluted from 5 mg/mL to 0.1 mg/mL. This finding was similar with the results presented by Podaralla et al., [123] who reported that a larger size (~400 nm of zein dispersion at 0.5% w/v) was observed when concentration of zein increased though a combination of Pluronic F68 and lecithin (2:1, w/w) was used for stabilization, possibly due to the subsequent increase of solution viscosity that delayed the nucleation process of nanoparticles. Followed by the current research trend, physical blending with stabilizers, such as surfactants or polysaccharides, has been extensively used for the stabilization of
zein colloidal dispersions, and the particle diameters were normally controlled by adjusting concentration of coating stabilizers as the content of zein was fixed. An inversely proportional relation was often concluded between the concentration of coating stabilizers and the mean particle size, regardless of biopolymers, polysaccharides or conventional surfactants. It indicated that a certain amount of coating stabilizers was often required in case of substantial aggregation and sedimentation of zein nanoparticles, possibly because electrostatic repulsion was not sufficient among them at the low concentration of stabilizers, or bridging effect caused the flocculation of zein nanoparticles when covering was not complete. For example, Chuacharoen et al. adopted lecithin: Pluronic F127 combination (0.045% : 0.09%, w/v) to improve chemical stability of zein nanoparticles, but a larger average particle diameter (208.8 ± 8.0 nm) was found as compared to nanoparticles without surfactants. Huang et al. also discussed that the concentration of pectin as coating polysaccharide had an influence on stability of the zein nanoparticles, with no less than 0.05% (w/v) of pectin needed to control the particle size below 300 nm. Compared to commonly used physical blending with coating stabilizers, chemical modification in our work can graft the stabilizers CMD onto the zein, in which way particle size is directly adjusted by concentration without requirement for minimum amount of added coating stabilizers.
Figure II-10. The effect of concentration and pH on particle size of Zein-CMD nanomicelles.

Besides, combined with the XPS results that 2 CMD molecules were averagely conjugated with one zein chain, mass weight ratio (w/w) of zein and CMD is about 2:1 in our modified Zein-CMD, assuming that the average molecular weight ratio of zein and CMD approximately equals to 4:1. This number is significantly lower than that from physical blending of zein and polysaccharides containing carboxymethyl groups based on previous research. For instance, Luo et al. was one of the first groups to use polysaccharides, such as carboxymethyl chitosan, to coat zein nanoparticles for colloidal stabilization and encapsulation efficiency improvement, while weight ratio of zein and CMC under optimal formulation was determined as 1:2.\(^{17}\) In addition, Liang et al. used sodium carboxymethyl cellulose (sodium-CMC) to cover zein nanoparticles, and optimal formula (zein and sodium-CMC wt. ratio was 1:3) was chosen for their cellular uptake experiments.\(^{93}\) Thus in comparison with research results of traditional physical blending above, the application of chemical conjugation enables equivalent or even better stabilizing effect with less amount of CMD on zein as the coating polysaccharides.

As the stability study of Zein-CMD colloidal system displayed in Table II-1, a slight growth was found for particle size and PDI post 7 days’ stage at 4 °C, followed by an further increase after 28 days’ storage period. Nevertheless, particle size remained stable below 200 nm after a long term of resting, which indicated that covalently conjugated CMD as the coater could efficiently prevent zein nanoparticles against precipitation. On the other hand, a pH-dependent relation was also observed for the Zein-CMD colloidal diameter. The pH points were designed as followed by the physiological environment of bio-relevant fluid, e.g. pH was close to 2.0 in simulating fasted state
gastric fluid, pH increased to 5.0 or 6.5 in simulating fed or fasted small intestinal fluid.\[127\] As shown in Figure II-10, colloidal diameter gradually increased as pH decreased from 6.5 to 2.0, due to the gradual protonation of carboxyl groups from CMD in acidic environment which may result in lower surface potential but bigger particle size.\[93\] It is noteworthy that stable Zein-CMD nano-micelles with smaller particle size was achieved at pH 6.5, which was very near zein’s isoelectric point (pH 6.2). This result implies CMD layers could efficiently protect Zein-CMD nano-micelles against instability under environment of simulating small intestinal, which would enable it as a potential nano-carrier for the lipophilic drug or nutraceutical delivery. To summarize, chemical modification in our work can give an novel solution to improving the stability of zein nanoparticles in aqueous solutions, meanwhile to reduce the dependence of coating stabilizers or surfactants.


Figure II-11 shows AFM and SEM images of Zein-CMD nano-micelles obtained from freshly prepared dispersion. At 0.1 mg/mL, quasi-spherical morphology with a smooth surface was observed by AFM (Figure II-11a, b) and SEM (Figure II-11c). The result is consistent with the work from Wang and Padua, which concluded that zein spheres were preferably assembled at low mass fraction.\[128\] By increasing Zein-CMD concentration to 2 mg/mL, however, the micellar shape was transformed from quasi-spherical to blocky-shaped or polygonal-shaped with a larger size (Figure II-11d). It agrees with the observation from Wang and Padua et al. that all other nano-scale or micro-scale structures of particles were based on zein spheres.\[108\]
Figure II-11. (a) 2D AFM topography image and (b) 3D AFM image of Zein-CMD micelles at 0.1 mg/mL with scale at 1um. SEM images of Zein-CMD micelles at (c) 0.1 mg/mL and (d) 2 mg/mL (scale bar is 2um and magnification is ×16000).

Figure II-12 shows a single core-shell, spherical structure of Zein-CMD micelle at 0.1 mg/mL by TEM, with darker region of hydrophobic zein core and lighter outer of hydrophilic CMD shell observed. This visual structure indicated that Zein-CMD molecules assembled into core-shell round-like micelle via anti-solvent method. The polar repulsion induced by aqueous surroundings would aggregate the zein domains of Zein-CMD as the hydrophobic core, with the CMD chains as the hydrophilic shell. Figure II-12b exhibits the preferable aggregation tendency for core-shell micelles of Zein-CMD, which might account for the transition of micellar shape from quasi-spherical to blocky-shaped when concentration of Zein-CMD increased.
4. Conclusions

In summary, this chapter work paves one way to improve the amphiphilic property of zein-based material as a potential delivery vehicle through tailored chemical modification of zein protein with functional polysaccharide. We successfully conjugated zein protein with hydrophilic carboxymethyl dextran (CMD) at the molar ratio of 1:2 by covalent linking, and found the reduction of ordered α-helix structure while elongated rod-like conformation remained in aqueous alcohol solution. As compared to the conventional methods of physical blending with massive dependence on other coating polymers or surfactants, our work can pave a novel way to stabilize zein-based colloidal delivery system by intrinsically enhancing its amphiphilic nature with much less charging of polysaccharide amount.
CHAPTER III. PREPARATION OF AMORPHOUS DIHYDROMYRICETIN WITH ZEIN-CARBOXYMETHYL DEXTRAN AS THE NANO-CARRIER.

1. Introduction.

Dihydromyricetin (DMY), extracted in a large scale from traditional herb *Hovenia dulcis*, has been seen as a potential bioactive phytochemical for the medicine use, such as alleviating alcohol intoxication, treatment of nonalcoholic fatty liver disease, etc. As one kind of flavonoids, DMY also has gained an increasing interest credited to its potential benefits, e.g. anti-oxidant/anti-inflammatory, anti-cancer, anti-bacterial, etc. However, DMY has typical poor aqueous solubility restricting its further clinical application. In order to solve this issue, various approaches have been tried to improve release performance of DMY. Although the method of inclusion complexes was tried in early work to achieve better dissolution performance of DMY than solid dispersion, it was faced with laborious preparation and limit of small dose complexation. In addition, co-crystallization approach instead of using soluble salts has been brought up due to the instability of DMY in alkaline aqueous solutions, but mass of additional efforts in preliminary co-crystal screening and impurity removal of co-former were still required. Recently, nanoencapsulation of DMY using cationic nanocapsules of Eudragits RS100® (supplied from the Corporation Evonik) has also been studied on behalf of its possible advantages on the increased solubility and stability in bio-relevant fluids. However, safety concern remained due to the scientific fact that a decrease in cell viability with moderate cytotoxicity was observed for blank Eudragits RS100® nanocapsules in a dose-dependent manner. Thus, future study on the formulation
development of DMY is still needed regarding on the dissolution enhancement of DMY in biological fluid.

One feasible strategy is to disrupt the crystalline structure of DMY using solvent and maintain DMY in amorphous status, resulting in faster dissolution and higher solubility. To this end, the application of proper amorphous media enables the DMY molecules to be ordered densely in the short range but randomly without any crystal forms. This can create a supersaturating solution of DMY and greatly facilitate the diffusion of more solute molecules during clinical drug-delivery applications. However, to maintain amorphous status of DMY is always one challenge considering that amorphous material could re-crystallize due to excess entropy, enthalpy and free energy \[^{137}\]. The principal solution is to choose one suitable polymeric matrix in which amorphous molecules are dispersed and stabilized. The commonly used polymers in the market include cellulose and its derivatives, poly (vinylpyrrolidone) (PVP) and its derivatives, acrylic acid-based enteric Eudragits, etc. \[^{138}\].

Nowadays GRAS (Generally Recognized as Safe) materials originated from plants have been widely developed to encapsulate phytochemicals instead of synthetic or semi-synthetic polymeric excipients \[^{139}\]. Among them amorphous zein protein is a promising star considering its amphiphilic characteristic and sequential flexible deformation due to polarity change of solvent \[^{3}\]. More importantly, phytochemicals preferentially interact with the residues of prolamins, and proline-rich zein shows good potential in the encapsulation of phytochemicals via hydrophobic interactions or hydrogen bonding \[^{140}, \, 141]\). Up to now a variety of phytochemical-loaded zein particulate delivery systems have been studied. For example, it is reported that curcumin was encapsulated in zein/sodium caseinate colloidal particles via strong hydrophobic interactions to enhance its dispersibility in aqueous solutions, and improve the stability against photolysis or
degradation in the intestinal environment \cite{15, 142}. Another well-researched natural phytochemical, Quercetin, was loaded in zein matrix to form spherical particles, which resulted in the disappearance of needle-like crystals of pure Quercetin and increased antioxidant activity in aqueous medium \cite{143}. Moreover, Tangeretin and Cranberry procyanidins have been proposed to prepare zein-based composite particles via hydrophobic interactions, on behalf of improved stability and bioavailability during controlled delivery \cite{144, 145}. Recently, zein-epigallocatechin gallate (zein-EGCG) conjugates that were outer covered by rhamnolipids have been prepared, for the co-delivery through hydrophobic interactions and hydrogen bonding \cite{146}.

The recent research trend already indicates that additional polymeric stabilizer or surfactant is required due to the strong hydrophobic attractions between zein and lipophilic phytochemicals, which can induce the precipitation rather than uniformly dispersed zein-based particulate delivery system when increasing loading of the target compound. For those physical modifications, however, complicated preparation steps are often required considering multiple factors to be considered in the formulation design, including stabilizers screening, specific pH condition, concentration of additives, etc. \cite{147, 148}. Hence in our work, we chose the modified zein-based biomaterial, zein-carboxyl methylated dextran (Zein-CMD), as the polymeric carrier to encapsulate and stabilize the amorphous DMY. Zein-CMD was prepared by conjugating carboxymethyl dextran (CMD) with zein and detailed synthesis procedure can be found in our previous work [refer paper 1]. As presented in our previous work, hydrophilic modified zein material could further reduce the risk of large aggregates or precipitation in zein-based delivery system due to the isoelectric point of 6.2.

In this chapter work, amorphous DMY was produced using Zein-CMD as the
carrier, which was abbreviated as DMY/Zein-CMD. The loading and encapsulation efficiency were measured sorted by initial DMY load. Its amorphous origin and accelerated stability were investigated via multiple solid-state characterizations, including FTIR, XRPD, PLM, and DSC (mDSC). The dissolution enhancement of DMY released from DMY/Zein-CMD was assessed as compared to pure crystalline DMY in biorelevant media.


2.1. Materials.

Dihydromyricetin (DMY) (purity >98.0%) was a gift from South China Agricultural University (Guangdong, China). The polymeric carrier zein-carboxymethyl dextran (Zein-CMD) was synthesized and synthesis procedure can be found from our previous chapter. α-zein (abbreviated as zein) was purchased from Wako Chemical Industries, Ltd. (Tokyo, Japan). Milli-Q water and pure ethanol were obtained from Food Science Department, Rutgers. (New Brunswick, NJ). All organic solvent were purchased from VWR, Ltd. (Bridgeport, NJ). SIF powder containing sodium taurocholate and lecithin was purchased from Biorelevant.com Ltd. (London, UK). Three different kinds of biorelevant media FaSSGF (Simulated Gastric Fluid), FaSSIF (Fasted State Simulated Intestinal Fluid) and FeSSIF (Fed State Simulated Intestinal Fluid) were prepared, simulating gut fluids before (fasted) and after (fed) feeding according to the literature [127].

For FaSSGF buffer (1L): Weigh 1.999 g of sodium chloride (NaCl) into a 1 L volumetric flask followed by adding 900 mL of deionized (DI) water. Adjust pH to exactly 1.2 using 1M Hydrochloric acid (HCl) and q.s.to 1 L by DI water. Then dissolve 0.597 g of SIF powder into the 1 L volumetric flask.
For FaSSIF buffer (1L): Weigh 0.420 g of sodium hydroxide pellets (NaOH), 3.438 g of monobasic sodium phosphate anhydrous (NaH₂PO₄) and 6.186 g of sodium chloride (NaCl) into a 1 L volumetric flask and then add 900 mL of DI water. Adjust pH to exactly 6.5 using either 1N NaOH or 1N HCl and q.s.to 1 L by DI water. Then dissolve SIF powder (2.24 g) into the 1 L prepared buffer.

For FeSSIF buffer (1L): Weigh 4.040 g of sodium hydroxide pellets (NaOH), 8.650 g of glacial acetic acid (CH₃COOH) and 11.87 g of sodium chloride (NaCl) into a 1 L volumetric flask followed by addition of 900 mL DI water. Adjust pH to exactly 5.0 using either 1N NaOH or 1N HCl and q.s. to 1 L by DI water. Then dissolve 11.20 g of SIF powder into the 1 L prepared buffer.

2.2. Preparation of DMY/Zein-CMD.

Preparation procedure was followed by different initial load of DMY as shown in Table 1. Briefly, 5 mg/mL of DMY ethanol solution and Zein-CMD 70% ethanol solution were pre-mixed to obtain the homogenous solution, and 1 mL of pre-mixed solution was added dropwise into 4 mL of anti-solvent water under ultra-sonication (Solid State Ultrasonic FS-28, Fisher Scientific). Then nitrogen flowed through the resulting dispersion solution while continuous stirring for 1 h, followed by compensation using water. The prepared dispersion solution was frozen at -60°C and then freeze-dried (FREEZONE 4.5 Freeze Dryer, LABCONCO) for 2 days to collect the final solid sample DMY/Zein-CMD.

2.3. Loading and encapsulation efficiency.

The obtained solids of DMY/Zein-CMD was washed by 2 mL 95% ethanol to remove un-encapsulated DMY. The washed solids were collected and DMY was extracted by 10 mL pure ethanol using vortex for 5 min. Then equivalent ethanol solution
containing DMY was transferred to a 96-well UV plate. The amount of DMY was determined in triplicate in spectra-photometrical way using a BioTek microplate reader and data was analyzed by KC4 data reduction software. Encapsulation efficiency (EE) and loading efficiency (LE) based on absorbance intensity at wavelength of 320 nm were calculated according to the equations below:

\[
\text{LE (w/w \%)} = \frac{\text{Mass of encapsulated DMY}}{\text{Mass of total solids}} \times 100
\]

\[
\text{EE (w/w \%)} = \frac{\text{Mass of encapsulated DMY}}{\text{Mass of input DMY}} \times 100
\]

2.4. Particle size and zeta potential.

The particle size of dispersed DMY/Zein-CMD was measured through dynamic light scattering (DLS) at room temperature. A laser at 658 nm was operated at a fixed vertical angle with the refraction index as 1.333 (Between 1.330 for pure water and 1.355 for pure ethanol). The data was obtained by Cumulant analysis of the intensity-intensity autocorrelation function, \( G(q, t) \). Zeta-potential measurement was conducted using Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcs, UK). Each measurement was conducted in triplicate.

2.5. Solid-state characterizations.

The potential interaction between DMY and Zein-CMD was studied by FTIR. Amorphous origin and physical stability of DMY/Zein-CMD under accelerated conditions were investigated via XRPD and PLM.

2.5.1. Fourier transform infrared spectroscopy (FTIR).

IR analysis was conducted on a Thermal Nicolet Nexus 670 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) and spectra was processed by using OMNIC 7.2 software. Solid samples of DMY/Zein-CMD were flattened to ensure good contact with
the ZnSe crystal. 128 scans were performed at wavenumber range of 4000-600 cm\(^{-1}\) with 4 cm\(^{-1}\) resolutions.

2.5.2. X-ray powder diffraction (XRPD).

XRPD tests were performed with Panalytical X’Pert3 Powder X-ray Diffraction on a Si zero-background holder. The 2θ position was calibrated against Panalytical 640 Si powder standard. X-Ray wavelength of K\(_{\alpha1}\) and K\(_{\alpha2}\) were 1.540598 Å and 1.544426 Å. The K\(_{\alpha2}\)/K\(_{\alpha1}\) intensity ratio was 0.50. X-Ray tube setting was at 45 kV, 40 mA. The scan mode was continuous with range (°2TH) of 3° - 40° and step size of 0.026. Total run time was 20 min.

2.5.3. Polarized light microscopy (PLM).

PLM, as one microscope tool complementary to XRPD, was used to detect any birefringent specimens representing crystalline component under polarized light. The PLM image was captured on Nikon ECLIPSE Ci-POL in auto-exposure mode at room temperature. Oil immersion was used to disperse the sample in case of any large aggregates.

2.6. Thermal analysis.

In order to collect thermodynamic information, DSC was performed for DMY/Zein-CMD and reference crystalline DMY using a Q2000 TA DSC instrument. 10 mg of sample was sealed in a crimped aluminum pan and purge rate of nitrogen was 50 ml/min. The heating rate was at 10 °C/min and DSC thermogram was collected from 25°C to 280°C.

Modulated differential scanning calorimetry (mDSC) was conducted for DMY/Zein-
CMD using the same model of instrument, to investigate the amorphous characteristic from reversible heat flow. The mode of mDSC was set up at ramp rate of 3 °C/min with period of 40 s and amplitude of ±0.636. About 10 mg sample was sealed in a crimped aluminum pan using nitrogen as the purge gas (50 mL/min) and the heat flow was recorded from 25°C to 280°C.

2.7. Morphology study.

The morphology of DMY/Zein-CMD was studied under tapping mode by Veeco AFM (NanoScope IIIA Multimode, Santa Barbara, CA). Briefly, 200 μL of fresh prepared sample solution was placed on a silicon chip and fast dried using nitrogen gas. The 2 N·m⁻¹ of spring constant of silicon tip was used.

SEM image of DMY/Zein-CMD was collected by a XL-30 Field Emission Scanning Electron Microscope (FESEM, Japan). Solid sample obtained through nitrogen dry was coated with 10 nm of Au on silicon chips to improve electrical conductivity. Accelerating voltage of field emission gun was 20 kV and the scale bar was 1 μm.

2.8. Dissolution study in bio-relevant media.

Firstly, the solubility of crystalline DMY was measured followed by United States Pharmacopeia (USP) [135]. FaSSGF (FaSSIF or FeSSIF) buffer was used as dissolution media. Excessive DMY was suspended in 5 mL of dissolution media under stirring of 100 rpm at 37 °C for 4 h. The suspension was taken out and filtered through 0.25 PTFE membrane for HPLC test.

Then, dissolution test of DMY/Zein-CMD was designed imitating the USP dissolution apparatus II with paddle at 100 rpm [67]. Kinetic solubility of DMY released from DMY/Zein-CMD was measured, and the result was compared with solubility of crystalline DMY under the same media condition. Briefly, samples (eq. to 100 mg of
DMY) were loaded in 100 mL of dissolution vessel at 37°C. Suspension solution was withdrawn at certain time points (15 min, 30 min, 1 h, 2 h, 4h) and 20 uL of supernatant obtained by filtration (0.25 µm PTFE filter) was injected into HPLC (DIONEX UltiMate 3000) for measuring the concentration of released DMY.

The typical parameters of HPLC method was set up as follows [150]. An Luna Carbon 18 column (250 mm × 4.6 mm, 5 µm) was used, with an isocratic mode used by consisting of 75% mobile phase A (0.1% phosphoric acid in water) and 25% mobile phase B (acetonitrile). The flow rate was 1.0 ml/min, and UV wavelength was 292 nm. The standard solution of DMY was accurately prepared at 0.420 mg/ml, which was termed as standard 100%. Calibration curves was established over the range of 21-420 µg/mL with regression coefficient greater than 0.9999. The nominal retention time of DMY was around 6.0 minute. The limit of detection (LOD, signal/noise ≥ 3) and limit of quantification (LOQ, signal/noise ≥ 10) were determined as 2.1 µg/mL and 4.2 µg/mL, respectively. The method validated on precision and recovery. The RSD of intra-day precision (n = 6) based on six replicated injections using 100% and 10% standards solution were 0.28% and 0.61%, respectively. The mean recovery ± RSD (n = 9) of DMY was 99.7% ± 0.9%. All measurements of samples were carried out in triplicate.

3. Results and Discussions.

3.1. DMY/Zein-CMD dispersion.

Table III-1. Loading and encapsulation efficiency by DMY load.
In the present research, DMY/Zein-CMD dispersion was first prepared through anti-solvent method under ultra-sonication, and then solid sample was obtained by freeze drying. As sorted by initial load of DMY, the loading efficiency and encapsulation efficiency for each sample were displayed in Table III-1. In principal, adding more DMY could lead to higher loading, and when up to 30 wt.% of DMY was added, the loading and encapsulation efficiency were measured to be 17.1%±1.3% and 57.1%±4.3%, respectively. However, visual turbidity was observed as DMY load was above 30%, possibly due to the strong hydrophobicity of DMY in aqueous solution resulting in overload of DMY and corresponding precipitation. Figure III-1 shows the visual images of three different formula dispersion at 30 wt.% of DMY. Obvious precipitation was found when aqueous ethanol solution containing DMY and pristine zein was added dropwise into water, indicating the failure of DMY encapsulation (Figure III-1b). Turbidity was also observed for another unsuccessful trail of using CMD to coat DMY-loaded zein colloidal particles (Figure III-1c). An uniform dispersion state was only achieved using Zein-CMD as the carrier (Figure III-1a), indicating that Zein-CMD outperformed the above two candidates in stabilizing DMY-encapsulated dispersion with a higher load.

<table>
<thead>
<tr>
<th>Initial load of DMY (wt.%)</th>
<th>Loading efficiency (%)(^a)</th>
<th>Encapsulation efficiency (%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%</td>
<td>17.1%±1.3%</td>
<td>57.1%±4.3%</td>
</tr>
<tr>
<td>20%</td>
<td>6.6%±1.3%</td>
<td>32.9%±6.4%</td>
</tr>
<tr>
<td>10%</td>
<td>2.2%±0.7%</td>
<td>21.7%±7.5%</td>
</tr>
</tbody>
</table>

\(^a\) Each value is a mean of three measurements ± SD.
**Figure III-1.** Visual images of three formula dispersion: (a) DMY/Zein-CMD in water, (b) DMY/zein in water and (c) DMY/zein in CMD solution. The initial load of DMY for all three formula is 30 wt.%

By DLS, DMY/Zein-CMD dispersion showed an average size of 136.2 ± 0.9 nm and PDI value of 0.217 ± 0.006, indicating its homogeneity at Nano-scale level. According to the previous work in Chapter II, Zein-CMD micelles with an average size of 125.3±0.9 nm and PDI of 0.161±0.025 were obtained under the similar preparation procedure. By comparison, the size of DMY/Zein-CMD increased within a reasonable range (by about 10%), suggesting the integrity of Zein-CMD micellar structure during encapsulation of DMY. The zeta-potential was measured to be -47.4 ±1.5 credited to the negative charges of carboxyl groups from the polymeric carrier Zein-CMD. The strong electrostatic impulsion induced by high negative surface charge could further explain the appearance of translucent homogeneity of DMY/Zein-CMD dispersion at nano-scale, as reported in other literature using carboxymethyl chitosan as outer material for stability of zein nanoparticles suspended in aqueous solution [17, 147, 151]. This suggests that Zein-CMD could form micellar carriers to efficiently encapsulate the core-located DMY and simultaneously stabilize the overall carrier by outer contact between hydrophilic CMD and water layer [18].
3.2. Interactions between DMY and Zein-CMD.

As shown in Figure III-2, IR was performed to study the dominant interactions between the carrier Zein-CMD and encapsulated DMY. For Zein-CMD, the typical absorption peaks belonging to amide I/II appeared at 1637 cm\(^{-1}\) or 1543 cm\(^{-1}\), respectively. The broad peak of 3300 cm\(^{-1}\) corresponded to stretching vibrations of hydroxyl groups (O-H). After DMY/Zein-CMD was prepared, however, a slight shift toward lower wave number at 1635 cm\(^{-1}\) was observed in amide I region, and more obviously, the absorption intensity of amide II had a sharp decrease. It suggested the hydrogen bonding formation at the carbonyl group which caused the intensity decrease in C=O characteristic double bond \(^{[149, 152]}\). Furthermore, the peak of 3300 cm\(^{-1}\) became wider without significant peak shift, indicating the formation of hydrophobic interactions between carrier and DMY. In addition, in the fingerprint region one predominant absorption peak of DMY was found at 1160 cm\(^{-1}\), indicating successful encapsulation \(^{[153]}\). By comparing FTIR spectra of carrier Zein-CMD and DMY/Zein-CMD, the significant change in regions of amide I and II can indicate the formation of hydrogen bonding, which could make a synergetic contribution to restraining molecular mobility of DMY molecules and improving the physical stability with conventional hydrophobic interactions. For an amorphous system, prevention of recrystallization is always a challenge considering thermodynamically instability and incline to recrystallize due to structural relaxation \(^{[31, 154]}\). Restrain of molecular mobility is the key point because in some cases, even below the glass transition temperature, amorphous system still could find enough mobility to turn back into crystalline material over investigated time period \(^{[154]}\). Therefore in our work, the polymeric carrier Zein-CMD is expected to reduce molecular mobility of amorphous DMY via hydrophobic interactions and hydrogen
bonding, subsequently achieving a better physical stability \cite{155,156}.

![FTIR spectra comparison](image)

**Figure III-2.** FTIR spectra of carrier Zein-CMD and DMY/Zein-CMD.

### 3.3. Accelerated stability test.

Physical stability of DMY/Zein-CMD under accelerated stress conditions was studied by XRPD and PLM (Figure III-3), considering that temperature and humidity are two main inducements for amorphous material to recrystallize due to the molecular mobility and plasticizing effect \cite{157}. According to the Bragg equation, regular diffraction patterns can only be obtained from crystalline component with periodic spatial lattice structure, thus diffraction peaks in XRPD spectra can represent the existence of crystalline structure while only hump-like amorphous halo can be observed for pure amorphous material \cite{158}. As can be seen in Figure III-3a, after exposure to 25°C, 40% relative humidity (RH) for 3 months, diffraction peaks were not observed for DMY/Zein-CMD confirming its amorphous origin. By comparison, the mixture of crystalline DMY and Zein-CMD (w/w, 3:7) showed typical diffraction peaks on top of amorphous halo originated from the carrier Zein-CMD. Furthermore, when relative humidity was increased from 40% RH to 75% RH at 25°C, no obvious diffraction peaks were found.
after 1 month’s exposure (Figure III-3b). It was possibly because DMY was encapsulated in the hydrophobic core of Zein-CMD, inhibiting further plasticization of encapsulated amorphous DMY molecules by moisture contact. However, significant diffraction peaks were observed when temperature was heated up to 40°C at 75%RH, indicating the occurrence of DMY recrystallization as displayed in Figure III-3b. It suggested that temperature was dominant regarding on the molecular mobility of DMY molecules under accelerated stress conditions, which could lead to the later recrystallization.

Figure III-3. (a) XRPD overlay of DMY/Zein-CMD under exposure to 25°C, 40%RH after 1 week, 1 month and 3 months. (b) XRPD overlay of DMY/Zein-CMD after 1 month’s exposure to three different storage conditions (40°C, 75%RH), (25°C, 75%RH) or (25°C, 40%RH). XRPD pattern of carrier Zein-CMD and the mixture of crystalline DMY and Zein-CMD (w/w, 3:7) are shown as the reference. (c) PLM image of mixture of crystalline DMY and Zein-CMD (w/w, 3:7). (d) PLM image of DMY/Zein-CMD after
3 month’s exposure to 25°C, 40%RH. (e) PLM image of DMY/Zein-CMD after 1 month’s exposure to 25°C, 75%RH. All PLM images were taken under magnification x200.

On the other hand, PLM under magnification of x200 was used as a complementary tool to capture crystallization potential of amorphous DMY/Zein-CMD considering that tiny crystals could pass the detection of XRPD \[31\]. PLM can detect birefringent areas from crystalline component that is supposed to appear as bright particles (such as colorful plates, needles, etc.) in the micrographs. As shown in Figure III-3c, birefringent plates were observed for the mixture of crystalline DMY and Zein-CMD (w/w, 3:7). But no birefringent area was found in PLM image of amorphous DMY/Zein-CMD, either after 3 month’s exposure to 25°C, 40%RH (Figure III-3d) or after 1 month’s exposure to 25°C, 75%RH (Figure 3e). The findings by PLM indicated that amorphous DMY was able to be stabilized in carrier Zein-CMD at room temperature but under high relative humidity, which was designed to plasticize the amorphous DMY and accelerate recrystallization.

Through accelerated stability tests by XRPD and PLM, it is suggested that temperature was predominant followed by relative humidity on the behalf of stability of amorphous DMY/Zein-CMD during storage and shipment.

3.4. Thermal analysis.

Figure III-4 shows the thermal properties of DMY/Zein-CMD by DSC and mDSC. As can be seen in Figure III-4a, DSC curve of pristine crystalline DMY showed an endotherm around 237.6°C (onset point) followed by thermal decomposition; whereas no visible endothermic peak was observed from that of DMY/Zein-CMD, suggesting that DMY molecules were dispersed in the carrier Zein-CMD in an amorphous status. In
general, the amorphous materials exhibit either solid-like (glassy state) or liquid-like (rubbery state) properties, and the temperature range of the transition from glass to liquid or vice versa is defined as glass transition ($T_g$). Unlike crystalline structure, DMY molecules in amorphous status are randomly packed without long-range orders, corresponding to typical glass transition without melting peak in the DSC curve of DMY/Zein-CMD.

**Figure III-4.** (a) DSC overlay of crystalline DMY and amorphous DMY/Zein-CMD. (b) mDSC curve of amorphous DMY/Zein-CMD.

Furthermore, glass transition ($T_g$) of DMY/Zein-CMD was determined using modulated DSC (mDSC). As the reversing heat flow shown in Figure III-4b, two small endothermic peaks related with the sample relaxation were observed before 100°C, and a glass transition around 187.7°C (middle-point) was detected. It has been reported that the
effect of molecular mobility on recrystallization would be negligible if 50 K below the $T_g$ \[^{[157]}\], thus $T_g$ (187.7°C) of DMY/Zein-CMD detected by mDSC could be an instructive reference regarding on the safe storage conditions.

3.5. Morphology study.

The morphology of solids (e.g. spherical shape, needle-like shape, etc.) would affect the flowing properties of powder material, subsequently affecting the later process and final critical quality attributes of products \[^{[159]}\]. Thus morphology of amorphous DMY/Zein-CMD obtained through freeze drying was captured by AFM and SEM, respectively.

**Figure III-5** presents the height and three-dimensional AFM image of surface morphology of DMY/Zein-CMD. The sphere solid particles with sharp surface were observed, and aggregation of solids was also found in **Figure III-5a**, which was consistent with SEM image of DMY/Zein-CMD.

As can be seen in **Figure III-6**, irregular spherical solids were observed in aggregation format. The size of individual solids was 100-150 nm based on the scale bar of 1 μm, which matched the previous DLS data.
Figure III-5. (a) Height and (b) 3D AFM image of DMY/Zein-CMD. The Z range was 50 nm.

Figure III-6. SEM image of DMY/Zein-CMD.
3.6. Dissolution study in bio-relevant media.

Table III-2. Kinetic solubility of DMY released from DMY/Zein-CMD or crystalline DMY in bio-relevant media

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>Solubility in FaSSGF (mg/mL)</th>
<th>Solubility in FeSSIF (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMY/Zein-CMD</td>
<td>DMY</td>
</tr>
<tr>
<td>15</td>
<td>0.849±0.022</td>
<td>0.123±0.013</td>
</tr>
<tr>
<td>30</td>
<td>0.884±0.018</td>
<td>0.145±0.009</td>
</tr>
<tr>
<td>60</td>
<td>0.887±0.015</td>
<td>0.161±0.008</td>
</tr>
<tr>
<td>120</td>
<td>0.877±0.015</td>
<td>0.160±0.015</td>
</tr>
<tr>
<td>240</td>
<td>0.886±0.019</td>
<td>0.159±0.018</td>
</tr>
</tbody>
</table>

Each data is a mean of three measurements ±SD

Table III-2 presents in vitro kinetic solubility data of DMY released from DMY/Zein-CMD or pristine crystalline DMY incubated in bio-relevant media including FaSSGF (simulation of stomach, pH=1.2) and FeSSIF (simulation of intestine in fed states, pH=5.0). The DMY solubility in FaSSGF (FeSSIF) reached to be 0.16 mg/mL (0.09 mg/mL) after 4 h equilibration, which indicated that higher solubility of DMY was achieved at lower pH. By comparison, the solubility of DMY released from DMY/Zein-CMD was 5.6 times (or 7.2 times) that of crystalline DMY incubated in FaSSGF (or FeSSIF), suggesting that amorphous DMY in carrier Zein-CMD created supersaturated solution and increase the solubility. Moreover, as shown in Figure III-7a, none of visually transparent solution was obtained after filtration (0.25 µm PTFE filter) for crystalline DMY incubated in FaSSIF (simulation of intestine in fasted states, pH=6.5). This finding was in agreement with the result from Ruan et al. [134], who concluded that
DMY was unstable in solution when pH > 6.0, as similar as the phenomenon in FaSSIF buffer at pH 6.5 in our work. It suggests that administration of DMY product in the fasted state is unfavorable. On the contrary, the filtered clear supernatant was achieved from DMY/Zein-CMD suspension (Figure III-7b). It implied that carboxymethyl groups of Zein-CMD could adjust pH at local micro-domain so that pH-induced degradation of DMY was minimized in FaSSIF.

![Figure III-7. (a) Image of turbid solution of crystalline DMY incubated in FaSSIF after filtration through 0.25 PTFE membrane. (b) Image of filtered clear supernatant of DMY/Zein-CMD incubated in FaSSIF. Time points from left to right were 15 min, 30 min and 60 min.](image)

**Figure III-8** shows the dissolution profiles of amorphous DMY in Zein-CMD as compared to crystalline DMY incubated in FaSSGF or FeSSIF. The dissolution rate of crystalline DMY was slow with <16% and <10% of DMY being dissolved in FaSSGF and FeSSIF within 15 min, respectively. The dissolution rate of amorphous DMY from DMY/Zein-CMD appeared much faster, with the percentage of dissolved DMY > 84% in
FaSSGF and > 64% in FeSSIF within 15 min. Furthermore, no significant ‘Spring & Parachute’ trend was observed for dissolution curves of DMY/Zein-CMD in both two buffers \[1^{160}\], and a supersaturating status was maintained with > 88% or > 66% of DMY being dissolved in FaSSGF or FeSSIF within 4 hours. It was evident that higher solubility and faster dissolution of DMY was achieved for DMY/Zein-CMD as compared to crystalline DMY alone in the same bio-relevant media, which concluded that disrupting crystalline structure into amorphous one could be an efficient strategy regarding on bioaccessibility enhancement of aqueous poor soluble phytochemicals.

**Figure III-8.** Dissolution curves of DMY/Zein-CMD or crystalline DMY alone in FaSSGF or FeSSIF. Each data point is a mean of three measurements ± SD.


In this chapter study, amorphous solids DMY/Zein-CMD were successfully produced. When initial DMY load was up to be 30 wt.%, the average particle size and
zeta potential of prepared DMY/Zein-CMD dispersion were 136.2±0.9 nm and -47.4±1.5, respectively. FTIR analysis illustrated that hydrogen bonding and hydrophobic interactions would be formed during DMY encapsulation, contributing to the restrain of molecular mobility and prevention of recrystallization. Combining XRPD and PLM results, amorphous origin of DMY was confirmed and recrystallization of DMY was found as temperature increased to 40°C at 70%RH. The reversing heat curve of mDSC showed one glass transition ($T_g$) around 187.7°C (middle-point) for DMY/Zein-CMD, and sphere solid particles with sharp surface were observed by AFM and SEM. The dissolution study revealed that solubility of amorphous DMY can be 5.6 times or 7.2 times that of crystalline DMY incubated in FaSSGF or FeSSIF. Besides, at least 5 times faster dissolution rate was achieved for DMY/Zein-CMD than that of crystalline DMY within the first 15 min. In summary, the amorphous DMY in Zein-CMD has achieved increased aqueous solubility and faster dissolution rate as compared to crystalline DMY in the same bio-relevant release condition.
CHAPTER IV. OIL-IN-WATER PICKERING EMULSIONS
STABILIZED BY LAURYL MODIFIED ZEIN PARTICLES

1. Introduction.

Because of superior stability against coalescence and surfactant-free character, Pickering emulsions (PE) using food-compatible material particles has attracted a lot of interests over the decade, considering their emerging application potentials in food, consumer or pharmaceutical field \cite{24, 161}. So far different types of naturally derived material have been proposed to stabilize PE, such as starch granules and its derivatives \cite{162}, cellulose nanocrystals \cite{163}, prolamin-based colloidal particles \cite{25, 164}, etc. Among them protein-derived Pickering stabilization particles such as zein \cite{26}, kafirin \cite{164}, soy protein \cite{165}, pea protein \cite{166} and gliadin \cite{167}, etc. have been developed to balance the partition and the interfacial tension in oil/water based on the empirical equation as following \cite{168}.

\[
\Delta E = \gamma_{ow} \pi R^2 (1 - |\cos \theta|)^2
\]

Where $\gamma_{ow}$ is the oil-water interfacial tension, $R$ is the radius of colloidal particle, $\theta$ is the contact angle among the three phases, $\Delta E$ is the energy for colloidal particles to be detached from the interface. For any potential colloidal particles, partial wettability with the $\theta$ range of 30º to 150º are expected for the irreversible adsorption into the interface, under which detachment energy is much higher than Brownian motion.

Zein protein, the commercial prolamin representative with abundant resource from the corn \cite{169}, has been built up as a model for massive studies of food-grade particles stabilized oil in water (O/W) Pickering emulsions since 2012 \cite{25}. Zein has sufficient
hydrophobic amino acids (e.g. leucine, proline) but relatively deficient in acidic or basic amino acid residues, determining its insoluble characteristic in both oil phase and water phase \[^{170}\]. Hence zein particles will be anchored at an oil/water interface. Moreover, the alcohol soluble nature can facilitate zein’s self-assembly into colloidal particles via anti-solvent method, in which zein-based binary or even ternary complexes have been prepared as colloidal emulsifiers as well \[^{27,28}\]. In fact, it agrees with the current trend that zein-polymer or zein-surfactant complexes are fabricated to stabilize Pickering emulsions as oil phase fraction (\(\phi\)) is increased, due to the PE instability if only zein particles as the stabilizer. It can be explained by classical Young’s equation \(\cos \theta = (\gamma_{so} - \gamma_{sw}) / \gamma_{ow}\)^\[^{171}\], where solid colloidal particles (s), oil phase (o) and water phase (w) intersect, and \(\gamma\) refers to the interfacial tension. For Pickering emulsions stabilized by one layer of zein particles, the contact angle has to be within the range of 15\(^\circ\) to 90\(^\circ\), which means that zein particles are more immersed in water phase over oil phase as \(\gamma_{so} > \gamma_{sw}\)^\[^{24}\]. It might result in the oil phase leakage or even phase inversion at high oil internal phase fraction, leading to the instability and final breakage of Pickering emulsions \[^{26}\].

In the face of this issue, tuning the hydrophile-lipophile balance (HLB) of zein particles at the interface has been proposed through physical complexation with surfactants or biopolymers (e.g. polysaccharides, proteins). For instance, Gao et al. used ionic surfactant sodium stearate to form complex with zein, but related stable Pickering emulsions were only prepared at \(\phi = 0.1\) when added surfactant concentration was above 10 mM \[^{172}\]. Feng et al. absorbed sodium caseinate onto the surfaces of zein colloidal particles to stabilize Pickering emulsions at \(\phi = 0.5\). However, numerous conditionality had to be considered such as the specific pH of complex formation at 3.0, the influence of
amount of added surfactant on the wettability of zein colloidal particles over oil/water phase, etc.\[^{29}\] In fact, it is the common problem for physical blending method of zein particles and other emulsion stabilizers, in which complex can be only fabricated under specific conditions through non-covalent bonds \[^{173}\]. Furthermore, it does not always work using complexes to stabilize Pickering emulsions as $\phi > 0.5$. It is found that merge of oil droplets occurred for Pickering emulsions stabilized by zein-chitosan particles when $\phi$ was increased to 0.7, suggesting that it was close to the phase inversion threshold for PE system \[^{174}\].

Recently, in order to further strengthen the PE structure at high oil internal phase, zein/gum complex have been introduced as emulsion stabilizers such as gum Arabic \[^{175}\], corn fiber gum \[^{176}\], etc. Dai et al. generated complex particles (1 wt%) of zein and gum Arabic to stabilize Pickering emulsions at $\phi = 0.7$ \[^{175}\]. Another research by Li et al. indicated as concentration of zein/gum Arabic colloidal particles was as high as 6.25% (w/v), the creaming index (that is used to indicate the creaming extent for Pickering emulsions at different oil internal phase fraction $\phi$) cannot even be obtained when $\phi > 0.5$ due to the flocculation \[^{177}\]. The contrary conclusions between these two works might be attributed to the different resource of gum Arabic, implying that further studies are still needed as zein/polysaccharide complex are introduced to stabilize Pickering emulsions.

In terms of stabilization of Pickering emulsions as oil internal phase fraction increases, chemical modification allows an alternative exploration to intrinsically tune surface wettability of zein-based colloidal particles, instead of conditional physical blending method. Although a great number of research work on chemically tailoring the surface of inorganic colloidal particles \[^{178}\], to the best of our knowledge, few work has reported chemically tuning wettability of zein colloidal particles for PE stabilization. Since zein-based colloidal particles are preferably wetted by water phase over oil phase,
we hypothesized that slight hydrophobic modification for zein protein material could result in the intermediate wettability in the oil-water interface.

Herein in this chapter, we present a study on the stabilization of Pickering emulsions by lauryl-zein conjugate which is obtained by grafting lauryl chlorides onto zein. The objective in our work is to determine the chemical modification extent confirmed by $^1$H NMR, where stabilization of Pickering emulsions based on zein conjugate particles can be realized at $\varphi = 0.7$. Moreover, a relationship between chemical modification extent and HLB of modified zein particles reflected by contact angle can be built up, as an instructive experience for precisely tuning the surface interface wettability of zein particles.


2.1. Materials.

The $\alpha$-Zein (abbreviated as zein) was purchased from Wako Chemical Industries, Ltd. (Tokyo, Japan). Lauryl chloride (ACS grade, Acros Organics) and trimethylamine (TEA, ACS grade) were purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Deuterated dimethyl sulfoxide (DMSO-$d_6$) was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Milli-Q water and pure ethanol were obtained from Food Science Department, Rutgers. (New Brunswick, NJ). All organic solvents (such as dimethylformamide, diethyl ether, etc.) were purchased from VWR, Ltd. (Bridgeport, NJ).

2.2. Preparation of zein conjugate.

Zein conjugate was prepared by chemically grafting lauryl chloride chains onto zein protein by acylation reaction with some modifications $^{179, 180}$. TEA (0.5, 1, 2, or 4 mmol) per gram of zein was firstly added into a 5% w/v solution of zein in DMF while
continuously stirring for 10 min. Then lauryle chloride (1.5, 3, 6 or 12 mmol) was added dropwise and the mixture solution was sealed and heated to 65 ºC for 3h under stirring. Subsequently, the reaction mixture was poured into diethyl ether under stirring to obtain the precipitated product and remove any residual small molecular reagents and organic solvent including TEA, unreacted lauryle chloride, DMF. The washing step was repeated three times. The final product zein conjugate samples were named as zein-C0.5, zein-C1, zein-C2 and zein-C4, according to the feed molar/mass ratios of lauryle chloride to zein.

Chemical bonds and the amount of lauryle per protein in the modified products were characterized by ¹H NMR and FTIR. The ¹H NMR tests were performed for all zein conjugate samples and pristine zein protein as reference in DMSO-δ6 at 25 ºC using a Varian VNMRS 500 MHz spectrometer. FTIR spectra were acquired using a Thermal Nicolet Nexus 670 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) and data was processed by using OMNIC 7.2 software. All zein conjugate samples and pristine zein were flattened to ensure good contact with the ZnSe crystal. 64 scans were used at wavenumber range of 4000 ~ 600 cm⁻¹ with 16 cm⁻¹ resolutions.

The dispersion status and conformation of the modified proteins were further characterized using solution small-angle X-ray scattering (SAXS). Zein and zein conjugate solutions were prepared with both 80% ethanol-water and acetic acid at ambient temperature within a concentration gradient. SAXS experiments were carried out at the Bio-CAT, 18-ID beam-line section of Advanced Photon Source (Argonne National Laboratory). Single exposure period was 1s and X-Ray wavelength was 1.03 Å, with. Samples were loaded into a cylindrical quartz flow cell of 1.5mm diameter with constant load rate (10 μL/s). A brass block with 25°C water flow was equipped around the quartz cell to maintain constant temperature. The scattering vector q ranged from 0.0052 Å⁻¹ to 0.38 Å⁻¹. All SAXS intensity profiles were collected by the mean of 15 measurements
post proper background subtraction.

2.3. Preparation of zein conjugate particles

1 wt% of zein conjugate particles were prepared via an anti-solvent method. 0.1 g of zein conjugate (zein-C0.5, zein-C1, zein-C2 and zein-C4) was dissolved in 1.5 mL of acetic acid to prepare stock solution, which was then added dropwise into 8.5 mL DI water under ultrasonication. Removal of acetic acid was performed by dialysis against DI water until the pH of suspension solution was no less than 3.0. Zein particles (1 wt%) was prepared using the same method above as the reference.

The size and hydrophilic-lipophilic balance (HLB) value of the prepared zein conjugate particles were characterized using dynamic light scattering (DLS) and water-in-air contact angle. The two critical attributes were also measured for prepared pristine zein particles.

The average particle size of freshly prepared suspension containing zein conjugate particles was measured in triplicate by DLS at room temperature after dilution. A solid-state laser was operated at 660 nm, and scattering measurement was made at a fixed vertical angle with the refraction index as 1.333 (Between 1.330 for pure water and 1.355 for pure ethanol). The data was processed by cumulate analysis based on intensity-intensity autocorrelation function, G (q, t).

For comparing the HLB of zein conjugate particles with different degree of grafting, water-in-air contact angle ($\theta_{aw}$) of zein conjugate casting films were determined as followed. Typically, each suspension was evenly casted onto the pre-cleaned glass slide and homogeneous zein conjugate (or pristine zein) film was obtained by evaporation at 60 ºC overnight. By depositing one water droplet onto the fleshly prepared film under ambient atmosphere, the water-in-air contact angle was determined using an AST Contact Angle System (AST Products Inc., MA). Each measurement was repeated over at least 6
times.

2.4. Preparation of zein conjugate PE

The preparation procedure of zein conjugate particles stabilized Pickering emulsion is described as following. 1 wt% zein conjugate was dispersed with vegetable oil at oil fraction of 0.5 or 0.7, and then the 10 mL of mixture solution was high-speed homogenized with a 10 mm homogenization probe (T25 homogenizer, IKA 190 Works, Inc., Wilmington, NC) at 14,000 rpm for 2 min. The droplets of each freshly prepared Pickering emulsions were added into DI water (oil phase) to identify the type.

The stability, microstructure observation and the rheological behavior of the Pickering emulsions were also studied.

The visual stability of PE stabilized by zein conjugate particles at ambient temperature were visually compared with that of zein after equilibration for specific period, e.g. 1 day and 1 week. Then the stabilizing ability of zein conjugate particles (or pristine zein particles) at $\phi = 0.5$ or 0.7 was quantified by stability index ($SI$), which was calculated based on the equation as following $^{[181]}$.

$$SI = \frac{H_{7d}}{H_{1d}} * 100$$

Where $H_{7d}$ is the height of emulsions post 1-week storage, and $H_{1d}$ is that of 1-day storage.

Microstructures were visualized using a Nikon microscope (10×objective, Eclipse TE 2000-U, Nikon Corporation, Tokyo, Japan). The droplet size was estimated by obtained image $^{[164]}$.

The critical rheological properties of zein conjugate particles stabilized Pickering emulsions, e.g. storage modulus and loss modulus, apparent viscosity, were measured using a strain-controlled ARES rheometer (TA Instruments, New Castle, DE) with a cone-and-plate geometry (0.04 rad of cone angle, 50 mm of diameter, 0.05 mm of gap).
Each freshly prepared Pickering emulsions was flattened onto the plate for 3 min equilibrium before measurement. The linear viscoelastic region was first determined by dynamic strain sweep at a fixed angular frequency of 10 rad/s. Then oscillatory frequency sweep experiment was conducted within 0.1-100 rad/s to measure storage modulus $G'$ and loss modulus $G''$ of Pickering emulsions depending on the frequency. In addition, the curves of apparent viscosity vs. shear rate (from 0.1 to 100 s$^{-1}$) were recorded by steady shear experiments. All of the measurements were carried out under ambient atmosphere. All measurements were performed in duplicate.

2.5. Statistical analysis

Each experiment was conducted in triplicate, unless otherwise stated. All statistical analysis was performed using software of OriginPro 2010.

3. Results and Discussions.

3.1. Characterization of zein conjugates

It has been reported that zein protein can be chemically modified for achieving the targeted material property via acylation reaction $^{[179, 182]}$, because reactive side amine group of lysine in zein providing potential reaction sites to form amide conjugations.

As shown in Figure IV-1, along the increase of lauryl, typical adjacent peaks at 2977 and 2884 cm$^{-1}$ credited to the vibration of long-chained methylene groups were intensified $^{[183]}$, which indicates the formation of conjugates and more lauryl chains are grafted onto the zein molecules. Meanwhile, the peak intensities of amide I band at 1650 cm$^{-1}$ and amide II band at 1540 cm$^{-1}$ were both decreased $^{[184, 185]}$, indicating that amino groups of zein protein, e.g. side amine group of lysine, could be the potential site for acylation reaction with the new amide group formed. The new emerged peak at 1260 cm$^{-1}$ was assigned to the stretching vibration of the C-N bond on the amide III band $^{[184]}$. The
typical peak due to stretching vibration of methyl group from the lauryl chain end was observed at 1380 cm\(^{-1}\), and multiple peaks at around 1220 - 980 cm\(^{-1}\) could be credited to fingerprint region of C-N bond in the aliphatic chains.

**Figure IV-1.** FTIR spectra of lauryl-modified and pristine zein. Characteristic bands for protein and lauryl chains are labeled.

Further, **Figure IV-2** shows \(^1\)H NMR spectra comparison between zein and zein conjugates, confirming the successful grafting of lauryl chains onto zein molecules. The molar ratio of lauryl chain to zein protein can be quantified by the integral area ratio of peaks at 1.2 ppm and 0.8 ppm. The baselines of all \(^1\)H NMR spectra were corrected by 3-order Bernstein Polynomial Fit before integration. The integral area of peak in CH\(_3\) (δ 0.8) was standardized as 1.0, and the integral peak area in CH\(_2\) (δ 1.2) was 0.5. In zein conjugates, a new emerging triplet peak was found to squeeze on the right shoulder of
original one in CH\textsubscript{2} (δ 1.2), which referred to methylene H from lauryl chains and its integral area was 0.4 with the standard area of 1.0 as reference. The area ratio of integral peak in new emerging triplet peak to the standard reference of 1.0 increased from 0.4:1.0 to 3.2:1.0 from conjugates zein-C0.5 to zein-C4, suggesting more lauryl chains were grafted onto the zein molecules. Assume that methyl groups CH\textsubscript{3} (δ 0.8) were all from zein protein, the number of lauryl chains per zein protein are 1, 2, 6 and 8 for these four conjugates.
Figure IV-2. $^1$H NMR spectra of overlay of zein and zein conjugate. $^1$H NMR spectra of both zein and zein-C0.5 around 0.7-1.3 ppm were zoomed in.

Then solution SAXS experiments were performed to investigate how modification affected the conformation and the dispersion of zein. As displayed in Figure IV-3, ellipsoid model was found the best to fit the intensity profiles of two zein conjugate
solutions (zein-C1 or zein-C4 in acetic acid), and perpendicular, parallel semi axis and the gyration radii (Rg) were extracted and plotted in Figure IV-3 (b) and (c). The result suggests that the modification did not change the rod like shape of single particle; modified zein particles are longer and larger than zein itself. Since the concentration range dose not reach C* (the overlap concentration of zein in acetic acid solution is 43 mg/ml [186]), zein particles are monodispersed in solution, but zein conjugates may aggregate with concentration increase. The fractal dimensions for solutions at 40 mg/ml reach 2.34 and 2.08 for zein-C1 and zein-C4, larger than that for pristine zein in acetic acid between 1.39 and 1.67 [32]. It suggests that lauryl grafting can decrease the solubility of zein in acetic acid, the aggregates of zein conjugates are more randomly distributed in acetic acid instead of rod-like aggregates for pristine zein. The influence of lauryl grafting on the conformation and the dispersion of zein in 80% alcohol solution was investigated by SAXS (Figure IV-4). As expected, acetic acid is dominant over ethanol/water mixing solution to dissolve zein, lauryl grafting decreased the solubility of zein in 80% alcohol solution and resulted in larger aggregates. Therefore, acetic acid was chosen to prepare zein conjugate particles due to better dispersing capability.
Figure IV-3. Intensity profile and ellipsoid model fit of zein-C1 (a) and zein-C4 (a’) zein conjugates in acetic acid. Variation trends of perpendicular semiaxis (solid point) and parallel semiaxis (hollow point) are shown in (b). In (c), solid triangles and squares are measurements of modified zein in acetic acid, the empty circles are measurements in acetic acid by Li et al.[186], the empty diamond is the measurement in aqueous methanol solution by Tatham et al.[187], whereas the empty squares are from zein in aqueous ethanol solutions collected by Matsushima et al.[188, 189]
Figure IV-4. Intensity profile and ellipsoid model fit of zein (a), 1:1 zein conjugate (a') and 4:1 zein conjugate (a'”) in 80% ethanol. Pair distance distribution function (PDDF) of zein (b), 1:1 modified zein (b’) and 4:1 modified zein (b’”). Variation trend of perpendicular semiaxis (c), parallel semiaxis (d) and radius of gyration Rg calculated using ellipsoid model (solid point) and GNOM (hollow point) (e).

3.2. Characterization of zein conjugate particles

Zein conjugate were prepared via anti-solvent method. After dialysis against DI water, only zein-C0.5 and pristine zein suspensions did not show visual precipitates, as illustrated in Figure IV-5. Other samples show clear precipitates which may be attributed from the large aggregates of them, as suggested from solution SAXS characterization. The larger aggregates of zein conjugates were also confirmed from DLS study. The 1 wt% zein-C0.5 dispersion has hydration radius of 238.8 ± 5.3 nm with PDI at 0.242 ±
0.007, while that for the pristine zein at identical condition is 138.4 ± 1.9 nm with PDI of 0.278 ± 0.005. The latter is close to previous reports \cite{16, 29}. For zein-C1, zein-C2 and zein-C4, DLS failed to present the size of particles because of visible aggregations. This might be due to the surging surface hydrophobicity when more lauryl chains were grafted onto zein molecules, which is similar with the phenomenon of depletion flocculation when zein particles are overly covered by excessive polysaccharides \cite{175, 191}.

**Figure IV-5.** Visual image of freshly prepared 1 wt% of (a) zein-C0.5 particle and (b) zein particle dispersions in water.

The HLB of zein conjugate particles is tunable depending on the degree of grafting, and it can be determined from the water-in-air contact angle ($\theta_{aw}$) where one water droplet onto the zein conjugate film was determined corresponding to wettability preference over water phase or oil phase. To date, it is believed that zein colloidal particles can be preferably wetted by water phase \cite{25, 29, 172}, with the average $\theta_{aw}$ of pristine zein film determined as 67.7° (**Figure IV-6a**). As displayed in **Figure IV-6**, the average $\theta_{aw}$ value increased from 67.7° to 97.7° when the degree of grafting increased
from zein-C0.5 to zein-C4. It indicates that grafting more lauryl chains onto the zein molecules could induce a wettability reversal of zein conjugate particles from water phase preference to oil phase preference. It is in agreement with the principle of physical blending method, that the wettability of zein particles in oil phase can be enhanced through incorporation of surfactants [29, 172] or bio-polymers [191, 192]. Furthermore, it opens one possibility to prepare water-in-oil (W/O) PE stabilized by more hydrophobic modified zein particles in the future. In our work, it is conceivable that oil-in-water (O/W) is the dominant type for zein or zein-C0.5 stabilized Pickering emulsions, but for zein-Cn (n ≥ 1) instable O/W Pickering emulsions might be inversed into W/O type.

Figure IV-6. Water-in-air contact angle ($\theta_{\text{aw}}$) image (one droplet on the film): (a) zein, (b) zein-C0.5, (c) zein-C1, (d) zein-C2 and (e) zein-C4. Each data is an average of six measurements ± SD.
3.3. Zein conjugate particles stabilized Pickering emulsions

Table IV-1. Stability characteristics of Pickering emulsions stabilized by zein conjugate or zein particles.

<table>
<thead>
<tr>
<th>Oil fraction (φ)</th>
<th>Particles</th>
<th>Emulsified volume fraction (%)</th>
<th>Stability index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 day</td>
<td>7 days</td>
</tr>
<tr>
<td>0.5</td>
<td>zein</td>
<td>75</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>zein-C0.5</td>
<td>90</td>
<td>75</td>
</tr>
<tr>
<td>0.7</td>
<td>zein</td>
<td>70</td>
<td>break</td>
</tr>
<tr>
<td></td>
<td>zein-C0.5</td>
<td>98</td>
<td>98</td>
</tr>
</tbody>
</table>

The O/W type was confirmed for PE stabilized by 1 wt% of zein-C0.5 conjugate or zein particles, as the emulsion droplets were found to be dispersed homogeneously in water but not oil. As shown in Table IV-1 and Figure IV-7 (a, b), similar to zein particles stabilized Pickering emulsions, zein-C0.5 conjugate Pickering emulsions were successfully prepared at φ = 0.5. Though a further shrink occurred after 7 days’ equilibration, a higher stability index was achieved for zein-C0.5 conjugate than that of zein particles stabilized Pickering emulsions. Furthermore, at φ = 0.7, 1 wt% zein-C0.5 conjugate could stabilize the Pickering emulsions very well with a 100% stability index after 7 days’ storage period (Figure IV-7c, d). For zein particles stabilized Pickering emulsions, however, coalescence accompanied by oiling off occurred with the storage time, and it already broke up at the storage time point of 7 days’ observation (Figure IV-7d).
Figure IV-7. Visual image of Pickering emulsions stabilized by 1 wt% of zein-C0.5 or zein particles at $\varphi = 0.5$ after storage period of (a) 1 day and (c) 7 days, or at $\varphi = 0.7$ after storage period of (c) 1 day and (d) 7 days.

Generally, increasing oil fraction can result in larger emulsified volume. Based on the study on kafirin particles stabilized Pickering emulsions, fully emulsified phase was finally obtained using 2 wt% of kafirin particles at $\varphi = 0.7$ \cite{164}. By comparison, zein-C0.5 particles can take the same effect at a lower concentration without use of any additional surfactants or bio-polymers. Moreover, the water contact angle ($\theta_{aw}$) for kafirin particles was measured to be $72^\circ$ \cite{164}, which is similar to the value for zein-C0.5 conjugate in our work. Based on measurements of $\theta_{aw}$, the HLB order should be zein > kafirin \approx zein-C0.5 conjugate. The contrast between zein’s failure and kafirin’s success indicates that HLB of material particles could possess a dominant role in the stabilization of Pickering emulsions at $\varphi = 0.7$. Herein, lowering HLB of zein particles through grafting lauryl chains can be a feasible way regarding on the stabilization issue.

The PE droplet microstructures by zein-C0.5 conjugate or zein particles are shown
in Figure IV-8 a-c. Spherical shape was observed for all three emulsion droplets without any apparent coalescence or flocculation. The droplet size for zein-C0.5 conjugate (1 wt%) appeared to be smaller as φ decreased from 0.7 to 0.5, possibly due to the limited number of stabilizing particles per oil droplet \[164\]. Rheology studies were conducted to help uncover the macroscopic properties behind the structure of Pickering emulsions. As seen in Figure IV-8d, the transcend of storage modulus $G'$ over loss modulus $G''$ was found over the entire frequency sweep range, and both $G'$ and $G''$ displayed weak reliance on the frequency, indicating that all three tested PE showed typical elastic behavior with a flocculated three-dimensional particle network \[171, 193\]. This finding matches the previous prolamin stabilized Pickering emulsions, that a dense pack of oil droplets on the structural base of colloidal particles leads to a gel-like status over liquid-like viscous behavior \[164, 194\]. In addition, a typical shear-thinning behavior was observed for all three Pickering emulsions (Figure IV-8e), indicating that a non-Newtonian flow-like network structure was formed through particles under low shear rate, but it was readily to break up when shear rate increased. The droplet size of three emulsions is in the order of zein-$\varphi=0.5 >$ zein-C0.5-$\varphi=0.7 >$ zein-C0.5-$\varphi=0.5$ by using power-law model to fit curves of viscosity vs shear rate \[195\]. As compared to pristine zein particles, zein-C0.5 conjugate can better contribute to the stability of Pickering emulsions against coalescence at $\varphi = 0.7$.

In general, increasing internal phase ratio can result in a higher emulsified phase with a greater gel strength, possibly because oil droplets would squeeze each other due to volume expansion at high oil internal phase fraction, leading to a more compact trapping network with strengthened rheological behaviors. To our best knowledge, however, there is few literature reporting that pristine zein particles at low concentration can stabilize high oil internal phase Pickering emulsions. It is because zein particles have higher HLB than kafirin, leading to the emulsion instability without use of surfactants or
polysaccharides. To properly adjust the HLB of zein particles might be the key solution, which has been fulfilled by lauryl modification in our work.

Figure IV-8. PE microscopic images for 1 wt% zein-C0.5 at (a) $\varphi = 0.5$ or (b) $\varphi = 0.7$. (c) PE microscopic image for 1 wt% zein at $\varphi = 0.5$. The scale bar of microscopic images was 100 μm in length. (d) Frequency sweep curves with frequency ranging at 0.1-100 rad/s. Storage modulus ($G'$) - filled, loss modulus ($G''$) - opened. (e) Apparent viscosity vs. shear rate (0.1-100 s$^{-1}$) fitted by power-law model.

3.4. Relationship among the degree of grafting, HLB and stability

As shown in Figure IV-9, the relationship among the degree of lauryl grafting onto zein protein, the HLB of conjugate particles and the PE stability was built up. The degree of grafting can be selective from 1 to 8 per zein, providing a wide range to control the wettability preference over the oil phase. Meanwhile, the extent of HLB modification was quantified by water contact angle ($\theta_{aw}$), providing a prospective guidance on visually targeting the candidate particles with appreciated HLB, e.g. $\theta_{aw}$ of zein-C0.5 was similar with that of kafirin. Hence, zein-C0.5, namely grafting 1 lauryl chain per zein, was
found to achieve a target HLB to accommodate 70% oil phase at 1 wt%. This finding is consistent with the current study that HLB of particles is seen as the paramount principle on stabilization of Pickering emulsions, regardless of naturally derived particles\textsuperscript{[161]}, physical colloidal complex\textsuperscript{[175]} or inorganic particles\textsuperscript{[178]}.

Not like conventional blending method that HLB of zein-based particles are adjusted by the ratio of zein and stabilizer, the limitation of saturated surface coverage can be avoided by chemical modification. For instance, sodium caseinate has been reported to adsorb and modify wettability of zein particles, but surface coverage was complete as zein: sodium caseinate ratio reached to be 10:3\textsuperscript{[29]}. When the ratio was up to 10:4, the measured three phase contact angle ($\theta$~85°) was same as that of plain sodium caseinate, indicating the limitation of modifying zein particles’ surface regardless of even more sodium caseinate (the ratio above 10:4) was added. On the contrary, lauryl modification in our work can continuously increase the water contact angle ($\theta_{aw}$) of zein conjugates from 67.4° ± 2.1° to 98.9° ± 1.9° without the limitation, and grafting 1 lauryl chain per zein can significantly enhance the inherent wettability preference over oil phase, which is very conducive to reducing the reliance of stabilizers on HLB adjustment of zein-based particles.
Figure IV-9. Relationship of degree of lauryl grafting and HLB. The red triangle is the measurement of water contact angle of kafirin by Xiao et al. [164].


Oil-in-water Pickering emulsions at oil phase fraction $\varphi = 0.7$ could be stabilized solely by lauryl-zein conjugate, with much better stability against coalescence than pristine zein particles. This is the first report of stabilization of Pickering emulsions at high oil fraction using modified zein conjugate with appreciated HLB, which was obtained by chemically grafting lauryl chains onto zein molecules. The HLB of lauryl-zein conjugate could be tuned within the range of 1-8 lauryl chains per zein, and at the same time, was visually quantified by water contact angle. Through this approach, Zein-C0.5 conjugate was found to stabilize Pickering emulsions at $\varphi = 0.7$ without any observation of creaming or flocculation in one week. This work can be an exploration into the development of Pickering emulsions stabilized solely by chemically modified food-compatible material, especially at high oil phase ratio and without any dependence on other surfactant additives.
CHAPTER V. A NOVEL ZEIN MICROSPHERE DELIVERY SYSTEM OF AMORPHOUS RESVERATROL

1. Introduction

Resveratrol, as a polyphenol phytoalexin naturally produced by plants in response to exogenous stress, has attracted lots of attention because of its wide distribution in the common dietary sources and promising functionalities at the aspect of therapy and healthcare \[68-71\]. Since the cancer chemo-protective activity of resveratrol was reported by Jang et al. \[196\], massive research have been conducted for recent two decades in a variety of medical and healthcare areas, like antioxidant \[197, 198\], anti-inflammatory \[199, 200\], antiplatelet \[201, 202\], cardiovascular protective effects \[203, 204\], even anti-obesity \[205, 206\]. Though a number of in vitro and in vivo trials above have indicated the therapeutic potentials of resveratrol, which mainly focused on the possible treatment or prevention against cancer and cardiac diseases, they have also revealed its low aqueous solubility and limited physicochemical stability leading to the poor oral bioavailability \[72, 73\].

On the one hand, resveratrol (CAS No. 501-36-0) is a lipophilic compound with high crystallinity and relatively high melting point (above 250 °C), resulting in the poor solubility in aqueous solution \[207, 208\]. Thus alcohol solution or organic cosolvent was often used to dissolve resveratrol in numerous research, e.g. oral administration of red wine to rats \[209, 210\], or resveratrol dissolved in 10% DMSO added in targeted cells \[211\], etc. Rather than using liquid formulation, solid dispersions can provide an alternative strategy by changing crystalline resveratrol into amorphous one to increase the solubility \[74\]. Wegiel et al. has studied impact of various polymeric materials on re-crystallization inhibition of amorphous solid dispersions of resveratrol, and found that strength of
interactions between resveratrol and polymers played a key role \[212\]. On the other hand, resveratrol is photo sensitive resulting in isomerization from trans- (E) to cis- (Z) under exposure to light as shown in Figure V-1 \[75\]. Trans isomer is the primary biologically active component in nature and cis isomer is not commercialized due to its relative chemical instability \[75, 76\]. Thus how to preserve resveratrol’s integrity as the trans form is another essential task for formulation development. Nam et al. used porous polymeric microspheres to stabilize resveratrol but the polymer used is not pharmaceutically accepted, let alone toxic organic solvent toluene, heptane, etc. \[77\].

In this work, we introduced zein protein as the commercial food-grade polymeric excipient to prepare amorphous formulation of resveratrol. Zein protein, as the GRAS (generally recognized as safe) and biodegradable polymer material \[1\], has been studied as the vehicle in delivery system of both nutraceuticals \[2\] and drugs \[3\]. Firstly, it contains high percentage of non-polar amino acids with amine residues \[1\], which could form a strong hydrogen bonding interaction with resveratrol to inhibit re-crystallization. Furthermore, zein microspheres have been reported to protect ivermectin from photodegradation and achieve in vitro sustained-release \[10\]. In recent years, zein-based microspheres have been developed for drugs or nutraceuticals delivery, mainly due to its easy scale-up process and relatively high load efficiency \[3, 10\]. In general, dispersed zein microspheres can be easily obtained via anti-solvent method credited to its characteristic
of alcohol soluble but insoluble in aqueous solution, with resveratrol encapsulated inside the core of zein microspheres by hydrophobic interaction.

Thus in this chapter a novel amorphous resveratrol-loaded zein microspheres delivery system was set up, to enhance dissolution under aqueous environment. The ability to stabilize amorphous resveratrol in zein microspheres was assessed by different weight ratio of resveratrol vs. zein microspheres. Multiple solid-state analysis were performed, including XRPD, PLM, particle size distribution (PSD), FTIR and DSC (mDSC), to screen the successful amorphous formulation and investigate their amorphous origin and physical stability during 3 months’ storage. For amorphous resveratrol-loaded zein microspheres, the in vitro dissolution behaviors in bio-relevant media were also investigated when compared to crystalline resveratrol alone.

2. Materials and Methods

2.1. Materials.

Resveratrol (purity >98%) was a gift from South China Agricultural University (Guangdong, China). The polymeric carrier α-Zein (abbreviated as zein) was purchased from Wako Chemical Industries, Ltd. (New York, NY). Milli-Q water and pure ethanol were obtained from Food Science Department, Rutgers. All organic solvent like Acetonitrile (HPLC grade) were purchased from VWR, Ltd. SIF powder was purchased from Biorelevant.com Ltd. Two bio-relevant media SGF (Simulated Gastric Fluid) and SIF (Simulated Intestinal Fluid) were prepared, simulating gastric and gut fluids respectively [127].

For SGF buffer (100 mL): Weigh 0.20 g sodium chloride (NaCl) and 90 mL of deionized (DI) water into a 100 mL volumetric flask. Adjust the pH to exactly 1.2 using 1 N Hydrochloric acid (HCl) and q.s.to 100 mL with DI water. Then dissolve 0.06 g of SIF
For SIF buffer (100 mL): Weigh 0.400 g of NaOH pellets, 0.865 g of acetic acid, 1.187 g of sodium chloride (NaCl) and 90 mL of DI water into a 100 mL volumetric flask. Adjust the pH to exactly 5.0 using either 1N NaOH or 1N HCl and q.s. to 100 mL with deionized water. Then dissolve 1.120 g of SIF powder into the prepared buffer.

2.2. Fabrication of resveratrol-loaded zein microspheres

Resveratrol-encapsulated zein microspheres were prepared through a fast phase separation method. As displayed in Table 1, experimental ID was named by initial loading of resveratrol, e.g. R20 indicated that initial amount of added resveratrol accounted for 20 wt. % of the total formulation. Briefly, 200 mg of zein and corresponding amount of resveratrol were dissolved in 10 mL of 80% ethanol, which was added immediately into 10 mL of anti-solvent water under stirring. After continuous stirring for 1 min, the produced suspensions were freeze-dried for 3 days to obtain microsphere powder. All prepared microsphere samples were stored in dry container at room temperature.

2.3. Loading and encapsulation efficiency

The loading efficiency and encapsulation efficiency of each microsphere sample were determined according to spectro-photometrical method with a few modifications. Typically, 10 mg of freeze-dried microsphere solids was flushed by 1 mL of pure ethanol three times to wash off non-entrapped resveratrol. After fast air dry, 10 mL of ethanol was added to extract resveratrol under vortexing for 10 min. Then sample solution was transferred to a 96-well plate, and its absorbance intensity was determined using a microplate reader (BioTek), with UV wavelength fixed at 350 nm. Triplicate measurement was performed for each sample. The loading efficiency (LE) and encapsulation efficiency (EE) were determined as per the equations below.
2.4. X-Ray Powder Diffraction (XRPD)

Zein microsphere powder was tested by XRPD to check whether amorphous formulation was obtained for different initial loading of resveratrol. For XRPD analysis, a PANalytical (X’Pert³ Powder) X-Ray powder diffractometer and Si zero background holder were used. The continuous mode was chosen in the scan range (°2Th.) from 3.0121 to 40.0133, and the step size was 0.026. The intensity ratio of Kα2/Kα1 was 0.50, with the X-Ray wavelength of Kα1 and Kα2 fixed at 1.540598 Å and 1.544426 Å. Testing time was 4 min for each sample.

2.5. Polarized Light Microscope (PLM)

The morphology of each microsphere sample was observed by PLM, to check the existence of any birefringent specimens under polarized light. Oil immersion was used to disperse the sample well on the slide and auto-exposure image was captured using Nikon ECLIPSE Ci-POL at room temperature.

Then, in order to investigate kinetic recrystallization of resveratrol upon solvent evaporation and corresponding inhibiting effect of zein microspheres at different loading ratio, 0.1 mL of freshly prepared suspension was placed on the slide under the monitoring of PLM. The image was captured at each interval of 2 min until the suspension was air dried. The magnification for all tests was fixed at ×100.

2.6. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra was collected in a frequency range from 4,000 cm⁻¹ to 600 cm⁻¹, using a resolution of 4 cm⁻¹ and 128 scans on a Thermal Nicolet Nexus 670 FTIR spectrometer
(Thermo Fisher Scientific Inc.). Typically, each microsphere powder was flattened by weighing paper to ensure good contact with the ZnSe crystal, and OMNIC 7.2 software was used to analyze the FTIR spectra.

2.7. Particle size distribution (PSD)

For PSD analysis, a Malvern (Mastersizer 3000) Particle Size Distribute with Hydro EV attachment was used. The distribution mode was in volume. Isopar-G was chosen as the dispersive solvent with refractive index at 1.33. The particle was assumed to be nonspherical with the particle refractive index at 1.51. About 50 mg of microsphere sample was stirred in Isopar-G at the flow rate of 2500 rpm, and the measurement was conducted in triplicate.

2.8. Morphology study

The morphology of batch sample R20 was studied by SEM using a XL-30 Field Emission Scanning Electron Microscope (FESEM, Japan). The sample powder was coated with 10 nm of Au on silicon chips to improve electrical conductivity before test. Accelerating voltage of field emission gun was 10 kV and the scale bar was 200 µm.

2.9. Thermal analysis

Firstly, the solid-state origin of target compound resveratrol was investigated by DSC (Differential scanning calorimetry) and TGA (Thermogravimetric Analysis). For DSC (Q2000, TA Instruments), a crimped aluminum pan was used with the purge rate of nitrogen fixed at 50 mL/min. DSC thermogram was collected in the ramp mode from 25 °C to 300 °C with the heating rate at 10 °C/min. For TGA (Q500, TA Instruments), an open platinum plate was used with the flow rate of nitrogen at 50 mL/min. Ramp rate was 10 °C/min and heating temperature was from 25 °C to 330 °C. In addition, two resveratrol-loaded zein microsphere samples (R10 and R20) were tested by DSC to exam
any endothermic peak related to crystalline domain in the sample.

Then, modulated differential scanning calorimetry (mDSC) was also performed for the microsphere sample R20 using the same Q2000 instrument. mDSC can divide heat flow of all the transitions into two parts. One is the non-reversible heat flow and the other is the reversible heat flow where glass transition and melting of tested materials can be obtained. Under the mDSC mode in this work, heating ramp rate of 3 °C/min within period of 40 s and amplitude of ±0.636 was set up. About 10 mg of sample was sealed in a crimped aluminum pan and purge rate of nitrogen was at 50 mL/min. The total heat flow was collected from 25 °C to 250 °C.

2.10. Dissolution study in bio-relevant media

Dissolution behaviors of amorphous resveratrol released from zein microspheres or crystalline resveratrol alone were studied in bio-relevant media of SGF and SIF [127]. Briefly, samples eq. to 20 mg of resveratrol in 100 mL of SGF and eq. to 40 mg of resveratrol in 100 mL of SIF were tested at 37 °C as per the USP dissolution apparatus II with paddle at 100 rpm. 10 μL of supernatant post filtration (0.25 μm PTFE filter) was injected into HPLC (Agilent 1100 Series) at 5 time points (15 min, 30 min, 1h, 2h and 4h). All HPLC samples were prepared under protection from the light.

The HPLC method was modified as follows [213]. Isocratic mode was used with the mobile phase of 0.1% PA solution: Acetonitrile as 50% : 50% and flow rate was at 0.5 mL/min. A Gemini Carbon 18 column (150 mm × 4.60 mm, 5 μm) was used, and the wavelength of UV detector was 306 nm. The nominal retention time of resveratrol was around 4.9 - 5.0 min. The limit of quantitation (LOQ) of resveratrol was 0.5 μg/mL with the absorbance intensity of sample 10 times baseline noise, and the linear detection range was 1.0 - 120.0 μg/mL with regression coefficient greater than 0.9999.
3. Results and Discussion

3.1. Basic characterizations of Resveratrol by TGA/DSC.

Table V-1. Zein microspheres with different initial resveratrol loading

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Initial loading of resveratrol</th>
<th>Loading efficiency (LE %)(^a)</th>
<th>Encapsulation efficiency (EE %)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R10</td>
<td>10%</td>
<td>6.3%±0.2%</td>
<td>63.1%±1.7%</td>
</tr>
<tr>
<td>R20</td>
<td>20%</td>
<td>11.0%±0.2%</td>
<td>55.0%±1.0%</td>
</tr>
<tr>
<td>R30</td>
<td>30%</td>
<td>12.6%±0.4%</td>
<td>42.1%±1.3%</td>
</tr>
<tr>
<td>R40</td>
<td>40%</td>
<td>12.4%±0.2%</td>
<td>30.9%±0.5%</td>
</tr>
</tbody>
</table>

\(^a\) Each data was mean of three measurements ± SD

Resveratrol is a hydrophobic phytochemical compound with high inherent crystallization tendency \(^{[214]}\). As shown in Figure V-2, the DSC curve of resveratrol displayed an endotherm at 265 °C (onset point) with specific heat capacity of 256 J/(g·K), which indicated its high energy of crystal lattice and subsequent high melting point. By TGA only 0.1% weight loss was observed before 100 °C, which showed that it was an anhydrous compound with hydrophobicity in normal ambient environment. Though highly crystalline and poorly soluble, the hydroxyl groups and π-π conjugation structure enables resveratrol to bind with a variety of polymers via hydrogen bonding, including food-grade zein protein \(^{[214]}\). Thus solid dispersion via anti-solvent method can be an effective strategy to stabilize the metastable amorphous resveratrol in the polymeric carrier, zein protein. In the present work, four batches of resveratrol-loaded zein microspheres were prepared by the ratio of initial resveratrol loading from 10% to 40% and assigned sample ID as R10, R20, R30, R40 respectively (Table V-1). Their performance in loading and encapsulation efficiency, inhibiting effect on re-crystallization, physical and chemical stability, maximum solubility, release rate, etc. were evaluated with crystalline resveratrol as the comparing reference.
3.2. Resveratrol loading and encapsulation efficiency.

A range of initial input of resveratrol (10% - 40%) were put in trial. As the results shown in Table V-1, even though initial loading was designed up to 40% (the sample ID, R40), the calculated loading of resveratrol via UV–Vis spectrometry didn’t show a linear increasing trend with the initial loading. The loading capacity was limited up to 12.6% possibly due to highly re-crystallizing character, which implied that a large proportion of resveratrol was stacked on the outer layer of zein microspheres, either in amorphous status or re-crystallized. Thus four batches of microsphere samples (R10, R20, R30 and R40) were first evaluated by XRPD, to exam the feasibility of amorphous formulation using zein microspheres to encapsulate and stabilize amorphous resveratrol.

3.3. Microsphere characterization by XRPD and PLM.

All four batches of microsphere samples were tested by XRPD. As shown in Figure V-3(a), the sample R10 and R20 both showed only amorphous patterns without significant diffraction peaks observed, but the other two sample batches with higher initial loading (R30 and R40) displayed the same diffraction peaks with amorphous halo, when compared to the equivalent mixture of resveratrol/zein (wt./wt. 1:9). The results indicated that amorphous formulation of resveratrol-loaded zein microspheres were
successfully obtained through the method of solid dispersions, but limited capability of zein microspheres to stabilize amorphous resveratrol was also found when initial loading was further increased, e.g. diffraction peaks that corresponded to re-crystallization of resveratrol were observed for sample R30. At the same time, re-crystallization of resveratrol could also limit the loading capability of zein microspheres, which resulted in the lower encapsulation efficiency at higher initial loading of resveratrol (Table V-1).

For two sample batches R10 and R20, the physical stability was also evaluated by XRPD after 1 month’s or 3 months’ stage at 25 °C and 40% RH, respectively. As revealed in Figure V-3 (b) and (c), no obvious diffraction peaks were found for these two batch samples after 3 months’ storage period, which indicated that amorphous resveratrol could be consistently stabilized in zein microspheres at lower loading.
Figure V-3. (a) XRPD overlay of four batches of freshly prepared microsphere samples (R10, R20, R30 and R40), compared with the crystalline pattern of equivalent mixture of resveratrol/zein (wt./wt. = 9/1). (b) XRPD overlay of R10 under exposure to 25 °C, 40% RH after 0 day, 1 month and 3 months. (c) XRPD overlay of R20 under exposure to 25 °C, 40% RH after 0 day, 1 month and 3 months.

PLM was used to further check the potential crystalline domains in small particle size that could not be detected by XRPD. The crystalline particles in the PLM micrograph would be supposed to appear bright and colorful due to their birefringent structure. As shown in Figure V-4, mass of birefringent plates were observed for R30 and R40, while no significant birefringence was found in the samples of R20 and R10.

Figure V-4. PLM image of four batch samples (R40, R30, R20, and R10). Magnification was × 100.
Furthermore, kinetic re-crystallization behaviors of resveratrol of R40 and R20 were monitored under PLM at magnification of 100 times. As can be seen in Figure V-5, for microsphere suspension of R40, it took only 6 minutes to air dry and obvious birefringent plates were observed due to high crystallizing tendency of resveratrol. For R20, however, it took a bit longer (15 minutes) to let the solution air dry the microsphere suspension, and no significant birefringence was seen under PLM. Besides, at the time point of 5 min and 10 min, bright spots surrounded by non-birefringent layers were observed, which revealed that potential recrystallization of resveratrol in local domain was restrained by the outer layer of amorphous zein microspheres, and in the final no birefringent aggregates occurred for R20 after 15 min’s air dry.

**Figure V-5.** PLM image of microsphere suspension of R40 and R20 during solvent evaporation at different time points.

3.4. *Interactions between resveratrol and zein microspheres.*

Interactions between resveratrol and zein microspheres were supposed to impact an
important influence on both encapsulation and stability of amorphous resveratrol, which were reflected by the slight change of wavenumbers in the IR analysis. The IR spectra of resveratrol displayed in Figure V-6 clearly indicated its typical absorption bands at 964 cm\(^{-1}\), 1380 cm\(^{-1}\) and 1581 cm\(^{-1}\) due to the C-C stretching \(^{[215]}\). After resveratrol was encapsulated in zein microspheres, the typical bands of resveratrol (964 cm\(^{-1}\) and 1380 cm\(^{-1}\)) were still observed for R40 and R20, with the intensity weakened significantly with the resveratrol loading decreasing from R40 to R20. However, the significant band of resveratrol at 1581 cm\(^{-1}\) disappeared and a new band at 1527 cm\(^{-1}\) was found, which was probably credited to the new formed hydrogen bond between resveratrol and zein microspheres. Moreover, another typical broad band corresponding to \(\text{–OH}\) stretching of resveratrol, shifted from 3193 cm\(^{-1}\) to 3278 cm\(^{-1}\) and 3286 cm\(^{-1}\) in the IR spectra of R40 and R20, respectively (Figure V-6). The significant band shift towards the \(\text{–OH}\) stretching band (3293 cm\(^{-1}\)) of pure zein protein also revealed the formation of hydrogen bonding \(^{[216]}\), which could be the dominant contribution to localize amorphous resveratrol in the zein microspheres.

![Figure V-6. FTIR spectra of resveratrol, R20, R40 and zein protein.](image-url)
3.5. Particle size distribution and morphology observation.

In general, particle size distribution (PSD) and morphology are amongst the critical attributes which can impact powder flowing and manufacturing process [217]. The PSD analysis in present work is based on the principles of laser diffraction, which measures angular variation in the scattered-light intensity when a laser beam passes through a dispersion of solid particles in Isopar-G. The size of measured particles is inversely proportional to the scattered angle of laser, and intensity data of angular scattering is collected to analyze particle size distribution based on the Mie theory of light scattering [218]. As displayed in Figure V-7, the microsphere particle size of R20 was 61 µm in D50 and 103 µm in D90, respectively. For R40, particle size was increased a bit with values at 68 µm (D50) and 119 µm (D90). It suggested that re-crystallization of resveratrol could produce crystals onto the outer layer of zein microspheres, that would induce the increase of overall particle size.

![Particle size distribution of R20 and R40](image)

**Figure V-7.** Particle size distribution (PSD) of R20 and R40.
Different morphology of particles, such as needle-like, spherical, irregular shape, etc. can also influence the subsequent flow properties of zein microsphere powder, thus the morphology of R20 was studied by SEM. Various smooth spherical microspheres were observed in the SEM image of R20 (Figure V-8), with a size distribution of around 20 ~ 150 μm obtained based on the scale bar of 200 μm. The visual observation from SEM image mainly conformed to the experimental results from PSD analysis (for sample R20), with the overall microsphere size within 200 μm and no extra-large microspheres found.

![SEM image of R20. Scale bar was 200 μm.](image)

3.6. Thermal analysis.

Differential scanning calorimetry (DSC), as one most common used thermo-analytical method, is very helpful to investigate thermal information, e.g. the melting point of crystalline drugs, glass transition of amorphous solids, etc. [216]. As can be seen from Figure V-9 (a), one large endothermic peak of crystalline resveratrol at the onset temperature of 264.9 °C was observed by DSC, while no significant endotherm was found for microsphere samples of R10 and R20. It indicated that amorphous formulation of resveratrol-loaded zein microspheres were produced for both of the two batches (R10
and R20), however, it is hard to find glass transition \((T_g)\) using conventional DSC due to interfering heating transitions of resveratrol and zein protein.

Therefore, modulated DSC (mDSC) was applied to detect \(T_g\) of amorphous solids, with R20 as the typical example. As shown in Figure V-9 (b), a possible glass transition around 145.9 °C (middle-point) was observed from the reversing heat flow. Similar to the result of standard DSC, no significant melting point (endotherm) was detected from non-reversible heat flow or total heat flow.

![Figure V-9](image)

**Figure V-9.** (a) DSC overlay of resveratrol, R20 and R10. (b) mDSC of R20.

### 3.7. Dissolution Study.

Considering the high membrane permeability of resveratrol as a BCS class-II compound \([219, 220]\), amorphous resveratrol stabilized in zein microspheres could be an efficient...
strategy to resolve its poor aqueous solubility issue. Thus dissolution profiles of R20 in bio-relevant media were plotted against time based on the equation as below, with crystalline resveratrol as the reference. Bio-relevant media used in this work included SGF (Simulated Gastric Fluid) and SIF (Simulated Intestinal Fluid, Fed state at pH = 5.0), according to the pre-formulation studies of resveratrol which suggested that it’s better to formulate resveratrol in a medium with pH of less than 6.0 \(^{221,222}\).

\[
\text{Dissolved resveratrol (\%) = } \frac{\text{Detected concentration of resveratrol per time point (\(\mu g/mL\))}}{\text{Possible maximum concentration (\(\mu g/mL\))}}
\]

As shown in Figure V-10, superb solubility and release profile of R20 were achieved as compared to those of crystalline resveratrol alone in bio-relevant media. In SGF, >35% of resveratrol was fast released from R20 in 15 minutes, while only less than 1% was dissolved for crystalline resveratrol alone. And though a ‘Spring & Parachute’ trend was observed for dissolution curve of R20 within 4h \(^{160}\), but still >22% of resveratrol was maintained in SGF, which was at least 4 times that of crystalline resveratrol. In SIF, dissolution rate of R20 was enhanced by 3.7 times when compared to that of crystalline resveratrol, and a supersaturating status was maintained within 4h with more than 78% of resveratrol dissolved for R20. The dissolution studies indicated that changing crystalline structure of resveratrol to amorphous status significantly improved its bioaccessibility in bio-relevant media, including increasing the solubility, enhancing dissolution rate, etc.
Figure V-10. Dissolution curves of D20 or resveratrol alone in bio-relevant media.


In this chapter work, amorphous resveratrol was successfully prepared and stabilized in zein microspheres via anti-solvent method. The efficiency of amorphous formulation was depended on the initial loading of resveratrol in zein microspheres. The solid-state characterization results revealed that zein microsphere delivery system had certain capability to inhibit re-crystallization of amorphous resveratrol, but its limitation was also found when the initial loading of resveratrol was more than 20%. In addition, the dissolution study indicated that a much better release profile of resveratrol from amorphous formulation was obtained, when compared to crystalline resveratrol alone.

Regarding on the BCS II characteristics of resveratrol, poor aqueous solubility could be a
major factor to induce its low bioaccessibility. Thus, our zein microsphere delivery system of amorphous resveratrol could provide an alternative way to improve the bioaccessibility of resveratrol in terms of oral administration.
CHAPTER VI. ENHANCING FELODIPINE DISSOLUTION AND BIOACCESSIBILITY THROUGH AMORPHOUS SOLID DISPERSIONS CONTAINING ZEIN.

1. Introduction

Oral administration of drugs from Biopharmaceutics classification system (BCS) class II or IV faces to poor aqueous solubility and slow release rate in the gastrointestinal (GI) tract, subsequently resulting in low bioavailability and negating efficacy for the drugs \[^{223}\]. In most cases, amorphization is an effective solution wherein crystalline structure of the drugs is disrupted and amorphous status is maintained to facilitate drug molecules into solute cavities. For each drug, the ratio of amorphous (A) and crystalline (C) solubility, \( \ln(\sigma_A^\text{A}/\sigma_C^\text{C}) \), is related to the free energy variation of those two states \( (\Delta G_T^\text{A,C}) \), as expressed by the equation below \[^{224}\].

\[
\Delta G_T^\text{A,C} = -RT\ln(\sigma_A^\text{A}/\sigma_C^\text{C})
\]

Where \( R \) is the gas constant, and \( T \) is the absolute temperature. It can be indicated that a higher solubility in amorphous state than crystalline form can result in re-crystallization tendency because of lower free energy of crystalline form. Thus, amorphous solid dispersions (ASD) can be introduced as a feasible strategy with drug molecularly dispersed in amorphous polymeric matrix (excipient) \[^{225}\]. In general, the polymeric excipient plays a crucial role in inhibiting re-crystallization of ASD during storage or downstream processing, such as mixing and tableting. Up to now a variety of polymers have been used for various functions, including cellulose and its derivatives, poly (vinylpyrrolidone) (PVP) and its derivatives, acrylic acid-based enteric Eudragits, etc. \[^{226},^{227}\]. The miscibility of drug-polymer takes a major influence on ASD
stabilization, considering that phase separation can promote nucleation and growth of crystals [228]. Therefore, numerous approaches have been tried to produce miscible solid dispersions and prevent any occurrence of phase separation, wherein the key step is to disrupt the lattice structure with external energy imparted to the process [229].

Spray drying was utilized in our work to produce amorphous solid dispersions, which is considered as an energy efficient, reproducible, and scalable manufacturing process to rapidly evaporate solvent and generate nano to micro size solid particles [230]. Compared to other commercial drying technologies, spray drying costs less and allows mild temperature conditions during process to avoid degradation of thermo-sensitive drugs. Basically, the process steps of spray drying consist of atomization of liquid, fast evaporation of droplet and particle collection [231]. A spray dried ASD has typically high drug-polymer miscibility and low moisture contents, resulting in long shelf life storage and effective protection from the environment [232].

Felodipine, a long-acting calcium channel blocker for treating hypertension, was chosen as a poor aqueous soluble drug model. Felodipine solid dispersions have been extensively studied using different commercial polymers in the last decade, e.g. synthetic poly (vinyl pyrrolidone) (PVP) [226] and Soluplus (SOL) [233], chemically processed hydroxypropyl methylcellulose (HPMC), hydroxypropyl methylcellulose acetate succinate (HPMCAS) [234], etc. It turned out different polymers could significantly impact the dissolution behaviors of ASD containing felodipine [234]. Recently, food-originated biopolymers are introduced as polymeric carriers in the preparation of ASD due to their natural merits, e.g. biocompatibility, biodegradability, low toxicity, etc. In reality, a series of GRAS (Generally Recognized as Safe) proteins originated from plants have been reported for the encapsulation of lipophilic bioactive ingredients [235, 236]. Among them zein protein is considered as a promising polymeric carrier due to its unique alcohol-
soluble characteristic and sequential flexible deformation with the polarity change of solvent \[^3\]. Moreover, non-ionic residues of zein (e.g. amino acid proline) can potentially provide non-covalent interaction sites with hydrophobic bioactive compounds, which could contribute to the intermolecular interactions (high dispersion and miscibility) between drug and polymer \[^{88, 147}\].

Lu et al. recently reported that zein protein as the carrier seemed better able to inhibit crystal growth of amorphous felodipine than other commercial polymers, however, only 20% (w/w) of felodipine were able to maintain amorphous using a rotary evaporator \[^{237}\]. There are few studies on the ASD preparation of zein through spray drying and in vitro dissolution evaluation in the early drug development. Thus in this work, spray dried ASD containing felodipine was prepared using zein as polymeric excipient (abbreviated as ASD Felodipine/Zein), and resulting samples were characterized via solid state methods, including PXRD, PLM and DSC (mDSC). The bioaccessibility of ASD Felodipine/Zein were investigated using simulated gastrointestinal fluid and TNO gastro-intestinal model-1 (TIM-1) model, respectively. For dissolution experiments, another two ASD batches of HPMC-AS and PVP-VA were prepared as the reference. Besides, preclinical formulation development of ASD Felodipine/Zein was performed to exam inhibiting effect of zein on recrystallization of felodipine in aqueous formulation on behalf of the future pre-clinical study.


2.1. Materials.

 Felodipine was purchased from Sigma-Aldrich Co. (MO, USA). The polymeric carrier \(\alpha\)-zein (abbreviated as zein) was purchased from Wako Chemical Industries, Ltd. (Tokyo, Japan). Hypromellose acetate succinate (HPMC-AS, AQOAT\(^{\text{®}}\)AS-MF) was
obtained from Shin-Etsu Chemical Co. (Tokyo, Japan). Polyvinylpyrrolidone/vinyl acetate copolymer (PVP-VA, Kollidon®VA64) was obtained from BASF Co. (NJ, USA). Methylcellulose (METHOCEL™ A4M) was obtained from Dow Chemical Co. (NJ, USA). Milli-Q water and pure ethanol were obtained from Food Science Department, Rutgers. All organic solvents were purchased from VWR, Ltd. (PA, USA). SIF powder was purchased from Biorelevant.com Ltd. (London, UK).

2.2. Preparation and Stability of ASD

ASD Felodipine/Zein was prepared using a Labplant SD-06 spray dryer (Goodwill Technology Ltd., HK, China) respectively. The initial load of felodipine was 30%. Typically, 5% w/v of drug-polymeric excipient was dissolved in 85% ethanol solution, which was then spray dried using nitrogen. Inlet (outlet) air temperature were 110 °C (60 °C). Feed pump rate was 9.0 mL/min and aspirator flow rate was 3.5 m³/min. The atomization pressure was 3.0 Bar, with plugging frequency set on fast mode. The spray dried solids were collected and then vacuum dried at 40 °C for 24h to remove residual solvent, and the yield of ASD Felodipine/Zein was 65%. Another two ASD batches of Felodipine/HPMC-AS and Felodipine/PVP-VA were also prepared at 30% felodipine load under the same preparation procedure.

Stability of Felodipine/Zein was investigated by storing the solid dispersions under accelerated conditions of 40 ± 1°C and 75 ± 3% RH. After 3M, samples were characterized in solid state to determine whether any recrystallization had occurred during storage.

2.3. Solid State Characterizations

2.3.1. Powder X-ray Diffraction (PXRD)
A Panalytical X’Pert³ Diffractometer (Malvern Panalytical Ltd., Malvern, UK) was used to perform PXRD for all samples on a Si zero-background holder. A Cu K-α X-Ray tube was set at 45 kV and 40 mA, with the wavelength of Kα₁ and Kα₂ were 1.54056 Å and 1.54443 Å. Kα₂/Kα₁ intensity ratio was 0.50. A scan from 3° to 40° (°2TH) was conducted at a rate of 0.062° (2θ) /s. Total run time was 10 min.

2.3.2. Polarized Light Microscopy (PLM)

The surface topography of felodipine and ASD Felodipine/Zein were both investigated using a DM 2500P Leica polarized light microscope (Leica Microsystems, Wetzlar, Germany). PLM image was captured under auto-exposure mode at room temperature, to visually check any birefringent plates of ASD sample as complementary to PXRD.

2.3.3. Thermal Analysis

Thermal characterizations of drug substance felodipine and ASD batch Felodipine/Zein were both performed by DSC and TGA. DSC thermogram was obtained by DSC Q2000 mode (TA instruments, New Castle, PA, USA). Before DSC test, calibration was carried out using indium and lead. Nitrogen was fixed at 50 mL/min as a purge gas, and about 5-10 mg of sample was sealed in a crimped aluminum pan at 10 °C/min from room temperature to 300 °C. TGA thermogram was obtained using a TGA Q5000 mode (TA instruments, New Castle, PA, USA). About 10 mg of sample on an open platinum plate was heated from room temperature to 300 °C at 10 °C/min. All DSC and TGA profiles were collected using Universal Analysis 2000 software provided by TA Instruments.

For ASD batch Felodipine/Zein, modulated mode (mDSC) was performed to detect
any possible glass transition from reversing heat flow. The mDSC thermogram was obtained by a DSC Q2000 mode (TA instruments, New Castle, PA, USA). Purge rate of nitrogen was fixed at 50 mL/min, and about 10 mg of sample sealed in a crimped aluminum pan was heated from room temperature to 250 °C at 3 °C/min within period of 40 s and amplitude of ± 0.636.

2.4. Dissolution Study

Firstly, the apparent solubility of felodipine in fed state simulated intestinal fluid (FeSSIF, pH 6.5) was determined at 37 °C. 50 mg of felodipine was dispersed in 500 mL of FeSSIF under stirring at 50 rpm, in which 500 mg of the selected polymer (HPMC-AS, PVP-VA or Zein) had been added. After stirring for 24 h, the solubility of felodipine in the suspension was measured using Agilent 1100 Series reversed-phase HPLC (Agilent Technologies, Santa Clara, CA, USA). All measurements were conducted in triplicate.

Then, dissolution profiles of the ASD Felodipine/Zein were obtained using a ZRS-8G dissolution tester (Tianjin Skylight Optical Instrument Co., Ltd., Tianjin, China) followed by USP II dissolution apparatus \([234, 238]\), and the result was compared with those of another two ASD batches, Felodipine/HPMC-AS and Felodipine/PVP-VA. Solid dispersions (eq. to 150 mg of felodipine) were put into 500 mL of dissolution buffer at 37 °C. The rotation speed was 50 rpm. About 1 mL of suspension was sampled at following time point (5 min, 15 min, 30 min, 1 h, 2 h, 4 h and 6 h) and then syringe-filtered using 0.25 μm PTFE filter membrane. 20 μL of supernatant was injected into Agilent 1100 Series reversed-phase HPLC to measure the solubility of felodipine per time point. The dissolution medium in this study was FeSSIF and the preparation procedure was described as the following \([127]\).

FeSSIF buffer (1 L): Weigh 4.00 g of sodium hydroxide pellets (NaOH), 8.65 g of
acetic acid, 11.87 g of sodium chloride (NaCl), and 900 mL of deionized (DI) water into a 1 L volumetric flask. Adjust pH to exactly 6.5 using either 1N NaOH or 1N HCl and q.s. to 1 L by DI water. Then dissolve 11.20 g of SIF powder into the prepared buffer. The prepared SIF buffer was sealed and stored at 4 °C for the dissolution test.

The HPLC method was set up as follows with modification \[^{[239]}\]. Isocratic mode was used with the 0.1% PA solution (20%): Acetonitrile (80%) at 1.0 mL/min. A Luna C18 column (250 mm × 4.60 mm, 5 μm) was used under ambient condition. The wavelength of UV detector was 238 nm. The concentration of felodipine standard solution was 200.0 μg/mL, which was termed as standard 100%. Calibration curves was established within range of 2.0 - 200.0 μg/mL with regression coefficient greater than 0.9999. The nominal retention time of felodipine was around 5.8 minute. The LOD and LOQ were determined as 0.2 μg/mL and 0.5 μg/mL, respectively. Validation was performed on precision and recovery. The RSD of intra-day precision (n = 6) based on six replicated injections using 100% and 10% standards solution were 0.04% and 0.13%, respectively. The mean recovery ± RSD (n = 9) of felodipine was 100.0% ± 0.1%. All measurements were conducted in triplicate.

2.5. Suspension of ASD Felodipine/Zein

In order to facilitate oral administration of solid dispersions, spray dried Felodipine/Zein was formulated in the vehicle 0.5% w/v Methocel A4M and its physical stability was studied by PXRD and PLM. Basically, 20mg (120 mg) Felodipine/Zein ASD sample was gently grinded with pestle in a mortar with 2 mL of 0.5% Methocel A4M solution gradually added, until well dispersed suspension was achieved at 3 mg (active)/mL and 18 mg (active)/mL respectively. The ASD suspension was stored at RT for 24 hours, and then was tested by PXRD and PLM at 24 h time point for checking the
occurrence of recrystallization.

2.6. TNO gastro-intestinal model-1 (TIM-1)

The in vitro digestion behavior of ASD Felodipine/Zein was investigated using the dynamic TNO gastrointestinal model-1 (TIM-1) (Zeist, The Netherlands), which can simulate the human upper gastrointestinal (GI) tract containing stomach, duodenum, jejunum, and ileum \[^{240}\]. The four compartments are infused with prepared gastric secretions, bile, pancreatic secretions and start residue to modify pH and mimic physiological digestive conditions. The temperature of system model was maintained at 37 °C under control by computer programs.

In this study, dispersed suspension of pure crystalline felodipine or ASD Felodipine/Zein in 0.5% Methocel A4M were mixed with demi water, gastric electrolyte solution and gastric start residue, to prepare 300 g of “meal” containing equivalent 0.1 w/w % of felodipine. The “meal” was then taken into the fed-state TIM-1 model and digestion behaviors were monitored through each compartment from stomach to ileum. The dialysate fluids of jejunal and ileal compartments were passed through semipermeable capillary membranes with 0.05 μm pore size, and were collected at 30, 60, 90, 120, 180, 240, 300 and 360 min. The jejunal and ileal filtrates were used for HPLC test. For the extraction procedure of felodipine, 1 mL of sample solution inoculated with an internal standard (nimodipine, 20 µg/mL) was mixed with 3 mL of ethyl ether under vortex for 5 min, and 2 mL of supernatant was obtained after centrifuge at 12000g for 10 min at ambient condition. The supernatant was removed through air drying overnight and it was diluted with Acetonitrile for the HPLC test. The experiments were carried out in duplicate and each sample was also analyzed in duplicate. The percent of cumulative bioaccessibility of felodipine was calculated based on the equation as below \[^{241}\].

\[
\%\text{Bioaccessibility} = \frac{\text{Weight of dissolved felodipine}}{\text{Weight of felodipine in the} \ldots}
\]
meal ×100%.

3. Results and Discussions.

3.1. Basic Characterizations of Felodipine and Zein

![Figure VI-1. DSC and TGA thermograms of felodipine.](image)

Basic characterizations were first performed to study the solid origins of felodipine and polymeric excipient zein protein. Characteristic DSC and TGA thermograms for felodipine are displayed in Figure VI-1. The DSC thermogram had a large endothermic peak at 142.3 °C which corresponded to the melting (Tm) of the drug substance, followed by thermal decomposition from 220 °C. Felodipine has been reported in BCS II with very low solubility due to its highly crystalline long-order range \[234, 242\]. By TGA, less than 0.1% of weight loss was observed for felodipine before 130 °C, which indicated that the model drug used in our work was typically anhydrous \[243\]. The crystalline nature of felodipine was further confirmed by PXRD as presented in Figure VI-2, with characteristic peak positions at 10.2°, 10.8°, 16.6°, 16.9°, 21.0°, 23.6°, 24.8°, 25.8°, 26.8°, 29.8°, 31.3°, 32.3° \[237, 244\], whereas amorphous state of zein protein was determined as only typical amorphous halo observed.
The preliminary data of thermal analysis were instructive regarding on the critical processing parameters (CPPs) of spray drying in the next step. For example, inlet temperature of spray drying should be controlled at least 20 °C below the melting point of felodipine, in case that any thermal degradation occurred with the flow of spray drying. In addition, plasticizing characteristic of felodipine at high weight ratio of drug-polymer mixture has also been reported previously \cite{245}, which implied that it’s better to monitor the spray drying temperature (both inlet and outlet temperature) in the real time, to prevent the phenomenon of soften and sticky physical mixture.

3.2. Solid State Characterizations of ASD Felodipine/Zein

Figure VI-3 (a) demonstrates PXRD pattern of ASD Felodipine/Zein with only amorphous halo observed after storage of 3 months under accelerated stability test. X-ray diffraction is used as one primary technique to identify pharmaceutical substances in the solid state owing to its excellent capacity to capture small structural changes \cite{246}. Unlike crystalline structure, amorphous powders have no long-range order so no Bragg diffraction were found in the PXRD pattern of ASD. It indicates that better stability performance was achieved by spray drying than the previous research work using rotary evaporation, that crystallization was identified by DSC after 1 month’s accelerated test.
when proportion of felodipine was at 30% \cite{237}. However, PXRD is not the only gold standard due to its insensitivity of detection when powder samples exist in a microcrystalline state \cite{247}.

**Figure VI-3.** (a) PXRD patterns of ASD Felodipine/Zein under condition of 40 °C and 75% RH for 0 M and 3 M. (b) PLM image of ASD Felodipine/Zein. Magnification ×500.

As shown in **Figure VI-3 (b)**, ASD Felodipine/Zein after 3 months’ accelerated test exhibited spherical particles at the size of 1-10 um without birefringence in the PLM image, which was used as a complementary technique to differentiate crystalline compartments from amorphous materials. When solid dispersions are amorphous with molecules randomly oriented, they possess only one principle refractive index which cannot result in colorful particles under polarized light. If partial crystallization occurred, the molecules in crystalline felodipine would be regularly arranged leading to various refractive indices and subsequent birefringence by polarized light. Thus under magnification of 500 times, absence of birefringence by PLM can be another strong evidence of solid dispersions Felodipine/Zein in amorphous state \cite{246, 248}. 
On the other hand, ASD felodipine/Zein was also evaluated by thermal solutions including DSC and TGA. Different from spectroscopic methods, thermal analysis can investigate the miscibility of felodipine in polymeric zein when developing ASD, which is an important predictive information regarding on the stability issue during storage and downstream process \cite{249,250}. The DSC and TGA thermograms for ASD Felodipine/Zein post 3 months’ accelerated test are shown in Figure VI-4 (a). ASD Felodipine/Zein showed only 0.9% weight loss upon heating to 100 °C by TGA, and no characteristic thermal events, such as endothermic melting peaks or exothermic crystallizing peaks, were observed in DSC curve, which illustrates the amorphous state of felodipine in the zein matrix. Typically, single glass transition (T\textsubscript{g}) for solid dispersions is an indicative of

**Figure VI-4.** (a) DSC and TGA thermograms of ASD Felodipine/Zein. (b) Modulated DSC thermograms of ASD Felodipine/Zein.
good miscibility and physical stability when domain size is in the micrometer scale. Thus modulated mode of DSC (mDSC) was used to separate reversible heat flow from all thermal transitions and detect the Tg of Felodipine/Zein. As illustrated in Figure VI-4 (b), one Tg around 128.6 °C was detected without other exotherms or endotherms observed, indicating the homogeneity and optimal stability of Felodipine/Zein.

3.3. Dissolution Study in FeSSIF.

Table VI-1. Felodipine Solubility in the simulated intestinal (SIF, pH 6.5) with or without added polymers at 37 ºC.

<table>
<thead>
<tr>
<th>Added Polymer (1 mg/mL)</th>
<th>Zein</th>
<th>HPMC-AS</th>
<th>PVP-VA</th>
<th>Without Polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility (ug/mL) of felodipine</td>
<td>2.41 ± 0.08</td>
<td>2.39 ± 0.08</td>
<td>2.90 ± 0.10</td>
<td>2.23 ± 0.07</td>
</tr>
</tbody>
</table>

Figure VI-5 indicates the release profiles of felodipine from solid dispersions (SD) containing three different polymeric excipients, which are zein, HPMC-AS and PVP-VA. All three SD resulted in much higher concentrations than equilibrium solubility of crystalline felodipine (see Table VI-1), suggesting the occur of supersaturation. The little variation of equilibrium solubility with or without polymers also revealed supersaturated status of felodipine could not be owing to the polymers. As two commonly used commercial polymers, solid dispersions of felodipine using HPMC-AS or PVP-VA has been massively studied, however, there is few report on whether zein protein as natural excipient can achieve competent performance as compared to the two formers.
Figure VI-5. Dissolution of felodipine from solid dispersions containing three different polymeric excipients: zein, HPMC-AS and PVP-VA.

Firstly, the initial dissolution rates were determined at the 5 min time point of dissolution, which were 16.9 µg/mL·min (zein), 23.2 µg/mL·min (HPMC-AS) and 26.6 µg/mL·min (PVP-VA), respectively. Moreover, the order of concentration peak being reached was PVP-VA (within 60 min), HPMC-AS (120 min) and zein (240 min). It is in agreement with the previous research that PVP-VA containing solid dispersions had faster dissolution rate than cellulosic solid dispersions [234], and zein containing solid dispersions showed sustained release of felodipine. Although dissolution occurs very rapidly for PVP-VA containing solid dispersions with a peak observed within 1 h, a significant drop in solubility occurred due to devitrification followed by spring and parachute effect [252]. It has been reported that HPMC-AS was found to be more effective
than PVP-VA in maintaining supersaturation solution of felodipine \textsuperscript{[234, 253]}, and there was no decrease in solubility within 4 h. Interestingly, the felodipine solution concentration in the presence of zein maintained growth over a 6-h period, although the initial dissolution rate was slower than that of another two polymers. The results can be rationalized by the slow digestibility of zein in gastrointestinal tract as follows: the disordered structure of amorphous felodipine results in a higher free energy (volume/enthalpy) as compared to crystalline form \textsuperscript{[254]}. But rapid nucleation of felodipine particles followed by decrease of apparent solubility would be triggered in more concentrated solution during initial fast dissolution period \textsuperscript{[255]}, such as PVP-VA containing solid dispersions.

On the other hand, the inhibition effects on crystallization by polymers have been documented \textsuperscript{[256, 257]}, that controlled release can maintain solution concentration at a stage where nucleation is not spontaneous \textsuperscript{[234]}. Because zein can resistant to digestive enzymes, it is accounted for enhanced capability of controlled release of felodipine \textsuperscript{[140]}, resulting in prolonged supersaturation within 6 h rather than a fast dropping parachute.

3.4. ASD Suspension.

PXRD patterns and PLM images of ASD suspension of Felodipine/Zein in the vehicle of 0.5% w/v methylcellulose are displayed in Figure VI-6. As displayed in Figure VI-6 (a), no crystalline XRD patterns were observed for the ASD suspensions at two concentrations of 3 or 18 mg/mL after 24 h at RT. Moreover, the PLM images of ASD suspensions showed 1-10 um particles with some aggregation, but no birefringence (crystalline pattern) was observed under 500 x magnification (Figure VI-6 b). The results indicated that ASD Felodipine/Zein were successfully formulated in 0.5% w/v methylcellulose suspension with a good physical stability, which gave superior syringeability over spray dried powder in the TIM-1 study. Pure crystalline felodipine was also formulated in the same vehicle before sampled into the TIM-1 model.
Figure VI-6. (a) PXRD patterns and (b) PLM images (magnification ×500) of suspension of ASD Felodipine/Zein in 0.5% w/v methylcellulose at the concentration of 3 mg/mL and 16 mg/mL.

3.5. In Vitro Digestion of ASD Felodipine/Zein.

The bioaccessibility of felodipine for both ASD suspension and Felodipine/Zein physical mixture suspension were understood through TIM-1 system, and the bioaccessible felodipine from jejunum and ileum dialysate were tested through HPLC and illustrated in Figure VI-7. As seen from Figure VI-7 (a), for ASD suspension, bioaccessible felodipine in jejunum was maintained at high level from 60 min to 240 min, owing to the sustained release of felodipine from zein matrix and consequent supersaturation. By contrast, the digested content from Felodipine/Zein physical mixture
in jejunum only showed a peak during 120 - 180 min, followed by a continuous dropping down to the 6h end. By comparison of Figure VI-7 (b) with Figure VI-7 (a), the bioaccessible felodipine in ileum dialysate was significantly lower than the amount detected in Jejunum, and even no bioaccessible felodipine was detected for crystalline one before 90 min. It is revealed that Jejunum was the dominant absorption site for felodipine in small intestine, and there were certain adsorption amounts for ASD suspension in ileum compartment, especially during 90 - 240 min. Furthermore, the overall bioaccessibility of felodipine was determined in the ileum effluent which combined jejunum and ileum dialysates. As shown in Figure VI-7 (c), the maximum concentration for both ASD suspension and crystalline reference occurred during 120 - 180 min, and remarkable enhancement of overall bioaccessible felodipine was observed during almost every time interval.

Figure VI-7. Bioaccessible felodipine accumulated in every 30 min or 60 min interval. (a)
Bioaccessible felodipine in jejunum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. (b) Bioaccessible felodipine in ileum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. (c) Total bioaccessible felodipine in both jejunum and ileum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation.

Meanwhile, a cumulative bioaccessibility content of felodipine in the jejunum or ileum dialysate, or combined dialysates was collected and compared between ASD and crystalline control. As plotted in Figure VI-8 (a), the cumulative amount of felodipine derived from ASD suspension was about 29.4%, which was at least 8 times as that of Felodipine/Zein physical mixture during 6 h TIM-1 test. The similar trend was found for the ileum compartment that 9.6% of bioaccessibility from ASD suspension was collected, while only 1.2% of bioaccessible felodipine was obtained from the crystalline control (Figure VI-8 b). Combining results of both jejunum and ileum dialysates as demonstrated in Figure VI-8 (c), the cumulative bioaccessibility of felodipine from ASD (39.1%) was still 8 times or more than 4.7% of Felodipine/Zein physical mixture. Therefore, it is concluded that amorphous solid dispersions using zein as polymeric matrix by spray drying could effectively enhance bioaccessibility of felodipine through in vitro TIM-1 model.
Figure VI-8. Cumulative bioaccessibility profile of felodipine (% of input) accumulated in every 30 min or 60 min. (a) Cumulative bioaccessibility of felodipine in jejunum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. (b) Cumulative bioaccessibility of felodipine in ileum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation. (c) Total cumulative bioaccessibility of felodipine in both jejunum and ileum dialysate from Felodipine/Zein physical mixture (w/w, 3:7) and ASD formulation.


In this work, amorphous solid dispersions of felodipine using zein as the polymeric carrier were successfully produced by method of spray drying. The multiple solid state characterizations demonstrated that amorphous origin of ASD was confirmed by PXRD with only amorphous halo observed under 3 months' accelerated stability test, and
meanwhile spherical particles of about 1 um were seen without any birefringence in the PLM image. Modulated mode of DSC (mDSC) result showed that only a single Tg was detected around 128.6 °C without observation of other exotherms or endotherms, indicating the good miscibility of felodipine in polymeric zein through spray drying. In in vitro dissolution test, another two ASD batches using HPMC-AS and PVP-VA as polymeric excipients were also involved for reference. The results illustrated that the sustained release from ASD Felodipine/Zein resulted in prolonged supersaturation during 6 h dissolution test, which was different from conventional spring and dropping parachute pattern as revealed in the polymer PVP-VA. Finally, based on cumulative bioaccessibility of felodipine through TIM-1 in vitro digestion model, it is concluded that an significant increased bioaccessibility from ASD has been achieved as compared to that of pure crystalline sample. In summary, spray dried amorphous solid dispersions using zein as polymeric excipient was proved to have maintained saturation solution of felodipine and enhanced its bioaccessibility in simulated upper intestinal tract.
CONCLUSIONS

In this thesis, potential applications of zein-based material was extended in both nano-encapsulation and Pickering emulsions by hydrophilic or hydrophobic modification. On one hand, zein was hydrophilic modified by conjugation with carboxyl methyl dextran (CMD) in order to enhance its colloidal stability regardless of its isoelectric point of 6.2. As compared to conventional physical blend with polysaccharides as the outer coating, hydrophilic modification can internally modify zein’s amphipathic property with less dependence on the additives. The synthesized novel amphiphilic zein-based material, Zein-CMD, showed easy self-assembly of nano-micelles by simple anti-solvent method, with a better stability in the aqueous solutions within the physiological pH range of 2-6.8 than pristine zein.

The nano-micelles composed of Zein-CMD was utilized to encapsulate typical hydrophobic phytochemical, Dihydromyricetin (DMY). Due to very poor aqueous solubility, DMY was found to fail the encapsulation solely using zein as the nano-carrier or physically blending with the CMD as outer coating. Nano-micelles prepared by amphiphilic zein-CMD paved one new way to both stabilize the loaded DMY and enhance the encapsulation efficiency. Besides, DMY encapsulated in the Zein-CMD micelles proved to be maintained in amorphous status possibly through synergetic effects of hydrogen bonding and hydrophobic interactions between DMY and Zein-CMD. This finding accounted for the enhanced dissolution behaviors of DMY released from Zein-CMD nano-micelles than equivalent crystalline DMY mixed with zein.

On the other hand, zein protein was also forwarded with hydrophobic modification on behalf of better stabilizing Pickering emulsions based on zein particles. The
hydrophile-lipophile balance (HLB) of modified zein conjugate particles could be tuned by the number of grafted lauryl chains per zein chain, and its hydrophobic modification degree was represented by water-in-air contact angle. The modified zein conjugate could better stabilize PE at higher oil ratio, overwhelming the one solely based on zein particles.

Lastly, two application fields using zein as the delivery carrier at scale-up level was paved through freeze drying and spray drying, respectively. A novel microspheres delivery system based on zein material was prepared by anti-solvent method and following freeze drying technique, to encapsulate and stabilize amorphous phytochemical resveratrol with low solubility. Zein material as the carrier showed the capability to maintain 20% initial load of resveratrol based on 3 months’ stability data, and dissolution performance of resveratrol released from zein microspheres was significantly enhanced as compared to equivalent crystalline resveratrol in bio-relevant media.

In the following, amorphous solid dispersions (ASD) of felodipine was successfully prepared using zein as the polymeric excipient by spray drying, and the amorphous origin of felodipine at 30 wt% load was confirmed upon accelerated stability evaluation. By TIM-1 in vitro digestion model, cumulative bioaccessibility of felodipine released from ASD was at least 6 times higher when compared with equivalent crystalline felodipine mixed with zein. The results upon microspheres or ASD suggested that zein as polymeric carrier was capable to manufacture and maintain amorphous bioactive compounds with poor solubility, which could result in the saturation status of targeted compound and enhance its bioaccessibility in bio-relevant media or simulated upper intestinal tract.
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