E2 protein nanocage as a pickering emulsifier and its applications

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**E2 PROTEIN NANOCAGE
AS A PICKERING EMULSIFIER
AND ITS APPLICATIONS**

MRIDUL SARKER

School of Chemical and Biomedical Engineering

2017

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A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of
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2017

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6. Biomedical Engineering Society 9th Scientific Meeting (BES9SM), May 2015, Hwa Chong Institution, Singapore.

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Abstract

Particle-stabilized emulsion or Pickering emulsion has drawn attention in recent years because of its several attractive features. Pickering emulsion offers higher stability in different conditions and it has found applications in a variety of industries including food, pharmaceuticals, and consumer products.

The selection of particles or Pickering emulsifier is crucial to engineering the characteristic features of Pickering emulsion. Inorganic, organic as well as hybrid nanoparticles have been used in stabilizing Pickering emulsions for different applications with some identified limitations. Inorganic particle is less preferred for the development of food and pharmaceutical products due to its limited biocompatibility. Organic particles have low stability in different conditions presenting a research gap and a quest for new organic materials to overcome these issues. In this work, a protein-based material, E2 protein nanocage, has been introduced as a new Pickering emulsifier. E2 is the first non-viral protein nanocage to be used in stabilizing Pickering emulsion.

E2 exhibits excellent surface activity because of the amphiphilic nature of its external surface. E2 adsorbs at the liquid-liquid interface and provides kinetic stability to the emulsion. The E2-stabilized emulsion exhibits pH-switchability for at least 5 cycles and shows stability in a wide range of pH (neutral to basic), ionic strength (as high as 250 mM NaCl) and storage temperature (up to 50°C). The increase in oil fraction in the emulsion composition results in the formation of gel-like structure. The rheological analysis of Pickering emulsion and gel-like structure confirmed the higher mechanical strength, long-term stability and elasticity of the gel-like structure

compared to the emulsion.

The structural integrity of E2 at the oil/water interface has been studied experimentally and theoretically. The adsorption kinetics of E2 on model hydrophobic surface confirms the formation of 26 nm in a thick monolayer of protein nanocage. In-depth study of the molecular structure reveals an intact tertiary structure of E2 at the interface while the secondary structure results in an increase in alpha-helicity. The trends of denaturation of E2 protein in different denaturation conditions confirm the trend of structural change of E2 at the interface. Furthermore, the theoretical analysis suggests that hydrophobic force dominates over surface force indicating the structural integrity of protein cage at the interface.

To explore a potential application in formulating functional food products, of E2-stabilized Pickering emulsion has been proposed as a platform to deliver active molecules in the gastrointestinal tract with two-step release mechanism. Micronutrients such as iron or calcium can be loaded in the E2 while lipophilic micronutrients in the oil phase of the emulsion. The digestion profile of E2 and E2-stabilized emulsion has been investigated *in-vitro* using gastric simulated fluid followed by the analysis of the micronutrients bioaccessibility in the first step in stomach phase.

In summary, E2 has been shown to stabilize emulsion with pH-responsive behavior. Examination of its structure suggests that it maintains a cage-like structure at the oil/water interface. The non-viral origin combined with the loading of active molecules in both the E2 interior and the oil phase makes E2-stabilized Pickering emulsion attractive as nanocarriers with two-step release mechanism for functional food formulation.

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List of acronyms and abbreviations

BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CD	Catalytic (acetyltransferase) domain
CNC	Cellulose nanocrystal
DLS	Dynamic light scattering
E1	Pyruvate decarboxylase
E2	Dihydrolipoamide dehydrogenase
E3	Dihydrolipoamide acetyltransferase
E2-WT	Wild-type E2
E2-LC2	E2 protein with 2 cysteines at inner surface of the cage
E2-LFtn	E2 protein incorporated with ferritin-mimicking iron binding peptide
EDTA	Ethylenediaminetetraacetic acid
ESI	Emulsion stability index
Far-UV CD	Circular dichroism spectra far from ultraviolet wavelength range
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared spectroscopy
GIT	Gastrointestinal tract
GuHCl	Guanidine hydrochloride
HRIPT	Human repeat insult patch testing
ICP	Induced coupled plasma
IEC	Ion-exchange chromatography
IPTG	Isopropyl β -D-thiogalactopyranoside
LIP	Lipoyl Domain
LSM	Laser scanning microscope
LVR	Linear viscoelastic region
MCC	Macroscopic fibers
MFC	Microfibril cellulose
NFC	Nanofibril cellulose
NP	Nanoparticle
OD	Optical density
OSA	Octenyl Succinic acid
OWR	Oil/water ratio
PDB-ID	Protein data bank Identifier
PDH	Pyruvate dehydrogenase
pE2-WT	Plasmid pET-11a inserted with wild-type E2 gene
PLGA	poly (lactic-co-glycolic acid)
PMF	Protein mass fraction
PNIPAm	Poly (N-isopropyl acrylamide)
PSBD	Peripheral subunit-binding domain
QCMD	Quartz Crystal microbalance and dissipation
QFF	Q-Sepharose fast flow

SAM	Self-assembled monolayer
SC	Stratum corneum
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SOM	Self-organizing map
TEM	Transmission electron microscopy
TF	Tryptophan fluorescence
Trp	Tryptophan

Chapter 1

Introduction

1.1. Pickering emulsion

Emulsion is a colloid mixture consisting of two or more immiscible liquids where one liquid phase is dispersed into another. Emulsions are usually stabilized by surfactant by reducing interfacial tension between immiscible liquids. The amphiphilic nature of surfactants—containing both hydrophilic head and hydrophobic tail—permits the surfactants to be adsorbed at the interface of two immiscible liquids. About a century ago, in 1903, Walter Ramsden observed that non-surfactant solid particle can generate a resistance film at the interface of two immiscible liquids that can inhibit the coalescence of the emulsion droplets.[1] Later, this idea of emulsification by non-surfactant particle was elaborated by Spencer Umfreville Pickering in 1907 in his report on the formation of an emulsion with basic sulfates of iron and copper.[2] Since then, the particle-stabilized emulsions are known as “Pickering emulsion”.

Although the concept of Pickering emulsion is about a century old, the research and applications of Pickering emulsion remain underrated over a long period of time. Interestingly, in last two decades, reports on Pickering emulsion has increased exponentially (**Figure 1-1**). The recent advancement in this technology has opened a new horizon in different potential fields of application such as food, pharmaceuticals, and cosmetics.

Figure 1-1: Number of publications associated with (A) Pickering emulsion, (B) Pickering emulsion and food, (C) Pickering emulsion and pharmaceuticals, and (D) Pickering emulsion and cosmetics in last two decades. Data are collected from Thomson Reuters Web of Science (website: <http://apps.webofknowledge.com/citationReport.do>) on 16th August 2017.

1.2. Challenges of various Pickering emulsifier

A wide range of particles from different sources has been used to stabilize Pickering emulsions. Nanoparticles (NPs) from inorganic sources e.g. silica NP,[3] latex,[4] palygorskite,[5] clay,[6] have been vastly used as stabilizers. Most of the inorganic particles have very well-defined shape and narrow size distribution. In addition, the surface of NPs is tunable for specific applications. However, the application of inorganic particle-stabilized Pickering emulsion has

limitations in the field of food or pharmaceuticals because of their poor biocompatibility and biodegradability.[7] In recent years, particles with biological origins have been applied in stabilizing Pickering emulsion. Cellulose,[8] chitosan,[9] lignin,[10] modified starch,[11-13] flavonoids[14, 15], lipid NP[16], water-soluble zein[17], soy protein[18], casein micelles[19] are some examples. Alongside organic NPs, microorganisms such as viruses [20-22] and bacteria [23, 24] have also been reported to stabilize Pickering emulsion. Organic molecules or NPs are mostly biocompatible and biodegradable. The disadvantages of adopting organic molecules are the polydispersity and the often requirement of surface modification to increase surface activity[25]. The colloidal and wetting properties of the bionanoparticles are poorly characterized for application in food products[18]. Moreover, the surface activity of most of the biomolecules can be easily affected by the environmental conditions such as pH, ionic strength, temperature, and storage time. In this context, monodispersed bionanoparticles with high surface activity and biocompatibility are highly desirable.

1.3. Scope of the thesis

Considering the desired properties of Pickering emulsifier according to different applications, in this thesis, E2 protein nanocage has been introduced as a Pickering emulsifier. E2 protein is part of the pyruvate dehydrogenase multi-enzyme complex found in thermophilic bacterium *Geobacillus stearothermophilus* [26] (Details in Section 2.5). E2 has a well-defined dodecahedron caged structure formed by the self-assembly of sixty subunits. Crystallographic studies show that the assembled structure of approximately 25 nm in diameter has a hollow core with 12 openings of 5 nm each (**Figure 1-2**;

PDB ID: 1B5S). The outer surface of the E2 is amphiphilic in nature containing both hydrophilic and hydrophobic patches. Therefore, the surface activity of E2 has been exploited in stabilizing Pickering emulsion.

Figure 1-2: (A) 2-fold, (B) 3-fold, and (C) 5-fold crystallographic representation of E2 protein nanocage.

1.4. Objectives of the thesis

The three objectives of this thesis are:

1. Formulation, optimization, and characterization of Pickering emulsion
2. Structural integrity of E2 upon adsorption on liquid-liquid interface
3. Investigation of the potential application of E2-stabilized Pickering emulsion in functional food formulation

These three objectives are designed to achieve different milestones in a different stage of the project to consolidate the platform for following part of the project. Investigation of the extent of surface activity of E2 followed by the formulation of Pickering emulsion are the key objectives for developing the platform of the project.

Formulation of the emulsion is followed by the molecular structural analysis in process of emulsification. As the structural integrity is of the key importance of any protein for their functionality, the investigation of structural

integrity of E2 protein nanocage in the process of emulsification is selected as a key objective to ensure the applicability of E2-stabilized emulsion in a different field of applications.

The application of E2-stabilised emulsion is explored in designing functional food for delivery of micronutrients. Estimation of loading capacity of E2 as a nanocarrier and bioaccessibility of the loaded molecule are key parameters in designing delivery media. Therefore, the objective of this part of the thesis is to investigate the digestion profile of E2-stabilised emulsion followed by calculation of bioaccessibility of micronutrition.

1.5. Organization of thesis

The thesis has been structured in the following style— Chapter 1 provides a general overview and current trend of Pickering emulsion including the main and detailed objectives of this work. Chapter 2 discusses the theoretical principles of emulsion formation and literature review of the application of Pickering emulsions. The materials and methods applied in this thesis are summarized in Chapter 3. The main results and findings are compiled in three core chapters— Chapter 4–1. The contents of these chapters are summarized briefly in the following paragraphs.

Chapter 4 is a version of a paper published in ACS Applied Materials and Interfaces. It reports the technique of formulation of Pickering emulsion using E2 as the Pickering emulsifier followed by the optimization of compositions to formulate stable emulsion. The chapter highlights the optimal formulation composition which is determined by varying protein mass fraction and oil/water ratio. The resulting stable Pickering emulsion has been investigated under different conditions. The chapter concludes by characterization of the structure

of Pickering emulsion by rheological analysis. It also reports the required energy of homogenization to disperse one phase into another to achieve a stable and monodispersed emulsion.

Chapter 1 discusses the molecular assembly and structural integrity of E2 upon adsorption at the liquid-liquid interface. The molecular assembly has been investigated by quartz crystal microbalance and dissipation (QCM-D) and transmission electron microscopy (TEM). The structural integrity of E2 has been confirmed by the tryptophan fluorescence (TF) and circular dichroism analyses. The interfacial structure of E2 has been compared to the denaturation pathway of E2 in different denaturation conditions. Finally, a molecular simulation and estimation of stabilizing forces have been performed to support the experimental findings.

The potential of E2-stabilized Pickering emulsion in designing functional food is explored in Chapter 1. An emulsion-based functional food for delivering micronutrients in gastric phase has been designed by encapsulating model micronutrients (e.g. Ca and Fe) in the cavity of E2 protein nanocage. A Pickering emulsion has been formulated by the mineralized E2 and subsequently digested in simulation gastric phase condition *in-vitro* to find the digestion kinetics of E2. The bioaccessibility of minerals in gastric phase has also been estimated to investigate the release efficiency of E2.

Chapter 1 concludes the thesis followed by a discussion on future directions for the research.

Chapter 2

Literature review

2.1. Pickering emulsion

Pickering emulsion is an emulsion stabilized by solid colloidal particle instead of conventional surfactants.[27, 28] Solid particles are partially wetted by immiscible liquid phases (e.g. oil and water) and act as an emulsifier by adsorbing at the liquid-liquid interface. Partial wetting is an indispensable property of the solid particle to be adsorbed and stabilize the interface.[29] Partial wetting depends on the relative affinity of the particles for both phases. The affinity of the particle towards any phase can be characterized by measuring contact angle (θ). For hydrophilic particles, the contact angle is $\theta < 90^\circ$ and the larger fraction of the particle will be exposed to the water phase. Similarly, for hydrophobic particles, the contact angle is $\theta > 90^\circ$ and the larger fraction of the particle will be exposed to the oil phase. The monolayer of adsorbed particles will bend the interface in such a way that larger fraction of the particle remains exposed towards the external side. Particles with contact angle $\theta < 90^\circ$ are suitable for forming an oil-in-water emulsion while particles with contact angle $\theta > 90^\circ$ are suitable for forming a water-in-oil emulsion. (Figure 2-1)

Figure 2-1 : Position of the solid particle at the interface of two immiscible phases with contact angle $\theta < 90^\circ$ (right), $\theta = 90^\circ$ (center), and $\theta > 90^\circ$ (left). Corresponding probable bending of the interface. For $\theta < 90^\circ$, solid particles stabilize oil-in-water emulsion while for $\theta > 90^\circ$, solid particles stabilize the water-in-oil emulsion. Figure is reproduced from review article published by McClements et al. [30]

2.1.1. Solid particles at the interface

The adsorption of solid particles at the interface associated with adsorption energy of particle due to the exchange of interfaces between the solid particle and the liquid phases. These interfaces are associated with interfacial tensions. Pioneering work by Bernard P. Binks [27] shows that for solid particles (s) of radius r —preferentially wetted by phase α , which is adsorbed on an interface between phase α and phase β , will result in the generation of s- β interface and loss of s- α and α - β interfaces. Therefore, the contact angle (θ_α) of the particle in phase α can be expressed by Young's law –

$$\cos \theta_\alpha = \frac{\gamma_{s-\beta} - \gamma_{s-\alpha}}{\gamma_{\alpha-\beta}} \dots \dots \dots (2-1)$$

For a small particle of micrometer size, the effect of gravity will be negligible. The energy of adsorption of a particle can be represented by- [27, 31]

$$E = -\pi r^2 \gamma_{\alpha-\beta} (1 \pm \cos \theta_\alpha)^2 \dots \dots \dots (2-2)$$

The negative sign in the bracket will be applied for $\theta < 90^\circ$ and positive sign in the bracket will be applied for $\theta > 90^\circ$. The energy is highest when the contact angle, $\theta = 90^\circ$. Either side of 90° will result in a sharp decrease in energy value. Therefore, the contact angle which is a representation of wettability of the solid particle is a major influential factor for the particle to be used as Pickering emulsifier.[27]

Figure 2-2: Variation of the energy of attachment, E (relative to kT), of a spherical particle of radius 10nm at a planner oil-water interface of interfacial tension $\gamma_{\alpha-\beta} = 36 \times 10^{-3} \text{ Nm}^{-1}$ with the contact angle θ , the particles makes with the interface at 298K. The figure is reproduced from article published by Binks et al.[32]

As the adsorption energy is dependent on the square of the particle radius, the adsorption energy decreases sharply for a particle of smaller radius. However, the adsorption energy is much larger than thermal energy, even when the particle size is in nm scale for the typical alkane-water interface [33]. For example, the

adsorption energy of a particle of 10nm radius, on an alkane-water interface ($\gamma_{\text{alkane-water}} = 50 \text{ mN m}^{-1}$) will be $E = 1.6 \times 10^{-17} \text{ J}$, considering the contact angle $\theta = 90^\circ$. This value is larger than thermal energy $-kT = 4.1 \times 10^{-21} \text{ J}$ at 293 K by few orders.

Figure 2-3: Variation of the energy required to detach a single spherical particle exhibiting a contact angle of 90° from a planar oil–water interface (of interfacial tension 50 mN m^{-1}) with particle radius at 298 K. Note the low energies for radii $< 0.5 \text{ nm}$ (similar to surfactant molecules) The figure is reproduced from article published by Binks et al.[27]

Moreover, Aveyard et al. discussed the effectiveness of the line tension is stabilizing Pickering emulsion using a particle of small radius.[34] The line tension acts in three-phase contact line around the adsorbed particle, which is negligible for the relatively larger particle. [34] Levine et al. [35-39] showed that, in the absence of a significant effect of line tension, particle stabilize emulsions are not thermodynamically stable. The physically realistic value of positive line tension can lead to exclusion of particle from droplet interface either by rendering the adsorption free energy positive or by creating energy barriers to adsorption. On the other hand, negative line tension will open the possibility of the existence of thermodynamically stable Pickering emulsion. The magnitude of line tension

is expected to be of the order of the product of an interfacial tension and a molecular dimension, and approximately 10 pN, according to theoretical estimates. However, the reported value of line tension exceeds this by 5 orders of magnitude or more.[40] The magnitude of this range has quite a drastic effect on the stabilization of emulsion by a particle of the radius of 20-30 nm range. The estimation of line tension is usually ambiguous [41] and the value and sign of line tension will be uncertain for specific systems. Therefore, it is certainly not possible to design a system with particular sign and value of line tension. Line tension has a critical value T_{crit} , at which free energy of adsorption becomes zero and line tension can reach a maximum value T_{max} . Between the contact angles correspond to T_{crit} and T_{max} , the system shows metastability. This particular range of contact angle is wider for larger particle and lower interfacial tension. For example, contact angle window is shorter (close to 90° only) for system with interfacial tension 50 mN m⁻¹ where the window of contact angle is much wider (71°–109°) for system with 10mM m⁻¹ (**Figure 2-4**) [34] Aveyard et al. also calculated the free energy of droplet formation for the systems with particle size of 15 nm, oil/water interfacial tension 50 mN m⁻¹ and line tension ± 50 pN. The results showed that the negative line tension could lead to forming thermodynamically stable emulsions and positive line tension could lead to thermodynamically stable emulsion if the contact angle is too remote from 90° (**Figure 2-4**). [34]

Figure 2-4: Free energy of drop formation in systems with particle radius =15 nm and initial drop radius (R) =1 μm. The points are for zero line tension; full lines are plots against θ and dotted lines are plots against θ_0 . The parameters selected are indicated in the figure. The figure is reproduced from an article published by Aveyard et al. [34]

Arising of capillary pressure in the thin film of continuous phase, near two colliding droplets, stabilized by solid spherical particle has a significant effect on the stability of the emulsion. [42-44] To rupture the film, it needs to exceed maximum (threshold) capillary pressure P^* , which is given by –

$$P^* = \frac{2\gamma \cos \theta}{ba} \dots \dots \dots (2-3)$$

Where, γ is oil/water interfacial tension, θ is the contact angle of liquid-liquid interface with particle measured in an internal liquid of the film, a is particle radius and b is constant for compact packing of the particle in the interfacial layer. For hexagonal close packing, b is 1.15 and for cubic packing b is equal to 1.41. The film thickness (h) corresponding to maximum/critical capillary pressure is critical thickness h_{cr} , at which the thin film between the approaching droplets will rupture.

Figure 2-5: Sketch of a film formed between two emulsion drops stabilized by spherical solid particles (a); $P^* = P_1 - P_2$ is the capillary pressure; the cases with $P^* = 0$ and $P^* > 0$ are depicted in (b) and (c), respectively. The figure is reproduced from article published by Ivanov et al. [42]

There are several factors that affect the critical capillary pressure. (i) Capillary pressure is proportional to $1/a$, i.e. the smaller the particles give more stable films, (ii) lower particle contact angles correspond to higher P_{cr} , i.e. to form more stable films contact angle must not be equal to or very close to 90° . [42] Kruglyakov et al. reported a maximum stability of emulsion stabilized by silica particle can be achieved in the region of intermediate quasi-equilibrium contact angles ($\theta = 55^\circ - 70^\circ$) [43] (iii) Contact angle hysteresis has a profound effect on P_{cr} , higher the hysteresis, higher the P_{cr} . [44] (iv) Packing of particle at the surface of droplets, lower the interparticle distance, higher the P_{cr} . Any defects in the packing array will sharply decrease the threshold capillary pressure to promote

rupture of the thin film.

Figure 2-6: Plot of the dimensionless capillary pressure p vs the dimensionless thickness of the liquid meniscus, h/a ; the full and the broken lines correspond to fixed contact angle θ and contact radius r_c , respectively. The inset represents the dimensionless maximum meniscus pressure, p^* , vs the contact angle hysteresis, θ_h . The figure is reproduced from article published by Ivanov et al. [42]

Besides these quantitative parameters e.g. wettability and size of the particle, and line tension, some other factors like shape, surface properties, surface roughness, and concentration also play a crucial role in stabilizing Pickering emulsion. [45] The effect of different particle shape (e.g. rod-like, fibers, cubes) on the stability of emulsion has been studied by Madivala et al. [46] Studies show that, the stability of emulsion increases with the increase of aspect ratio of the particles. [47, 48]

Surface property of emulsifier is another factor that affects the stability of Pickering emulsion. Large droplets tend to aggregate and separate from the continuous phase. To stabilize the emulsion, the presence of electrostatic

repulsion or steric hindrance between the droplets are necessary. However, the force between particles will prohibit the assembly of particles on the interface which is known as activation barrier. [29]

Surface roughness is another significant factor that affect the adsorption, motion and particle-interface interaction at liquid-liquid interface.[49] Surface roughness strongly affect the wettability of colloidal particles.[50] Surface roughness results the pinning of contact line and consequently arrests the adsorbed-particle in metastable condition which can be as far as from equilibrium as effectively turning a hydrophilic particle into a highly hydrophobic one. Contact line-pinning also induces the deformation of interface which further traps the particle in metastable state.[49]

Klumperman et al. established a theoretical model to determine the optimal concentration of particle required to obtain both partial wetting and colloidal stability. The limitation of the theoretical model is the lack of consideration of curvature effect, which has a significant effect on the packing of particles on the interface. [33, 34] Harwell et al. reported that the surface coverage of droplets by silane group have a more profound effect on the structure of emulsion than the contact angle of the particles. [35] The increase of the concentration of particle results in the decrease droplet size, the increase the surface coverage and also the formation of network structure around the droplets which further improve the emulsion stability. Binks et al. have shown that droplet size of the emulsion, stabilized by SiO_2 , decreases with the increase of particle concentration up to 3%.[28] At a concentration higher than 3%, the droplet size will not decrease but the extra particles tend to form a network between dispersed phases. Midmore et al. have shown that a stable emulsion can be achieved with

surface coverage as low as 29%—stabilized by SiO₂ and hydroxypropyl cellulose where he proposed the formation of two dimensional gel-like array of silica particle at oil/water interface.[51]

Roke et al.[52] showed that the effect of curvature on surface coverage by nanoparticles in the nanoscopic curved surface is much lower than the surface coverage on a planar surface. The curvature effect of the droplet also introduces defects in HCP packing of particles as reported by Binks et al.[53] Therefore, the calculated surface coverage value assuming a planar surface is much lower than that of full coverage. The concept has also been consolidated by Murray et al. [54] that the surface coverage by active particle does not need to be completed to stabilize Pickering emulsion as long as the adsorbed layer forms a rigid network.

Fernando Leal-Calderon et al. [55] proposed two possible mechanisms of forming stable Pickering emulsion with low surface coverage. When two droplets approach to each other, the particles adsorbed at both interfaces spontaneously rearrange in such a way that the particles at one interface fill the interstices of particle lattices of the opposing interface which follows by the aggregation of particles at the interface to form a bridging monolayer. Another mechanism is related to dipole-dipole interaction between adsorbed particles from two approaching interfaces. As a charged particle adsorbed between two polar and non-polar media produces an effective dipole because of the surface charge difference in the two different media. The effective dipole-dipole interaction energy is always attractive for contact angle between 0° to 90°. This simple dipole-dipole interaction allows the accumulation of particles between the approaching interfaces. Piazza et al. explained a similar mechanism, known as “relocation effect” as the droplets of low surface coverage approach each other,

the redistribution of adsorbed particles in the contact region between adjacent droplets will inhibit coalescence. [56]

2.2. Stability of Pickering emulsion

In the presence of conventional surfactant or surface active molecules, an energy barrier is created between the dispersed phases which prohibit the breakdown of the emulsion and the emulsion becomes kinetically stable. For emulsion system stabilized by solid particle, the reduction of interfacial tension does not occur. The capillary force between the liquid phases and the adsorbed particles becomes the governing factor in destabilizing the system.[57] The breakdown of any emulsion is driven by flocculation, coalescence, Ostwald ripening, or a combination of all these processes.

Figure 2-7: Schematic representation of the various destabilization pathways of the emulsion.

2.2.1. Flocculation

Flocculation is most subtle among other destabilization techniques and most complicated to control because of the sensitivity of the process to different factors. [58] Flocculation directly affects other destabilization processes (e.g. creaming, coalescence) and is often considered as the precursor of coalescence. [59] Two most common mechanisms of flocculation are depletion and bridging.

2.2.1.1. Depletion flocculation

In the presence of unabsorbed surfactants in the continuous phase of the emulsion, depletion flocculation can occur. Unabsorbed surfactants or emulsifier induce osmotic pressure gradients within the continuous phase surrounding the droplets which results in a high-density packing of the emulsion droplets. The force can be expressed by measuring the concentration of unabsorbed surfactants and radius of gyration of the surfactant molecule. The depletion force ω_{dep} can be expressed as–

$$\omega_{dep}(0) = \frac{-3kT c R_v}{2\rho} \left(1 + \frac{1}{2} \frac{c R_v}{\rho}\right) \left(\frac{\gamma_d}{\gamma_g} + \frac{2}{3}\right) \dots\dots\dots (2-4)$$

Where, c is the concentration of surfactant in (kg/m^3), γ_d and γ_g are the radius of the emulsion droplet and gyration respectively, ρ is the density of the surfactant molecule and R_v can be expressed as–

$$R_v = \frac{4\pi\gamma g^3 \rho N_A}{3M} \dots\dots\dots (2-5)$$

Most droplets are flocculated when the potential of depletion exceeds $4kT$. Depletion force driven flocculation is generally weak, flexible and reversible.[60] Therefore, the stability of emulsion can be restored or manipulated.

2.2.1.2. Bridging flocculation

Bridging flocculation occurs due to the electrostatic interactions between oppositely charged emulsion droplets and surfactants.[61] The electrostatic interaction between droplets has very high strength, lower flexibility and usually not reversible. The emulsions form bridging flocculation has very high storage modulus and higher elasticity also which reflects the formation of bridging networks between emulsion droplets.

2.2.2. Coalescence

Coalescence occurs when the thin film of continuous phase between two adjacent droplets is drained out leading the nucleation of a pore. As the pore reaches a critical size, it starts to grow faster and two droplets are merged.[62] Coalescence of Pickering emulsion can occur either by the formation of the liquid bridge which grows continuously to merge the droplets or by the formation of a bridge via an oscillating mechanism which does not grow due to geometric restrictions and the two droplets merge as soon as the stable bridge forms.[63] Partial coalescence is another type of destabilization pathway in which two droplets start to merge together and arrest in midway giving rise to a peanut or dumbbell-like shape.[64] This phenomenon occurs when the surface of the droplets are not fully covered and adjacent droplets start to coalesce to reduce the interfacial area. As the adsorbed particles do not detach from the interface, coalescence process stops as the full coverage obtained. This process also depends on the concentration of the particle at the interface.

2.2.3. Ostwald ripening

When dispersed phase is partially soluble in a continuous phase of the emulsion, Ostwald ripening takes place. Due to the Laplace pressure which is

higher on a curved surface than a flat surface, the highly curved surface of the smaller droplet will dissolve and larger droplets will grow. Some literature reported that the rate of Ostwald ripening is very low for Pickering emulsion. Nevertheless, Ostwald ripening can be a key mechanism to destabilize Pickering emulsion where the surface coverage is low.[6] The size of the droplets increases as long as the higher surface coverage is achieved. The solubility of two phases can be manipulated by the presence of micelles which may lead to Ostwald ripening for the Pickering emulsion as well.[65]

2.2.4. Creaming or sedimentation

Creaming and sedimentation occur in emulsion system depending on the droplet size of the dispersed phase and the density difference between the phases. The emulsion separates into two parts containing continuous phase in one part and concentrated emulsion in another part. Sedimentation occurs when the sizes of the droplets are large enough that the effect of gravity cannot be avoided. Creaming occurs when the density of the dispersed phase is lower than the density of continuous phase. The rate of sedimentation and creaming can be reduced by reducing droplet size and increasing the density of continuous phase. Another possible way to reduce the sedimentation is increasing the concentration of dispersed phase. At higher concentration of dispersed phase, the system becomes stable by forming a gel network between the droplets and shows higher resistance against sedimentation and creaming.[32]

2.3. Particles used as a Pickering emulsifier

Emulsions are a thermodynamically metastable system which tends to separate into individual components over a period of time. In an emulsion, different surface active molecules or particles are used to prevent or delay the

separation by reducing the surface free energy of the interfaces. For Pickering emulsions, solid particles with partial different wettability are used to stabilize the emulsion system. The particles have so far been used for stabilizing Pickering emulsions can be categorized based on the sources as—inorganic, organic and Janus particles.

2.3.1. Inorganic particles

In last few decades, inorganic particles have been extensively used in stabilizing Pickering emulsion. Inorganic particles such as silica, [66-69] clay, [6, 70-72] graphene oxide,[73, 74] quantum dots[75, 76] and metal oxides[77, 78] are commonly used.

2.3.1.1. Silica particles

Silica NPs are the mostly used inorganic particle in stabilizing emulsion and foams because of its flexible properties. Silica NP is a mesoporous material which can be synthesized with different surface properties.[79] Silica particles of a wide range of sizes are commercially available (from few nanometer to few micrometer). Surface modified hydrophilic silica NP[66, 67], hydrophobic silica NP[68], fluorescent silica NP[69] have been used in stabilizing Pickering emulsions and foams. The versatility of silica particle makes it an excellent candidate for stabilizing Pickering emulsion.

2.3.1.2. Clay particle

Clay particles such as bentonite[70], Laponite[6], montmorillonite[71], kaolinite[72] either in natural form or in surface-modified form, has been used in stabilizing Pickering emulsion. Surface modification of clay particle by grafting, ion-exchange or adsorption can produce a particle with different hydrophobicity or polarity. As the surface modification can manipulate the contact angle of the

particle, emulsions of various stability can be formulated by clay particles. Moreover, both nano-sized (e.g. 30 nm synthetic Laponite)[6] and micro-sized (e.g. montmorillonite; typically 0.1 to 2 μm)[71] are reported as an emulsion stabilizers. Clay particles are also natural and cost-effective for large-scale production.

2.3.1.3. Metal oxides and others

The metal oxides are another kind of particles that have been used in stabilizing Pickering emulsion. Metal nanoparticles such as Fe_2O_3 [77, 78], palygorskite [5], are reported to formulate stimuli-responsive Pickering emulsions. Negatively charged carbon black particles[80] and graphene oxide[81] have also been reported as surface active particles which can stabilize Pickering emulsion. Carbon black particle-stabilized emulsion can exchange the adsorbed particle from the interface with ionic and non-ionic surfactants. Graphene oxide-stabilized emulsion shows excellent stability, originated from the amphiphilic behavior, containing both unoxidized benzene ring and ionizable ($-\text{COOH}$) group.

2.3.2. Organic particles

Many biomacromolecules exhibit surface activity by stabilizing liquid-liquid or air-liquid interfaces. Therefore, besides the application of inorganic particle, particles of biological origin are vastly used in stabilizing Pickering emulsion. These particles can be categorized based on their origins such as carbohydrates-based, protein-based, and lipid-based particles. Organic particle stabilized Pickering emulsions have a large application in the field of food and pharmaceuticals industries. The applications of organic particle stabilized Pickering emulsion is described in details in Section 2.4.

2.3.2.1. Carbohydrate-based particle

Starch, cellulose and chitin/chitosan are the most commonly used organic Pickering emulsifier. Most of these biomacromolecules are hydrophilic in nature. Therefore, surface modification is often required to enhance the hydrophobicity of the particle.

Starch particles, the most abundant carbohydrate in the human diet, can be derived in a wide range of size and shape depending on the preparation technique. The starch particle is hydrophilic in nature which mostly stabilizes an oil-in-water emulsion. However, octenyl succinic acid (OSA) modification increases the starch hydrophobicity which can stabilize water-in-oil emulsion at a higher fraction of oil (>0.65). [82] Rayner et al. reported that 2–3% OSA modification of quinoa starch particle shows optimum emulsifying activity. [83] Esterification with acetic anhydride or phthalic anhydride and dry heat treatment and crystallization could be other methods to increase the hydrophobicity of starch granules. Li et al. showed that starch nanocrystal stabilizes emulsion without further modification. [84]

Cellulose, a linear polysaccharide, is the most available biopolymer in nature and can be derived from plant cell walls as well as from some bacteria e.g. *Gluconacetobacter xylinus*. [85] Cellulose in different forms like cellulose nanocrystal (CNC), macroscopic fibers (MCCs), microfibril cellulose (MFC), or nanofibril cellulose (NFC) have been reported as an emulsion stabilizer. [86] The stability of the emulsion is highly dependent on the size and the concentration of the cellulose. Stability increases with the increase of cellulose concentration and with the decrease of particle size. [86] Like starch, cellulose is also hydrophilic in nature and can be modified to increase hydrophobicity to formulate water-in-oil

emulsion. Anderson et al. used silylation to modify the hydrophobicity of cellulose to formulate oil continuous emulsion.[87] Moreover, the thermally-responsive emulsion has also been formulated by grafting of poly (N-isopropyl acrylamide) (PNIPAm) from the surface of nanocrystals.[85]

Chitin, another linear polysaccharide, is the second most abundant in nature. Natural chitin has a semi-crystalline structure which can be modified into crystalline form. A rod-like crystalline form of chitin shows excellent surface activity to stabilize the emulsion.[88] Chitosan, a deacetylated form of chitin, shows pH dependence at the interface. Moreover, chitosan is biocompatible and have antibacterial properties which make chitosan a common material for drug encapsulation. Wei et al. demonstrate the synthesis of poly (lactic-co-glycolic acid) (PLGA) particles from the emulsion stabilized by chitosan.[9] Chitosan has also been used in stabilizing pH-responsive Pickering emulsion.[9]

2.3.2.2. Lipid-based particle

Lipid-based particles like fat crystals have been widely used in stabilizing water-in-oil Pickering emulsion.[89] Fat crystals are relatively hydrophobic that makes the particles adsorbed on the oil/water interface with a contact angle higher than 90°.[90] Other lipophilic particles like flavonoid,[14] phytosterol,[91] lactylate or glyceryl stearyl citrate have also been used in stabilizing Pickering emulsion. Ye et al. recently reported an emulsion-stabilized Pickering emulsion in which casein was used to stabilize alkane droplet.[19]

2.3.2.3. Protein-based particle

Proteins are amphiphilic biomolecule which has a wide range of applications in biological functions. The amphiphilicity of proteins allows the molecules to adsorb on a liquid-liquid interface and form steric barriers against

coalescence. Different protein-based particle—from both plant and dairy sources— has been successfully used in stabilizing oil-in-water Pickering emulsion. Relatively hydrophobic soy[18] or zein proteins[92] can be dispersed in the aqueous phase by thermomechanical treatments like stirring–heating at 95°C or ultrasonication to be used as Pickering emulsifier. Lactoferrin particle[93] both with and without surface modification are used as Pickering emulsifier. Proteins from dairy sources like whey protein isolates, casein have also been reported as Pickering emulsifier.[94] Heat treatment of whey protein can increase their emulsifying properties.[95]

Alongside globular proteins, structured protein (e.g. cage-like proteins) are also used in stabilizing Pickering emulsion. Both viral and non-viral proteins are reported to be adsorbed on an oil/water interface to formulate Pickering emulsion earlier. Russell et al. reported the use of viruses such as tobacco mosaic virus[20], cowpea mosaic virus[21], and turnip yellow mosaic virus[22] to stabilize oil-in-water emulsions. Fujii et al. reported the application of non-viral, iron-storing protein nanocage, ferritin, as an effective Pickering emulsifier. [96] Though these structured proteins have several advantages over other biomolecules, the studies of stabilizing Pickering emulsion using bio-nanoparticles are still limited. [96]

2.3.2.4. Other biological entities

Other biological particulates like viruses, bacteria, and spore particles— have very well-defined shapes and size— are used as Pickering emulsifier as well. Dorobanatu et al. reported the application of four different hydrocarbon-degrading bacteria of different hydrophobicity in stabilizing Pickering emulsion.[97] Rosenberg et al. studied the ability of different strains of

Acinetobacter calcoaceticus to adsorb on oil/water interface.[98] Binks et al.[99], Ballard et al.[100] studied the ability of Moss spore (*Lycopodium clavatum*) to stabilize Pickering emulsion.

2.3.3. Janus particles

Janus particles are renamed after the two-faced Roman god of doorways as these particles display at least two distinct surface properties. Amphiphilic Janus particles are derived by combining both hydrophilic and hydrophobic particle together. These non-uniform particles have two different contact angles which have considerable advantages in stabilizing emulsions. The amphiphilic nature of these particles can be used in stabilizing both oil-in-water and water-in-oil Pickering emulsion. However, the synthesis of Janus particles is more difficult than conventional nanoparticles.[101]

2.4. Applications of Pickering emulsions

Pickering emulsion has potential to be used as a functional emulsion in various applications. In the last few decades, the use of Pickering emulsions has increased extensively in the formulation of functional food, pharmaceuticals, and cosmetics products.[89, 102-106] In the following sections the recent advancements of Pickering emulsion in these fields are reviewed in details.

2.4.1. Applications in pharmaceuticals

Applications of Pickering emulsion in pharmaceuticals include the studies of drug administration through oral or topical route using emulsion as a delivery platform. Yang et al. studied the intravenous injection of paclitaxel-loaded Pickering emulsion stabilized by deformable poly (N-isopropyl acrylamide-co-allylamine) (PNIAM-co-AA) nanogels[107]. Only limited studies on intravenous injection of Pickering emulsion have been reported. Most of the

reported mode of administrations have been oral and topical.

Chevalier et al. reported the transdermal administration of hydrophilic drugs using water-in-oil emulsion stabilized by silica particle.[108] Results showed that the efficiency of penetration through pig skin is at least three times higher than conventional emulsion because of the strong adhesion force between Pickering emulsions and skin. However, the delivery of lipophilic drugs using water-in-oil Pickering emulsion was not significantly different from the conventional emulsion.[109] Wahlgren et al. used the starch stabilized Pickering emulsion of different oils for topical application.[110] The higher flux of diffusion through the skin was obtained compared to usual solution formulation.

The delivery of drugs or active compounds through oral route has been studied extensively by using Pickering emulsion. Nitin et al. encapsulated curcumin in silica nanoparticle stabilized the emulsion and showed 100 times more stability compared to curcumin solution.[111] The epithelial cell has up taken the Pickering emulsion droplet very efficiently and showed excellent cell viability in presence of emulsion. Lesmes et al. formulate a silica particle stabilized emulsion with excellent stability at pH value 3–7, ensuring the protection of lipid droplet in gastrointestinal tract (GIT) conditions.[112]

Pickering emulsion has also been used as a template for the formulation of drug carrier. Maas et al. reported the formulation of colloidosomes by using particles instead of conventional surfactants which avoid the use of toxic stabilizers.[113] The colloidosomes are versatile and can be used as a carrier of antibodies, proteins or any drug molecules. Nan et al. formulate chitosan stabilized Pickering emulsion to use it as a template to create insulin-coated colloidosomes.[114] Poly(N-isopropyl acrylamide) (PNIPAm) nanoparticles

stabilized emulsion has stimulus-responsive characteristic at a different temperature which triggers the release of loaded molecules in the emulsion.[115] However, the delivery mechanism from the stimuli-responsive emulsion is associated with the destruction of shell around droplet. Zhou et al. reported a novel technique to formulate stimuli-responsive colloidosomes which can show stimuli response without deformation of the shell.[116]

The potential application of Pickering emulsion in bio-imaging i.e. photoacoustic imaging, biosensing and in using as a bioreactor has also been reported. O'Donnell et al. used gold nanoparticle stabilized Pickering emulsion as an agent for enhancing contrast in photoacoustic imaging.[117, 118] Maas et al. formulate a Pickering emulsion using both magnetic iron oxide and fluorescence silica particles which incorporate both fluorescence and magnetism in the same emulsion.[119] These properties can be exploited and applied in bioimaging or biosensing.

2.4.2. Application in food formulation

Food is a complex colloidal system containing various kinds of molecules. The droplets of the food system are stabilized by small-molecule surfactants, proteins, polysaccharides or other types of emulsifying agents. The application of nanoparticle in stabilizing functional food has recently drawn large attention. More than 95% of the publications related to the application of Pickering emulsion in food formulation have been published in last decade (**Figure 1-1**)[25]. The stability of food formulation is largely dependent on the type of emulsifying agent used.

The organic nanoparticle, as well as modified inorganic nanoparticle, have been used in developing functional food complex based on emulsion.

Surface modification can be done in different ways, e.g. binding colloidal particle with oppositely charged smaller surfactants. Binks et al. reported the formulation of gel-like complex with enhanced microstructure stability stabilized by anionic silica particle + cationic CTAB[120] and alumina-coated silica particle + anionic SDS[121]. The stability can be affected by surfactant/particle ratio[122] and sequence of addition.[123] The synergistic mechanism was proposed to explain the long-term stability of Pickering emulsion based on surfactant and particle mixtures.[124] The addition of a small surfactant can be avoided by increasing wettability of the nanoparticles by mixing oppositely charged nanoparticles in a binary mixture which will result in a hetero aggregated particle before emulsification.[125] Besides surface modification, rheology of food formulation can also be controlled by tuning the pH and ionic strength.

Oil-continuous Pickering emulsion can be formulated by using wax particle—natural (beeswax) or synthetic (polyethylene based)—as Pickering emulsifier.[126, 127] Besides the primary stabilization mechanism of adsorption on the interface, wax particles make a particle network to immobilize coarse water droplet in the colloidal system to prevent sedimentation. Various types of commercially available wax have been reported to be used as Pickering emulsifier in food formulation. Pickering emulsion stabilized by fat crystal also stabilize in the similar mechanism of forming three-dimensional particle network.[128]

2.4.3. Application in cosmetics

The use of Pickering emulsion for topical application in the form of skincare product is emerging very recently. The emulsion-based cosmetic products are usually stabilized by conventional surfactant molecules which may

cause the disruption of the skin, toxicity and may alter the pharmacokinetics of co-administered drugs. The particle-based emulsion has great flexibility to tune the properties and control the penetration of deliverable molecules through or into the skin.

Both inorganic and organic particle is used in designing personal care products based on Pickering emulsion. The silica nanoparticle-stabilized Pickering emulsion was studied to investigate the rate of flux of hydrophilic model drug, caffeine. The flux of caffeine from Pickering emulsion was at least three-fold higher than the conventional emulsion.[108] The higher flux is associated with the higher adhesion of silica particle with the surface of the skin. The delivery of the hydrophobic model drug, retinol, was also studied by the same group.[109] The result showed the penetration depth of retinol is higher from conventional emulsion compared to Pickering emulsion. Retinol reaches the deeper layer of the skin that is the dermis when carried by conventional emulsion. In contrast, the retinol penetration depth is reduced to reaching only the outermost layer of the skin that is the stratum corneum (SC) layer of epidermis when carried by silica-based Pickering emulsion. The possible reason could be the droplets of the Pickering emulsion is less flexible than conventional emulsion. Another silica-based Pickering emulsion showed higher flux compared to conventional emulsion—studied using arbutin as a model drug. TiO_2 is another well-known nanoparticle used in the formulation of skin care products which has two much-desired properties. TiO_2 has had much less skin irritancy and can increase sun protection factor (SPF) of the sunscreen cream as well.[129] A Pickering emulsion stabilized by TiO_2 has been successfully used in sunscreen formulation to protect the skin from UVB/A.[130] The emulsion has also been

used in stabilizing and delivering melatonin. A separate study by Daniels et al. showed the effect of different surface modified TiO₂ on the type and stability of Pickering emulsion for topical application which reported the higher stability of w/o emulsion with the increase of contact angle of the TiO₂ particle.[131]

The recent trend of the formulation of green or biocompatible products in skincare industries opens the opportunity to design Pickering emulsion-based cosmetic products stabilized by organic particles. Marku et al. designed a Pickering emulsion using OSA modified quinoa starch particle to deliver active ingredients—methyl salicylate.[110] The highly stable (8 weeks) product was formed with oil fraction between 41–70% which had the homogeneous appearance and high permeability (8.0 µg/ (cm²/h)). Alongside these studies, the stability and storage conditions of Pickering emulsions, formulated with different oils and different particles, are also investigated.

Cyclodextrins (CD) has been used in designing Pickering emulsion-based cosmetic products because of its few desired properties e.g. drug solubility, high photostability, less skin irritancy. α-CD and β-CD are the most used in the development of products for topical application for prevention and treatment of skin disorder.[132, 133] β-CD (<10 µm) stabilized emulsion showed higher stability compared to α-CD (>20 µm) stabilized emulsion due to higher surface coverage and packing of smaller nanocrystal at the droplet interface.[134]

The characterization of toxicological analysis, pharmacological analysis, and analytical evaluation are also necessary to select appropriate emulsifier for cosmetic application. Marto et al. investigated the sensitivity and skin irritancy of starch-stabilized emulsion on a human by HRIPT assay which showed no skin irritation in the initial 3 weeks of exposure.[135]

2.4.4. Oil recovery

Surfactant-stabilized emulsions are heavily used in petroleum industries to enhance the efficiency of oil recovery. However, the application of particle stabilized emulsion in oil recovery is the comparatively less studied area.[136] The relatively smaller droplet size and higher viscosity enhance the advantages of using Pickering emulsion in oil recovery. The relatively smaller droplet size allows the oil to flow through small pores of the rock and the viscosity creates back pressure that allows sticky or highly viscous oil to flow. Gao et al. studied the application of pH-responsive magnetic nanoparticle to separate oil and water from the emulsion and the nanoparticle was recycled at the end of the separation.[137]

2.4.5. Catalyst recycling

The biphasic condition of Pickering emulsion has been exploited to enhance solvent compatibility to increase the reaction rate. Stimuli-responsive Pickering emulsions are most favorable in such applications as the separation technique of reaction products is more convenient. Yang et al. compared the reaction efficiency of biphasic catalytic reaction between the conventional biphasic system and Pickering emulsion system which shows the efficiency of Pickering emulsion system is comparable to the stirrer-driven biphasic system.[138] Chen et al. reported a light-responsive Pickering emulsion stabilized by upconversion nano phosphors which significantly enhances the biocatalytic activity of *A. faecalis* ATCC 8750.[139]

2.5. E2 protein nanocage: characteristics and potential as a Pickering emulsifier

The unique structural characteristics of protein cages elevate the functions

and diverse applications in the field of nanotechnology. Protein cages have been applied as nanocarrier of drugs or other foreign molecules. The protein cages originated from viral sources, are not considered for delivering drug and the non-viral protein cages are not well established for their emulsifying ability. However, designing of emulsion-based delivery platform requires both emulsifying and delivering ability of protein nanocages. Therefore, in this Ph.D. work, emulsifying properties of protein nanocage is explored to design an emulsion-based drug delivery platform. Previously reported viral protein cages have been shown excellent emulsifying properties in terms of surface activity to act as a nanoparticle to stabilize Pickering emulsion. This Ph.D. work is dedicated to the use of nonviral protein nanocage—E2— to explore its surface activity and emulsifying properties.

Figure 2-8: Model for active-site coupling in a hypothetical E1-E2-E3 complex. Three E1 tetramers (purple) and three E3 dimers (yellow) are shown located in the outer protein shell above the inner icosahedron (grey) formed by 60 E2 catalytic domains. Image is reproduced from Milne et al. [140]

E2 protein is part of the pyruvate dehydrogenase (PDH) multienzyme complex found in thermophilic bacterium *Geobacillus stearothermophilus*. The PDH multienzyme complexes play a pivotal role to catalyze the oxidative decarboxylation of pyruvate to form acetyl-CoA and to link glycolysis and the tricarboxylic acid cycle.[26, 141] PDH multienzyme complex is consists of multiple copies of three enzymes—Pyruvate decarboxylase, E1; dihydrolipoamide acetyltransferase, E2, and dihydrolipoamide dehydrogenase, E3. E1 and E3 enzymes are assembled around E2 by forming a tight bond with it. (**Figure 2-8**)[142, 143] Depending on the origin of species, the structure of E2 has two different symmetries. In gram-negative bacteria, 24 subunits of E2 form an octahedral cage structure wherein gram-positive or in eukaryotes, 60 subunits of E2 forms an icosahedral structure. The detail 3-D structural analysis of PDH complex with 60-mer E2 enzyme has been performed by crystal diffraction.[140]

Each 60-mer E2 have three mechanistic domains—a. N-terminal 9kDa lipoyl domain (LIP, ~80 amino acid residues in length—depends on the species, b. 4kD a peripheral subunit-binding domain to which E1 and E3 are bound mutually exclusively (PSBD, ~35 amino acid residues) and c. C-terminal 28kDa catalytic (acetyltransferase) domain (CD, ~250 residues). These domains are linked with each other by a highly flexible linker peptide (~ 25-30 amino acid residues)—rich in alanine, proline or other charged amino acids.[144, 145] LIP and PSBD domains are responsible for binding E1 and E3 dimers with the E2 core, where the CD domain assemble the pentagonal dodecahedral core and its active sites for acyltransferase.[140, 143]

The PDH multienzyme complex has been engineered to isolate the acetyltransferase catalytic domain from E1 and E3 which has well-defined

dodecahedron cage structure.[146, 147] Crystallographic studies show that the assembled structure is highly symmetric—approximately 25 nm in diameter—and has a hollow core inside with 12 openings in a different facet of the cage—each of these openings is 5 nm in diameter.[142, 143]

The unique properties of cage structure of E2 have drawn attention for using the cage in the field of biomedical application as a nanocarrier of drugs. E2 has been previously reported for loading of foreign molecule e.g. fluorescence dye[147], doxorubicin[148, 149] and iron molecule[150] in the inner cavity of the cage structure by forming a covalent bond with cysteine mutation sites or by electrostatic interactions. Both wild-type (WT) and genetically modified E2 have been investigated to encapsulate foreign molecules without disturbing the assembly mechanism of cage structure.[146, 151] The thermophilic origin of E2 ensures its stability at a higher temperature.[152] The robustness of E2 allows the flexibility in genetic modification of E2 for specific applications.

Figure 2-9: The surface properties of E2; (A) distribution of hydrophilic (cyan) and hydrophobic (orange) residues in E2 shown along the 3-fold axis, (B) distribution of positively (blue) and negatively (red) charged residues in the E2 show along with a 3-fold axis.

The exterior surface of the E2 protein nanocage contains both hydrophilic and hydrophobic amino acid residues which ensure the amphiphilic behavior of E2. The relative hydrophobicity can be measured by assigning a relative value to all amino acid. Matthews et al. estimated the hydrophobicity of 20 amino acids based on the solvent transfer free energies from octanol to water.[153] According to the assigned value, the relative hydrophobicity of E2 Protein is measured to be ~520kJ/mol. Though this method cannot differentiate between the hydrophobic residues that are located on the surface and the core of protein structure. If only surface residues are taken into consideration, the “effective hydrophobicity” of E2 will be ~125kJ/mol. **(Figure 2-9 A)**

The distribution of hydrophilic and hydrophobic residues on the exterior surface reveals the amphiphilic nature of E2. The amphiphilic behavior of E2 has been exploited in this work to establish E2 as a Pickering emulsifier.

Chapter 3

Materials and methods

This chapter describes the materials and methods used in the thesis.

3.1. Materials:

The materials used for conducting different experiments are described below-

3.1.1. Protein overexpression, purification, and characterization

The plasmid (pE2) containing E2-loop cysteine 2 (E2-LC2) and E2-loop-Ferritin (E2-LFtn) sequence, constructed by Dr. Peng Tao, were supplied from BeANs Lab, Nanyang Technological University.[150] *E. coli* strains BL21 (DE3) were used as host cells for overexpression of plasmid. Ampicillin antibiotic and Isopropyl β -D-1-thiogalactopyranoside (IPTG) were purchased from 1st Base, Singapore. Buffer reagent Tris and sodium phosphate were purchased from USB Corporation. Ethylenediaminetetraacetic acid (EDTA) and sodium azide were purchased from Fluka.

3.1.2. Emulsion formulation and characterization

Pickering emulsion is formulated with Rosemary oil, Milli-Q water, and protein nanocage as an emulsifier. Rosemary oil (Kosher (FCC)) was purchased from SAFC, Sigma-Aldrich (Catalogue no. W299200). The HLB value of surfactant required to stabilize oil/water (o/w) emulsion with minimum droplet size using rosemary oil as an oil phase is reported as 15, and the refractive index of rosemary oil is reported as 1.468.[154] Milli-Q water, purified using Milli-Q

Synthesis A10, Merck, was used as one of the formulation components of the Pickering emulsion. Alexa Flour 488- C5-Maleimide and Nile red were purchased from Life Technologies and Sigma-Aldrich, respectively, and used as obtained.

3.1.3. Preparation of model hydrophobic surface for QCMD experiment

Sulfuric acid (98%), 30% hydrogen peroxide, 1-Dodecanethiol were purchased from Sigma-Aldrich, Singapore. Gold-coated AT-cut piezoelectric quartz crystals were purchased from Analytical Technologies Pte Ltd, Singapore.

3.1.4. Mineralization and simulation of gastric phase digestion: *in-vitro*

Ferrous sulfate (Cat. No. F8048) and calcium chloride (Cat. No. C3306), potassium dihydrogen phosphate (Cat. No. 244023), ammonium chloride (Cat. No. 213330), sodium carbonate (Cat. No. 497-19-8) were purchased from Sigma-Aldrich. Potassium chloride (Cat. No. 1.04936.1000), sodium chloride (Cat. No. 1.06404.5000) were purchased from Merck, Singapore. Magnesium chloride (Cat. No. 195304) was purchased from MP Biomedicals. Pepsin from porcine gastric mucosa (Cat. No. P7000) was also purchased from Sigma-Aldrich to carry out the simulation of gastric phase digestion: *In-vitro*. Visible stain dye Coomassie blue G-250 has been obtained from Bio-Rad, Singapore to stain the protein in SDS-PAGE and during spectrophotometric analysis of digestion kinetics. All the chemicals were used as it is obtained unless mentioned otherwise.

3.2. Methods

3.2.1. Protein overexpression, purification, and characterization

E2-LC2 mutant protein has been made by replacing two amino acids, aspartic acid and glycine (amino acids #381 and #382), on wild-type E2 (E2-WT) (PDB ID: 1B5S). These two amino acids were replaced with 2 cysteine amino acids by site-directed mutagenesis. E2-LC2 was used to carry out all experiments of chapter 4 of the thesis. Another genetically mutated form of E2-WT renamed as E2-loop Ferritin (E2-LFtn) was used to carry out all the experiments of chapter 5 and 6 of this thesis. An amino acid motif consisting of six amino acids Gln, Glu, His, Glu, Asp and Glu (QEHEDE) was introduced in the amino acid sequence of E2-WT by replacing four amino acids Arg, Asp, Gly and Glu (RDGE) from position #380-#383 by site-directed mutagenesis. The E2-LC2 and E2-LFtn genes were cloned separately into pET-11a vector (pE2-LC2 and pE2-LFtn respectively) between NdeI and BamHI cutting sites.

3.2.1.1. Overexpression of protein

E2-LC2 and E2-LFtn were produced in *E.coli* strain BL21 (DE3) recombinantly and purified following a protocol described by Dalmau et al.[147] The *E.coli* cells were cultured in Luria-Bertani medium supplemented with 100mg/L ampicillin. The gene expression was induced by addition of 1mM IPTG in the culture when the optical density (OD₆₀₀) reaches 0.6-0.8. Cells were harvested 4 hours after induction by centrifugation at 4500×g for 45 min and stored at -80°C. The cells were re-suspended in Tris buffer (20 mM Tris, 5 mM EDTA, and 0.02% sodium azide, pH 8.7) and lysed by ultrasonication in vibracell cell disrupter. (Model VC505, 500 W, 20 kHz) for 25 min at 37% amplitude with pulse setting 5 sec on/off. Energy input was given through tip (630-0220, Sonics

& Materials Inc.) containing a piezoelectric crystal with a probe diameter of 13 mm. Disrupted cells were heat treated for 20 min at 75°C to precipitate out the heat-sensitive native proteins. Subsequently, disrupted cells were centrifuged at high speed (145510×g) for 1 hour to remove all insoluble fractions of cells and precipitated proteins using ultracentrifuge (Optima L-90K, Beckman Coulter, CA) and rotor Ti 50.2. After centrifugation, the only supernatant was collected for further purification.

3.2.1.2. Purification of protein

E2 protein was purified using fast protein liquid chromatography (FPLC) by using the principle of ion-exchange chromatography. The soluble fraction of proteins (E2-LC2 and E2-LFtn) were filtered and loaded onto an anion-exchange chromatography column (HiPrep QFF 16/10, GE Healthcare), which had been equilibrated with Tris buffer (20mM Tris, 5 mM EDTA and 0.02% NaN₃, pH 8.7) on a fast protein liquid chromatography (FPLC) system (ÄKTA Pure, GE Healthcare). Purified E2 was pooled from flow-through fraction and bound proteins were eluted by flowing Tris buffer with 1M NaCl in the column.

3.2.1.3. Characterization of protein

Purified E2 nanocages were concentrated by using 100kDa cut-off filter (Amicon, Merck). The concentration of E2 was determined using BCA Protein Assay Kit (Pierce) using bovine serum albumin (BSA) as a standard. A SDS-PAGE analysis was performed by running the samples on 4–20% Tris–HCl gels. The hydrodynamic diameter of the purified protein cages was determined by measuring 1 mg/mL of the protein sample in a buffer (20 mM Tris pH 8.7, 5 mM EDTA, and 0.02% sodium azide) using dynamic light scattering (DLS) technique on a Zetasizer Nano ZS instrument (Malvern Instruments). The cage assembly

was further confirmed by transmission electron microscopy (TEM) (JEOL JEM-1400). Protein samples (0.1 mg/mL) were negatively stained for 3 min with 1.5% uranyl acetate on carbon-coated electron microscopy grids (Formvar-carbon film on 300 mesh copper grids, Electron Microscopy Science), and images were obtained with transmission electron microscope operating at 100 kV.

3.2.2. Emulsion formulation and characterization

3.2.2.1. Formulation of emulsion

Pickering emulsions were prepared by mixing rosemary oil and water at a certain volume ratio in a 15 mL clear screw top vial (Sigma-Aldrich). E2 solution (20 mM Tris pH 8.7, 5 mM EDTA, and 0.02% sodium azide) was added to the oil and water mixture drop by drop while the mixture was stirring on a magnetic stirrer. This process results in a heterogeneous coarse emulsion with large droplet diameter. Subsequently, the coarse emulsion was subjected to homogenization by ultrasonication[155-157] using a Vibracell cell disrupter (Model VC505, 500 W, 20 kHz) for 2 min at 40% amplitude. Energy input was given through stepped microtip 1/8 in. (630-0422, Sonics & Materials Inc.) containing a piezoelectric crystal with a probe diameter of 3 mm.

Optimization of E2-stabilized Pickering emulsions: The oil/water ratio and the mass fractions of the E2 emulsifier are the most important factors that affect the formation and stability of the Pickering emulsion. The minimum required an amount of E2 to acquire stable Pickering emulsion is determined by varying the relative amounts of the rosemary oil, water, and the mass fraction of the E2. The oil/water ratio was varied from 0.11 to 0.67 (v/v), and the mass fraction of E2 was varied from 0.05 to 0.35 (wt %).

Determination of emulsion stability index (ESI): A study of the kinetic stability of emulsion[158] was carried out by measuring the extent of gravitational phase separation. Following the Stokes law, the cream phase will be observed at a certain age of an emulsion of a certain droplet size because of the balance of drag and gravitational forces. For the measurement of kinetic stability, emulsions were formulated and stored in 15 mL clear screw top vials at room temperature for 10 days. ESI is defined as the percentage of the emulsion phase in the sample and was calculated using the following equation:

$$ESI = \frac{h_E}{h_T} \times 100\% \dots \dots \dots (3-1)$$

Where h_E is the height of the emulsion phase and h_T is the total height of the sample. The measurement was performed in triplicate and an average value was taken for analysis.

3.2.2.2. Characterization of emulsion

Type of emulsion (drop dilution test): The emulsion type (oil-in-water or water-in-oil) was determined qualitatively by drop dilution test. In the test, the miscibility of the Pickering emulsion with pure water and rosemary oil were checked qualitatively. A drop of the freshly formulated emulsion was mixed with a drop of Milli-Q water and rosemary oil separately on a microscope slide. Dilution in either phase determined the continuous phase of the emulsion. Miscibility of Pickering emulsion type was also confirmed by optical microscopy.

The hydrodynamic diameter of emulsion droplet: Hydrodynamic diameter of dispersed phase droplet of the emulsions was measured using DLS technique (Zetasizer Nano ZS, Malvern Instruments). Freshly formulated

emulsions were too turbid to be measured using the light scattering technique and resulted in the high probability of multiple scattering events. Hence, the emulsions were diluted 100 times with water to reduce the turbidity and to minimize the probability of multiple scattering. The effect of dilution on the droplet size was investigated by measuring the droplet size of a sample at different dilutions. The droplet size was consistent over a wide range of dilutions, which implies that diluting the samples before the measurements did not sacrifice the quality of the measurements. All measurements were made in triplicate, and the reported values represent the averages.

Measurement of surface coverage: The total mean interfacial area (S_o) in a given volume of emulsion was evaluated from the average droplet size and volume of oil emulsified using the following relationship-

$$S_o = \text{Surface area of droplet} \times \text{Number of droplet} \\ = S_d \times \frac{M_o}{V_d \times \rho_o} \dots \dots \dots (3-2)$$

Where, S_d is the surface area of one droplet considering the diameter of the dispersed phase oil droplet, M_o is the mass of emulsified oil, V_d is the volume of the droplet considering the diameter of the oil droplet, and ρ_o is the density of the rosemary oil at room temperature which is reported to be 903 g/dm³. The total area that the protein can cover (S_p) was estimated, assuming a monolayer of hexagonal closed packing of protein nanocages on oil-water interface, from the mass of the protein nanocage (m_p) used in the formulation of the emulsion.

$$S_p = \frac{\text{No. of cages} \times \text{Surface area can be covered by a single cage}}{\text{HCP packing factor}} \\ = \frac{\left[\frac{m_p \times N_A}{60 \times M_p} \times (\pi \times D_p^2) \right]}{0.907} \dots \dots \dots (3-3)$$

Where, N_A is the Avogadro's number, D_p is the diameter of the protein, and M_p is the molecular weight of the E2. For the estimation of the surface coverage, protein nanocages are modeled as hard spheres adsorbing onto a planner surface of the oil droplet. The surface coverage (Γ_p) is calculated as,

$$\Gamma_p = \frac{S_p}{S_o} \times 100\% \dots \dots \dots (3-4)$$

Measurement of the zeta potential: The zeta potential of the emulsion droplets was measured with Zetasizer Nano ZS (Malvern Instruments) using a disposable capillary cell DTS1070 (Malvern Instruments). Sample preparation technique was similar to the preparation for droplet size measurement. The sample was loaded into the capillary cell using a 1 mL syringe after inverting the cell to avoid the formation of air bubbles in the cell. The sample was analyzed at a scattering angle of 173°, and the effective electric field applied to the capillary cell was 150 V. Zeta potential measurements were carried out as a function of pH, ionic strength, storage time, and temperature. At least three measurements were performed for each sample.

Microstructure analysis of emulsion: The microstructure of liquid–liquid interface was examined using a confocal laser scanning microscope (Zeiss LSM 710 META) system with a 63× oil immersion objective lens. The oil phase was stained with Nile red while the E2-LC2 was visualized by conjugating AF488 to the interior. The dyes were excited by Argon ion laser at wavelengths of 514 and 488 nm. Samples were placed on a confocal microscope slide (25.4 × 76.2 mm; 1–1.2 mm thick; Sail Brand) and covered with a coverslip (24 × 50 × 0.17) mm; VFM Coverslips, Mochdre Enterprise Park), before imaging.

The microstructure of Pickering emulsion sample was also investigated by TEM microscopy. Samples were diluted 10 times before exposing to carbon grid to reduce the number of droplets in solution. The droplet of the emulsion was kept on the carbon grid for 5 minutes and dried it in the air. Subsequently, a droplet of 1.5% uranyl acetate was kept on to the carbon grid for another 5 minutes followed by air drying for few minutes. After drying the carbon grid was kept in a humidity controlled cabinet for at least 6 hours before imaging. All the micrographs were obtained with transmission electron microscope operating at 100 kV.

3.2.2.3. Stimulus-response of E2-stabilized Pickering emulsion

The E2-stabilized Pickering emulsion is investigated under different stimuli-responsive conditions to find out the optimum environmental condition to formulate and maintain the stable emulsion.

Ionic strength: Emulsions were prepared at different ionic strengths from 10 to 500 mM to investigate the effect of ion concentration on the stability of the E2-stabilized Pickering emulsion. The final volume of the emulsions was kept equal by adding Milli-Q water to the emulsion. Zeta potential of Pickering emulsions was measured at 2 and 10 days after the emulsion formation, and the droplet size of the dispersed phase was measured at days 2, 6, and 10. The samples were stored at room temperature.

Storage temperature: To investigate the thermal stability of E2-stabilized Pickering emulsion, the freshly formulated emulsions were stored at room temperature and in incubators at 37 and 50 °C. The stability of the emulsions was determined by measuring the zeta potential and droplet size of the dispersed phase. Zeta potential was measured within 1 h after preparation and at 10 days

from the formulation. Droplet size of the emulsions was measured at 2, 6, and 10 days from the formulation.

pH: All emulsions were prepared at neutral pH. Subsequently, the pH of the emulsions was tuned (~pH 2–11) by the addition of either HCl or NaOH. The stability of the emulsion was investigated by visual inspection and by measuring droplet size and zeta potential using DLS. The pH switchability of E2-stabilized Pickering emulsion was also investigated by changing the pH of the freshly prepared emulsion. After each cycle, the droplet size of the dispersed phase was measured by DLS. Measurements were performed within an hour of altering the pH of the emulsions.

3.2.2.4. Rheological analysis of emulsion

The rheological analysis was performed for E2-stabilized Pickering emulsions formulated using two different oil/water ratios of 0.11 and 0.66. Linear viscoelastic region (LVR) was determined by performing the oscillatory strain sweep test. All subsequent experiments were performed in the respective LVR region of each sample. All rheological measurements were performed in a controlled stress rheometer AR2000 (TA Instruments, Delaware, USA). A Peltier system at the bottom plate provided fast and accurate temperature control.

Analysis of flow behavior: Analysis of steady-state flow was conducted on the emulsion samples using a 20mm cone plate (cone angle: 0:59:53) geometry. The shear rate was applied from 0.1sec^{-1} to 500sec^{-1} . 10 points per decade were collected with 5% percentage of tolerance. Samples were deposited on the Peltier plate with a spatula in 10% excess which was trimmed after setting the gap to $1000\mu\text{m}$.

Oscillatory sweep test: The oscillatory rheological measurement was performed in the linear viscoelastic region to measure the storage modulus (G'), the loss modulus (G''), and the loss tangent ($\tan \delta$) by oscillatory frequency sweep tests, carried out for angular frequencies between 0.1 and 100 rad/s at a constant strain of 1%. 25mm parallel plate geometry was used to carry out the experiment.

Creep-recovery analysis: Creep-recovery test is a measurement of the response of samples in terms of percentage strain in application or relaxation of instantaneous stress and the strain increase over a certain period of time. A shear stress of 1Pa was applied to the sample for 60 seconds and the recovery period lasted for another 60 seconds.

Temperature ramp analysis: Long-term stability and the behavior of emulsion at different temperature were investigated by temperature ramp analysis. Firstly, the temperature was increased from 25–70°C for heating the Peltier plate with the heating rate 5°C per minutes. Subsequently, the temperature of Peltier plate was decreased to 25°C at the same rate. The percentage strain was constant at 0.01%. A single frequency of 1Hz was specified to ensure all conditions remain at equilibrium.

3.2.3. Integrity of protein structure after emulsification

The integrity of the cage-like structure of E2 upon adsorption on a liquid-liquid interface in the process of emulsification was investigated by analyzing the adsorption kinetics, adsorption layer properties and molecular structure of E2 at the liquid-liquid interface.

3.2.3.1. Measurement of adsorption kinetics

The investigation of adsorption kinetics of E2 on a liquid-liquid hydrophobic surface was performed by QCMD analysis which consists of two

steps. Firstly, the formation of the model hydrophobic surface on an AT-cut piezoelectric quartz crystals surface and secondly, the formation of an adsorbed layer of E2 on the hydrophobic surface while monitoring the frequency and dissipation shift due to adsorption in real-time.

3.2.3.1.1. Preparation of model hydrophobic surface

AT-cut piezoelectric quartz crystal of fundamental frequency 4.95Hz, coated with gold, was used to prepare a hydrophobic surface by 1-dodecanethiol modification. The sensor was cleaned with a highly oxidizing solution, known as “Piranha” solution (a mixture of 3:1 ratio of 98% sulfuric acid and 30% hydrogen peroxide), at high temperature $\sim 70^{\circ}\text{C}$ for 10 min followed by rinsing with hot Milli-Q water. Subsequently, the sensor was dried with nitrogen gas and placed in a UV/ozone (185nm and 254 nm) chamber (Bio-Force Nanosciences, USA) for another 10 min. UV/ozone-cleaned sensor was modified to form a self-assembled monolayer (SAM) with 2mM 1-dodecanethiol following the protocol reported by Lebec et al.[159] Briefly, the sensor was dipped in a solution of 2mM 1-dodecanethiol in ethanol for 16 hours while the solution was kept stirring on a magnetic stirrer. Subsequently, the sensors were rinsed with ethanol to remove any loosely bound molecules. The contact angle of the modified sensor surface was measured using the sessile drop technique in FTA 200 contact angle analyzer (First Ten Angstroms, VA, USA) to estimate the extent of hydrophobicity in terms of the contact angle of the water droplet.

3.2.3.1.2. Formation of adsorbed layer of E2

The adsorption of E2 on the SAM of 1-dodecanethiol was performed using a QCM-D system (Q-Sense E4 system, Q-Sense, Biolin Scientific, Sweden), connected to a peristaltic pump (ISMATEC® IPC High Precision

Multi-channel Dispenser, IDEX Health & Science, Germany). The baseline of the signal was obtained at the starting of each experiment with the solvent (100mM HEPES) in which protein was dissolved. A solution containing 10 mM E2 was fed to the hydrophobic sensor through the peristaltic pump at the flow rate of 50 μ l/min until the signal of frequency shift reaching a stable value. Unadsorbed or loosely bound protein cages were washed off by feeding another round of solvent (100mM HEPES) to the sensor surface and continued until frequency shift reaches to a stable value. All measurements were performed at room temperature.

3.2.3.2. Tertiary structural analysis upon adsorption

Tryptophan Fluorescence (TF) spectra were obtained using a spectrophotometer, Spectramax M5 (Molecular Devices, Sunnyvale, CA). The freshly prepared emulsion samples and protein solutions were taken into a quartz cell (Hellma GmbH & Co.) of 10mm path length. The excitation wavelength was set at 295 nm. This excitation wavelength was chosen over 280 nm, which is excitation wavelength of both tryptophan and tyrosine, to selectively excite the Trp residue only. The emission fluorescence spectra were collected in the wavelength range of 310–450 nm with 5nm step size and at a scan speed of 60nm/min. Each individual emission spectrum was the average of three independent readings and was obtained at room temperature.

3.2.3.3. Secondary structural analysis upon adsorption

All far-UV spectra of E2 in bulk solution (100mM HEPES) and E2-stabilized Pickering emulsion were obtained using Applied Photophysics circular dichroism spectrophotometer (Applied Photophysics Ltd, Leatherhead, UK). Quartz cell from Hellma GmbH& Co. was used to carry out the measurements.

The path length of the cell was 0.01cm. The baseline for protein in bulk solution was corrected by spectrum from bulk solvent (100mM HEPES) and the baseline for E2-stabilized Pickering emulsion was corrected by emulsion prepared with a sodium dodecyl sulfate (SDS) of same droplet size. All emulsions were centrifuged at 7378g and separated into cream phase from serum phase. As serum phase contains all the unadsorbed protein cages, the only cream phase was taken for measurement in CD. All emulsions were diluted 10 times before scanning in CD. The far-UV circular dichroism spectra were collected between 190nm and 260nm in 1 nm steps, with 1s averaging time and 3 scans for each sample. All spectra represent the average of all 3 scans from 3 different set of samples. Spectra were baseline subtracted, averaged and smoothed by using 5 points Savitzky-Golay[160] smoothing filter using the Chiralscan software (Applied Photophysics Ltd, Leatherhead, UK). The HT voltage was less than 1 kV over the range of wavelength to ensure the transmitted light level to the detector is adequate for accurate measurement. The spectra were converted to molar ellipticity (theta) by using following equation.

$$[\theta]_{molar\ ellipticity} = \frac{100 \times [\theta]_{deg}}{c \times l} \dots \dots \dots (3-5)$$

Where $[\theta]_{deg}$ is the circular dichroism in degree, c is the concentration of protein in molarity, l is the path length cell in cm. The proportion of each secondary structure components were estimated by deconvolution of each spectrum in K2D2, a web server to estimate secondary structure content of a protein. K2D2 is an artificial intelligence program used to find out correlations in data using self-organizing map (SOM) algorithm, a type of neural network.[161] It estimates the percentages of alpha helix and beta strand of a

protein from its far-UV CD spectrum where a range of working wavelength is 190-240 nm.[161, 162]

3.2.3.4. Molecular dynamics simulation of trimer-trimer interface

All simulations and analyses were performed using the GROMACS5.1.4 simulation package [163-165] with the GROMOS54a7 [166] force field (FF). The structure and coordinates of E2-WT were downloaded from the Protein Data Bank (PDB code: 1B5S). [167] To mimic the interactions between trimers in the assembled 60-mer protein nanocage, two monomers were initially clustered and solvated with ~70000 SPC waters in a periodic box of size 13 nm/side. To achieve neutrality, 4 Cl⁻ ions were added to the system. A temperature of 298 K and a pressure of 1 bar were maintained by applying the velocity-rescale thermostat [168] and the Parrinello-Rahman barostat [169] in an NPT ensemble. A real space cutoff of 1.2 nm was applied for Lennard-Jones and electrostatic forces with the inclusion of particle mesh Ewald summation for long-range electrostatics. [170] The LINCS algorithm was used to constrain the bond lengths [171, 172]. Simulations were performed for 200 ns with a time step of 2 fs on computational facilities supported by the National Institute of Supercomputing and Networking/Korea Institute of Science and Technology Information with supercomputing resources including technical support (KSC-2016-C3-036).

3.2.4. Formulation and digestion of functional food

E2-stabilised Pickering emulsion has been used to design a functional food for delivery of micronutrients. In the formulation of functional food, E2 protein cages were loaded with micronutrients e.g. minerals and mineralized E2 was used to stabilize Pickering emulsion. Subsequently, the Pickering emulsion

was digested in simulated gastric phase condition. The brief protocol is described in following sections.

3.2.4.1. Mineralization of E2

A genetically modified version of E2-WT renamed as E2-LFtn is reported to have an iron binding affinity in the inner cavity of the cage.[150] The details of this modification have been described earlier in Section 3.2.1. In this work, two different minerals e.g. iron and calcium have been encapsulated in E2-LFtn following the protocol after slight modification—described by Peng et al.[150] The stock solutions of both ferrous sulfate (200 mM) and calcium chloride (200 mM) were prepared freshly just before mineralization of E2. The stock solution of minerals was added step by step to the E2-LFtn solution (0.5 mg/ml) with 15 minutes gap between the steps. The solution was added dropwise to the protein solution while the solution was kept stirring on a revolver lab rotator. The loaded protein was incubated at room temperature for 1 hour followed by overnight (16hrs) incubation at 4°C. The loading of minerals was carried out when the E2 was dissolved in 20 mM Tris buffer (pH 8.7). The minerals that remain as unbound were separated by buffer exchange by desalting column (PD-10, GE Healthcare, for lower volume) or (Hiprep desalting 26/10, GE Healthcare, for higher volume). An analysis by inductively coupled plasma mass spectrometry (ICP-MS) was carried out to quantify encapsulated iron and calcium. The mineralized-E2 protein cages were characterized by TEM and DLS techniques to investigate the molecular assembly of cage structure.

3.2.4.1.1. Characterization of mineralized E2

A number of parameters have been described by I.J. Joye et al.[173] to characterize the mineralization efficiency. These parameters include

encapsulation capacity and encapsulation efficiency. These parameters were estimated to characterize the mineralization efficiency of E2. The maximum percentage of loaded minerals that can be incorporated into a protein cage is calculated as —

$$EC = 100 \times \frac{m_N}{m_P} \dots \dots \dots (3-6)$$

Here m_N is the mass of loaded cargo within E2 and m_P is the total mass of E2. The encapsulation efficiency was estimated as—

$$EE = 100 \times \frac{m_{N,E}}{m_{N,T}} \dots \dots \dots (3-7)$$

Here, $m_{N,E}$ is the amount of cargo encapsulated and $m_{N,T}$ is the total mass of cargo in the system to be loaded. The higher the value of these parameters ensures the efficiency of the delivery system. The EC and EE were estimated based the amount of loaded mineral—measured by ICP-MS—after separation of unbound minerals from the system.

3.2.4.2. Simulation of gastric phase digestion: *in-vitro* analysis

Simulation of gastric phase digestion of E2 protein nanocage and E2-stabilized emulsion were investigated by *in-vitro* analysis. The standardized static *in vitro* digestion method and GIT conditions are well described in an international consensus published by Minekus et al. in 2014.[174] A slightly modified protocol was followed in this work to investigate the gastric phase digestion profile of E2 and E2-stabilized emulsion.

Table 3-1: Composition of different ions in simulated gastric fluid (SGF) for final volume 500 mL

Components	Concentration		Volume used
	Molarity	mg/mL	
KCl	0.5	37.3	6.9
KH ₂ PO ₄	0.5	68	0.9
NaCl	1.5	87.66	11.8
MgCl ₂ (H ₂ O) ₆	0.15	30.5	0.4
(NH ₄) ₂ Cl	0.5	26.745	0.5
CaCl ₂ (H ₂ O) ₂	0.3	44.1	0.0025
Pepsin	91.4×10 ⁻⁶	3.2	-

3.2.4.2.1. Preparation of simulated gastric fluid (SGF)

To simulate the gastric phase digestion, a simulated gastric fluid (SGF) was prepared following the composition and protocol described by Minekus et al.[174] after a slight modification. The concentration of different ions in the gastric fluid is achieved by mixing the different salts in following composition—described in

Table 3-1. The pH of the solution was maintained at <3 by the addition of 1M HCl to replicate the gastric phase digestion conditions. A final stock solution of SGF always prepared freshly by dissolving pepsin (3.2 mg/mL) in 1 ml of the solution—containing the salts (pH <3).

3.2.4.2.2. Analysis of digestion kinetics

The digestion was carried out by mixing E2 (dissolve in 20mM Tris

buffer, pH 8.7) or E2-stabilized emulsion with SGF by 1:19 ratio. The mixture was kept stirring for 2 hours in an incubator at 37°C. A control experiment was carried out in parallel where E2 and E2-stabilized emulsion were mixed with SGF solution separately, prepared without pepsin. The size of E2 and droplet of the E2-stabilized emulsion was measured at different stages of digestion by quenching the activity of pepsin by adding sodium carbonate (0.2M).

The investigation of digestion kinetics was carried out by spectrophotometric analysis which was carried out in a 24 well plate adopting a modified digestion protocol. 1.9 mL of SGF was incubated at 37°C for at least 30 min before the experiments. 300 μ L Coomassie blue (CB) G-250 was added to the SGF followed by the addition of 100 μ L of E2 ([E2-LFtn] = 3.5mg/ml) and E2-stabilized emulsion in the reaction mixture. The absorbance of the protein-CB complex was measured at 595 nm on a SpectraMax M5 plate reader for 2 hours with 30 sec interval by keeping the chamber temperature constant at 37°C and by shaking the plate in every 30sec.

A standard curve was plotted showing the absorbance of E2-CB complex at a different concentration of E2. Similarly, another standard curve was plotted to show the absorption of the E2-stabilized emulsion-CB complex at a different dilution of the emulsion. Pepsin also has a specific interaction with CB. Therefore, the contribution of absorption from the pepsin-CB complex was subtracted from overall absorption. Finally, the amount of E2 digested and percentage of digestion were estimated by using the absorbance difference of E2-CB or E2-stabilized emulsion-CB complex before and during the digestion.

3.2.4.2.3. *Estimation of bioaccessibility*

Estimation of bioaccessibility was carried out by measuring the

concentration of released minerals in the digested chyme. The simulation of digestion in gastric phase, as described earlier, was followed by the separation of released minerals by flowing the digested chyme through PD-10 desalting column. The elution fractions from the desalting column were collected to estimate the concentration of released minerals by Induced Coupled Plasma Mass Spectrophotometry (ICP-MS). Finally, the bioaccessibility was estimated by the calculation of the percentage of minerals released in gastric phase digestion.

Chapter 4

Protein nanocage as a pH-switchable

Pickering emulsifier

4.1. Introduction

Encapsulation of active compounds in Pickering emulsions using bio-derived protein-based stabilizers holds potential for the development of novel formulations in the fields of foods and cosmetics. Most of the Pickering emulsifier from inorganic sources are not favored for application in food and pharmaceuticals industries because of their poor biocompatibility and biodegradability. Therefore, in recent years, stabilization of Pickering emulsion has been shifting from using inorganic particles to particles of biological origin.[7] However, the surface activity of Pickering emulsifiers from organic sources are susceptible to alter the environmental conditions such as pH, ionic strength, temperature, and storage condition. Moreover, the size and shape of the most reported organic emulsifier are not homogeneous. In this context, monodispersed bionanoparticles with high surface activity and biocompatibility are highly desirable. Therefore, in this chapter, we employ E2—a dodecahedron

This chapter is a modified version of the previously published work.

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hollow protein nanocage as a Pickering emulsifier.

The structural details of selecting E2 for this study is described in Section 2.5. Rosemary oil has been selected as oil phase for the formulation of Pickering emulsion. Rosemary oil, an essential oil, has been used as the lipophilic phase of the emulsion. Rosemary is an aromatic herb that belongs to the mint family, *Lamiaceae*. It has been used as a culinary herb and as an ingredient in personal care products for the treatment of congested respiratory tract problems[175] and arthritic pain reduction[176]. Rosemary oil has antibacterial [177, 178] and antiviral[179] properties.

In this chapter, the formulation of E2-stabilized emulsion has been established by optimizing energy input, compositions of rosemary oil, water, and E2 to develop a stable Pickering emulsion. The stability of resulting emulsion has been investigated at different pHs, ionic concentrations, and storage temperatures. This is the first detailed study of a nonviral E2 protein nanocage of biological origin used as a Pickering emulsifier.

4.2. Results and discussion

4.2.1. Overexpression, purification, and characterization

Site-directed mutagenesis of E2-WT results in the inclusion of two cysteine amino acids in each E2 protein subunit and introduces multiple conjugation sites in the inner cavity of the protein nanocage (**Figure 4-1A**). As the modified sites are on the internal surface of the protein core, it is expected that the external surface activity of the nanocages will remain unaltered.

Figure 4-1: (A) Three-fold crystallographic representation of E2-LC2, 25 nm in outer diameter with 12 openings, and each of the opening is 5 nm. Sites for conjugation to molecules are highlighted in red (aspartic acid, amino acid #381, D381) and blue (Glycine, amino acid #382, G382), (B) Transmission electron microscopy (TEM) image of dodecahedral hollow cage shape of the E2-LC2, (C) confocal microscopy fluorescence image of a coarse Pickering emulsion. The oil phase has been stained with Nile red and the E2-LC2 protein cages were conjugated with AF488 (emitting green light).

The purity of E2-LC2 is confirmed by performing SDS-PAGE analysis (**Figure A-2**). The theoretical molecular weight of E2-LC2 is 26.426 kDa.[180] A single band around 27 kDa on SDS-PAGE confirms the purity of E2-LC2 obtained from one of the flow-through fractions of ion-exchange chromatography (IEC). The hydrodynamic diameter of E2-LC2 is 25 nm with polydispersity index < 0.2 , consistent with previously published data for the wild-type E2 (E2-WT).[147] This result also suggests that the E2-LC2 properly self-assembles into a cage structure similar to that of the E2-WT. The assembly and the dodecahedral hollow shape of E2-LC2 are further confirmed by TEM (**Figure 4-1B**). The isoelectric point for E2-LC2 is determined to be 3.73 by measuring its zeta potential at different pH values (**Figure A-2B**).

The surface activity of E2-LC2 has been explored by mixing the protein with two immiscible liquids, water, and rosemary oil. **Figure 4-1C** shows a

scanning confocal fluorescence image of the resulting coarse Pickering emulsion, which confirms the presence of a distinct layer of AF488-conjugated protein nanocages (green) covering the surface of the rosemary oil droplets stained with Nile red (red). The zeta potential of the formulated emulsion droplets coated with E2-LC2 protein nanocages increased negatively to about -35 to -50 mV (depending on the formulation composition), while the zeta potential value of E2-LC2 dispersed in 20 mM Tris buffer (pH 8.7) is about -27 mV. The change in zeta potential may be explained by structural changes which may occur upon adsorption of E2-LC2 on the water/rosemary oil interface. Several studies have reported that globular proteins may deform at the water/oil interface.[53, 181, 182] As E2-LC2 is soluble and stable in aqueous condition and hence hydrophilic by nature, it may be expected to deform and undergo conformational changes that would expose hydrophobic patches on its surface to achieve the maximum favorable interaction with rosemary oil, discussed in details in the following chapter. Nonetheless, it is clear that the E2-LC2 adsorbs at the interface between water and rosemary oil and stabilizes the resulting Pickering emulsion.

4.2.2. Formulation of E2-stabilized Pickering emulsion

A Pickering emulsion was formed by homogenization of oil/water mixture by using ultrasonication.[156] The high pressure from the collapse of bubbles due to cavitation radiates shock wave to disturb the liquid in the neighborhood of a sonicating tip, causes the break-up of droplets and converts the coarse emulsion to micro- or nanoemulsion. The type of the resulting emulsion was determined by the drop dilution test. According to the Finkle's rule,[183] protective barrier around dispersed phase droplet is enhanced by particles that are preferentially wetted in the continuous phase of the emulsion.

In the current study, E2-LC2 is used as an emulsifier and according to drop dilution test, the formulated emulsions are oil-in-water emulsion as the emulsion is readily miscible with an aqueous phase (**Figure 4-2**).

4.2.3. Optimization of formulation composition

The composition of the formulation has been optimized to obtain stable Pickering emulsions by varying the rosemary oil/water ratio and the mass fraction of E2-LC2. To quantify the stability of the Pickering emulsions, emulsion stability index, ESI and the droplet size of the dispersed phase have been measured.

Figure 4-2: The visual representation of E2-LC2-stabilized Pickering emulsion shows (A) immiscibility of the emulsion with rosemary oil phase and (B) miscibility of the emulsion with the aqueous phase.

The effect of the mass fraction of E2-LC2 and the effect of rosemary oil/water ratio on the emulsion after 10 days of shelf life at ambient condition have been assessed qualitatively by visual inspection. Several emulsions experienced a creaming effect, i.e., full separation of the emulsion phase occurred on top of the serum (or aqueous) phase. The results of these experiments are shown in **Figure 4-3A** in terms of ESI of the Pickering emulsions of different compositions.

Figure 4-3: Effect of the oil/water ratio and the protein mass fraction on (A) the emulsion stability index (ESI) and (B) the droplet size for Pickering emulsions after 10 days of shelf life measured at ambient condition. Inset is the zoom-in of the area marked with a red rectangle.

Emulsions with oil fraction 0.1–0.2 (v/v) and E2-LC2 mass fraction 0.30–0.35 (wt %) showed higher stability with maximum ESI = 100. Lowering the E2-LC2 mass fraction to less than 0.3 (wt %) results in lower stability as evidenced by the separation of emulsion phase from the serum phase occurring within a few days. A sharp decrease of the emulsion stability occurs when the E2-LC2 mass fraction is kept constant at 0.3 and 0.35 (wt %) and the rosemary oil/water ratio increases. This destabilizing trend continues until the rosemary oil/water ratio reaches 0.35 (v/v). Beyond the oil/water ratio of 0.35, the stability of emulsion increases even though the rosemary oil/water ratio is increasing. We hypothesized that at this particular oil/water ratio, 0.35 (v/v), a gel structure forms which stabilizes the emulsion via network formation between the individual droplets. The formation of a network was subsequently analyzed by rheology.

The droplet size of the dispersed phase of the emulsion was measured at 10 days of shelf life for all emulsions with different rosemary oil/water ratios and mass fractions of E2-LC2, and the results are presented in **Figure 4-3B**. The visual images of Pickering emulsions at different protein mass fraction (PMF) (OWR= 0.11) and at different oil/water ratio (OWR) (PMF =0.35 wt %) are

shown in **Figure A-3** (Appendix-A) which shows the kinetic stability of Pickering emulsions after 10 days of storage period at ambient condition.

The droplet size increases as the oil/water ratio increases at a lower E2-LC2 mass fraction. When the amount of E2-LC2 in the formulation is low, the coalescence of oil droplets results in the larger size of droplet above 10 μm , while at a higher E2-LC2 mass fraction, the surface coverage of the droplet increases, which prohibits the droplets to coalesce (**Figure 4-4**). As a result, at a higher E2-LC2 mass fraction, the droplet size of the Pickering emulsions remains below 2 μm . The Pickering emulsion with the lowest droplet size of 200–400 nm is formed when the E2-LC2 mass fraction is 0.30–0.35 (wt %) and the oil/water ratio is between 0.10 and 0.20 (v/v). In this region, the droplet size of the dispersed oil phase resembles a nanoemulsion. At a higher E2-LC2 mass fraction and smaller oil fraction droplet, a higher surface coverage is achieved, which results in lower droplet size and ultimately in higher stability. The highest surface coverage achieved for stable Pickering emulsion is $\sim 30\%$ at oil/water ratio of 0.11 and protein mass fraction of 0.35 wt %. This could be the results of curvature effect and packing defects at nanoscopic oil/water interface. Roke et al.[52] showed that surface coverage by nanoparticles in the nanoscopic curved surface is much lower than the surface coverage on a planar surface. The curvature effect of the droplet also introduces defects in HCP packing of particles as reported by Binks et al.[53] Therefore, the calculated surface coverage value assuming a planar surface is much lower than that of full coverage. Similar calculation approaches also result in the surface coverage of 54 and 40% for silica particle[184] and sulfated cotton cellulose nanocrystal (CNC)[185], respectively, on oil/water interface. The concept has also been consolidated by Murray et

al.[54] that the surface coverage by active particle does not need to be completed to stabilize Pickering emulsion as long as the adsorbed layer forms a rigid network. We found that the emulsion with the rosemary oil/water ratio of 0.11 and the E2-LC2 mass fraction of 0.35 wt % shows the highest stability, with ESI values of 100 after 10 days of shelf life at room temperature. For this particular emulsion formulation, the droplet size was approximately 300 nm.

Figure 4-4: Surface coverage (%) of rosemary oil droplet by E2-LC2 protein nanocage at different mass fractions of E2-LC2 (wt %) at constant oil/water ratio 0.11. The error bar represents the standard deviation in measurement.

4.2.4. pH Switchability of E2-LC2-Stabilized Pickering Emulsion.

E2-stabilized Pickering emulsion can be destabilized by switching the pH of the freshly formulated emulsion from 8 ± 0.2 to 4 ± 0.2 . At lower pH values, the emulsion separates into emulsion and serum phases, which is mainly consists of water phase with unabsorbed protein molecules. Subsequent restoration of the pH to 8 ± 0.2 results in stable emulsion with the highest ESI (**Figure 4-5**, inset). The microstructure of these two states has been investigated by optical microscopy (**Figure A-5**). This behavior is repeatable for at least five cycles which illustrate the reversibility of the process and formation of a pH-switchable

E2-stabilized Pickering emulsion. It is to be noted that the droplet size distribution in the destabilized state of the emulsion was highly polydispersed and was immeasurable by DLS technique. Therefore, **Figure 4-5** presents only the size of droplets for the stabilized state of the emulsion at the completion of each cycle. After completion of several cycles, the Pickering emulsion could not be restored to its stable state even at pH 8. Similar observations have been reported by Ding et al. for Pickering emulsions stabilized by palygorskite particles[186] and by Li and Stover[187] for emulsions stabilized by small organic molecule with charged groups.[184] The pH-responsive behavior of the Pickering emulsion occurs mostly at pH's close to the isoelectric point of the emulsifier, which is determined to be 3.73 for E2 protein nanocage (**Figure A-2 B**). At the isoelectric point, the surface charge decreases; therefore, the hydrophobicity increases. As the droplet surface is being covered by adsorbed protein nanocages, the surface charge of oil droplet also follows the trend of the surface charge of protein showed in **Figure 4-6A**. Therefore, lowering the amount of the surface charge results in a reduction of the electrostatic repulsion forces between oil droplets which trigger extensive flocculation and coagulation. The proposed mechanism for the irreversibility, after several cycles, is that the droplets may also coalesce in addition to wide-spread flocculation. The coalesced droplets require energy input to regain their initial smaller diameters. Since there is no energy input during the restoration of pH, as the cycle is repeated more droplets coalesce, and at a certain point, the emulsion is irreversibly separated. The optical microscopic images are shown in **Figure A-5** (Appendix-A) reveals the flocculation of Pickering emulsion at pH 4. However, at pH 8 the droplets remained dispersed in water and regained the stability. The irreversibility may

also result from the continuous increase of the ionic strength after each cycle because of the addition of HCl and NaOH to tune the pH values. Therefore, we performed a detailed characterization of the emulsions at different pHs and ionic concentrations.

Figure 4-5: Droplet size at pH 4 at the end of each destabilization and stabilization cycle. The droplet size is an average of at least 6 experiments. The error bar represents the standard error in measurement.

4.2.5. Effect of pH, ionic strength, and storage temperature on the stability of Pickering emulsion.

Surface charge is vital for the stability of the Pickering emulsion.[15] As the surface charge is susceptible to change when the pH changes, the stability of Pickering emulsion stabilized by E2 may be considerably affected. In the current experiment, the effect of pH on the stability of E2-stabilized Pickering emulsion (rosemary oil/water ratio 0.11 (v/v) and mass fraction of E2, 0.35 (wt %)) was investigated by varying the pH from 2 to 11 while keeping other variables, i.e. salt concentration and storage temperature, constant.

Qualitative analysis performed by visual inspection of the emulsions at different pHs suggests that Pickering emulsion is very stable at higher pHs,

neutral to the basic range, at pH ~ 4 the Pickering emulsion becomes unstable. The visual appearance of Pickering emulsions at different pHs is shown in **Figure A-4**. (Appendix-A) Our initial qualitative assessment was confirmed quantitatively by measuring the zeta potential and droplet size of freshly formulated Pickering emulsions at various pHs (**Figure 4-6A**). The absolute value of the zeta potential was less than 30 mV at pH values in the acidic range, while at pH > 7 , the absolute zeta potential value was higher than 30 mV. The similar increasing trend was observed in the measurement of the absolute value of zeta potential of E2 in the 20 mM Tris buffer solution (**Figure A-2B**). It can be concluded that E2 adsorbed at the interface controls the surface charge of the emulsion droplets. This result also agrees with the qualitative assessment. The droplet size continues to decrease with increasing pH. However, an increase in droplet size was observed at pH > 9.5 , and the Pickering emulsion was stable with a zeta potential value > 30 mV. The lowest droplet size of ~ 280 nm was observed at pH 9. The droplet size of the dispersed rosemary oil phase as well as the zeta potential was measured at different shelf life to determine the effect of the ionic concentration on the stability of the E2-stabilized Pickering emulsion.

Figure 4-6: Effect of pH on (A) zeta potential and (B) droplet size of the dispersed phase of a freshly prepared Pickering emulsion with 0.35% protein

mass fraction and oil/water ratio of 0.11. The error bar represents the standard error in measurement.

Figure 4-7A shows the dependence of the zeta potential on the ionic concentrations at a different aging time. The zeta potential of Pickering emulsions was decreased with the increase of the ionic concentration measured at different storage times. This phenomenon can be explained by the effect of salt screening. The increase of ionic concentration results in the increase of the concentration of counter ions in the colloidal solution. The counter ions immediately neutralize the oppositely charged species on the droplet surface, which leads to the reduction of the electrostatic double layer thickness. This phenomenon has been explained in the theory of electric double-layer compression with the increase of ionic strength.[188]

The characteristic thickness of a double layer is called the Debye length, k^{-1} , which is reciprocally proportional to the square root of the ion concentration. As the thickness of the double layer decreases with the ionic strength, the zeta potential of the Pickering emulsions also decreases with the increase of ionic concentration. The inset in **Figure 4-7A** depicts slight separation of the emulsion phase from the serum phase at an ionic concentration of 250 mM, while the emulsions with ionic concentrations of 25 and 50 mM showed no separation. The dispersed phase droplet size of E2-stabilized Pickering emulsions of different ionic concentrations was measured at different storage periods of 2, 6, and 10 days (**Figure 4-7B**). It is observed that the droplet sizes of the emulsions increase with the increase of ionic strength.

These results suggest that the pH and ionic strength play an important role in stabilizing Pickering emulsions by modulating the surface charge of E2 as well as the resulting droplets. A pH value close to the isoelectric point, the net surface

charge of the protein decreases which results in the decrease of interparticle electrostatic repulsion. The surface charge of emulsion droplets also follows the behavior of the surface charge of E2. Therefore, the inter-droplet electrostatic repulsion will be reduced. This phenomenon also occurs when salt is introduced into the emulsion. The salt ions reduce the surface charge by making a bond with counterions on the surface of E2. Therefore, the decrease in pH or increase of ionic concentration results in the reduction of inter-droplet electrostatic repulsion which results in the reduction of stability of the emulsions by flocculation leading to phase separation.¹⁸ At $\text{pH} \geq 7$ and ionic strength less than 250 mM, emulsions with the lowest droplet sizes are formed and have the highest stability index value.^[53, 189, 190]

Figure 4-7: (A, B) Effect of ionic concentrations on zeta potential and droplet size of Pickering emulsion at different storage periods, (C, D) Effect of storage temperature on zeta potential and droplet size of Pickering emulsion at different storage periods. Pickering emulsion with 0.35% E2-LC2 mass fraction and rosemary oil/water ratio 0.11 at pH 8.7 was used to conduct this investigation. Inset in A is the image of the samples at day 2. The error bar represents the standard error in measurement.

Figure 4-7C shows that despite a minute reduction, the absolute value of the zeta potential is greater than 30 mV at all temperatures, suggesting that the Pickering emulsion is stable for 10 days when incubated at temperatures up to 50 °C. At 25 and 50 °C, the droplet sizes were similar. The slight increase in the droplet size at 37 °C may have resulted from the reduction of the zeta potential as well as from the reduction of the surface charge. However, such reduction is not expected to affect the stability of the emulsion.

4.2.6. Rheological analysis of emulsion and gel-like structure

During the optimization of the emulsion formulation, we have hypothesized the formation of emulsion gel-like structure beyond a certain oil/water ratio. These emulsions, with a higher oil/water ratio, start to form gel-like structures and are stabilized by network formation. We have confirmed this hypothesis by comparing the rheological properties of emulsions prepared with different oil/water ratios of 0.11 and 0.66. The linear viscoelastic region had been determined before conducting the oscillatory strain sweep test (data not shown).

4.2.6.1. Flow behavior analysis

Shear stress versus shear rate curves from a flow analysis of emulsion and gel-like samples are illustrated in **Figure 4-8**. Shear stress versus shear rate curve has been fitted with power law equation as follows:

$$\sigma = k\gamma^n \dots \dots \dots (4-1)$$

Where σ is Shear stress (Pa), γ is the shear rate (s^{-1}), k is consistency index, and n is power law index. The value of n reflects the behavior of the system. If $n < 1$, then the system shows shear thinning behavior. The system has shear thickening behavior if $n > 1$, and Newtonian behavior when $n = 1$.

Figure 4-8: Rheological analysis of (A) emulsion oil/water ratio = 0.11 and (B) emulsion with the gel-like structure (oil/water ratio = 0.66) by flow analysis plotted as shear stress vs shear rate. owr = oil/water ratio.

The value of power law index, n , was calculated from both shear stress versus shear rate (**Figure 4-8 A-B**) and viscosity versus shear rate curve (

Figure A-6) for emulsions with different oil/water ratio. The values of power law index, n , were found to be 0.36 and 0.98 for emulsions with oil/water ratio = 0.11 and 0.66, respectively. Emulsion with low oil/water ratio is showing Newtonian behavior as the value of power law index is very close to 1, while emulsion having a high percentage of oil depicts shear thinning behavior as the value of power law index is less than 1 and close to zero. Shear thinning behavior in emulsion indicates the presence of weak attractive forces between the emulsion droplets, which give rise to the formation of weak emulsion gel-like structure in emulsion with higher oil/ water ratio.[191] A resistance to flow is provided by the network and arises from weak interaction forces. If the shear stress is lower than the attractive forces between droplets, then the shear energy will be stored

as an extension of bonds between dispersed phase droplets. This phenomenon increases the resistance of the system. As a result, the system will not flow, and it will show elasticity. When the stress becomes larger than the resistance forces, the system starts to flow. The application of stress causes the droplets to move from each other.

4.2.6.2. Oscillatory sweep test

Oscillatory rheological measurements of elastic or storage modulus (G') and viscous or loss modulus (G'') can indicate the mechanical strength of the emulsion system. Values of the phase angle shift δ can also provide information about the nature of the viscoelastic response of the emulsion system. In elastic structures, δ is 0° , and in purely viscous liquids, δ is 90° . The closer δ is to 0° , the more elasticity or elastic behavior will be shown by the emulsion system to the application of the shear stress. Thus, the more developed gel-like colloidal structure will be formed.

Figure 4-9: Frequency sweep analysis plotted as loss and storage modulus vs angular frequency; (A) with oil/water ratio (v/v) = 0.11 and (B) with oil/water ratio (v/v) = 0.66.

The magnitude of elastic modulus (G') is higher than viscous modulus

(G''), and both are independent of the frequency of the emulsion formulated with high rosemary oil/water ratio, 0.66 (**Figure 4-9**). This behavior of viscous and elastic modulus is a clear indication of network formation[191]. The phase angle value was also independent of the frequency range and remained constant for a wide range of frequencies, which indicates that the emulsion gel-like structure tends to show an elastic response to shear. However, the emulsion formulated at low rosemary oil/water ratio, 0.11, shows the complex behavior of elastic and viscous moduli, and the values are not independent over the range of frequencies. Thus, the emulsion formulated with low rosemary oil/water ratio has none or very little network formation between dispersed droplets.

Figure 4-10: (A) Flow behavior and (B) oscillatory sweep test of gel-like Pickering emulsion formulated with different protein mass fractions (PMFs) (Oil/water ratio = 0.66).

The formulation of gel-like was further optimized by using different protein mass fractions (PMFs) to determine the gelation conditions. Flow behavior and oscillatory frequency analysis have been performed to investigate the network formation of gel-like Pickering emulsion. Gel-like emulsions at

oil/water ratio 0.66 show shear-thinning behavior as discussed earlier (**Figure 4-9A**). However, the formation of gel-like structure or formation of a network between emulsion droplets was not observed instantly. An oscillatory sweep test (**Figure 4-10B**) confirms the formation of the gel-like structure at higher protein mass fraction. The formulation of the gel-like structure is observed with a minimum of ~0.30 wt% as indicated by the higher storage modulus compared to the loss modulus and the values are independent of angular frequency. The emulsions, formulated with 0.20–25 wt%, also show similar trends in dynamic modulus in the lower angular frequency range. However, the storage and loss modulus crossed over at higher angular frequency range, showing the low strength of the emulsions. We hypothesized that, at these low PMFs, emulsions have low surface coverage and started to form a network between the droplets which are not strong enough to bear high frequencies. The emulsions with minimum PMF of 0.3 wt% showed higher stability in increased frequencies as well.

4.2.6.3. Temperature ramp test

Long-term stability is a critical assessment to establish the potential of the emulsions in designing cosmetic or pharmaceuticals products. [192] The assessments are often done in a shorter period of time by accelerating the aging process. The most widely used standard procedure is temperature ramp where the dynamic modulus is measured at a fixed temperature—usually 40°C—over a period of time.[193] Recent studies showed that the dynamic temperature ramp can be considered a valid method to simulated forced aging test for cosmetic emulsions. [192]

A temperature ramp test has been performed to investigate the long-term

stability of gel-like emulsion stabilized by E2. The results showed the general trends of dynamic modulus of emulsions with different PMFs during heating and cooling cycles. **Figure 4-11** shows the growing strength of emulsion sample with heating as the dynamic modulus was increasing with temperature. The observed sharp increase of dynamic modulus at a higher temperature may result from the drying up of the continuous phase of the gel-like emulsion. The drying up of emulsion with age increases the dynamic modulus which is irreversible during the cooling process. The dynamic modulus remains unchanged during the cooling period. The gel-like emulsion formulated with PMF 0.30wt% shows the maximum stability with temperature ramping or aging. The sharp increase of dynamic modulus has occurred at low temperature as 20–30°C for emulsion with PMF 0.02–0.25wt% where the emulsion formulated with PMF 0.30% shows the stability up to ~50°C. From the result, it can be hypothesized that, the emulsion with higher PMF will show enhanced stability in long term storage. Therefore, the E2-Stabilized Pickering emulsion will be a good candidate for formulation of cosmetic and food product.

Figure 4-11: Temperature ramp analysis of gel-like Pickering emulsion formulated with different protein mass fractions; (A) PMF = 0.2 wt%, (B) PMF = 0.25%, and (C) PMF = 0.3% (oil/water ratio = 0.66).

4.2.6.4. Creep-recovery analysis

Creep-recovery test—a basic rheology analysis performed to investigate the viscoelasticity of gel-like emulsions. [194] Creep occurs when the strain of material increases over time after the application of an instantaneous stress. Recovery is the process of the decreasing strain due to subsequent removal of stress. [195] Two parameters can be extracted from the creep-recovery test—the maximum creep and the relative recovery. [196] The maximum creep is the peak strain obtained at the end of the creep procedure and the relative recovery is a percentage of strain recovered at the end of the recovery stage as compared to the peak strain. Lower peak strain indicates a harder structure with lower flow ability while a lower relative recovery is associated with a lower elasticity in structure. [197] **Figure 4-12** shows the results of creep-recovery analysis—performed on emulsion formulated with different PMFs. The highest relative recovery is observed for emulsion with PMF 0.2 wt% which is 7.9% where for emulsions with PMF 0.25–0.3 wt%, relative recovery is lower after 10 days of storage time. Lower recovery indicates that the gel-like emulsions were deformed to some extent during stress and the deformation is permanent. The emulsions with higher PMF shows less viscoelasticity. Lower viscoelasticity is preferred for the formulation of pharmaceutical and cosmetic products for topical application. [198] However, this study will be extended further to find time-temperature superposition to predict long-term properties using short-duration tests. By creep test data in one or two months, creep behavior after some years can be predicted with acceptable accuracy.

Figure 4-12: Creep-recovery analysis of gel-like emulsion formulated with different PMF and constant oil/water ratio 0.66.

4.3. Conclusion

E2 protein nanocage is a surface-active nanoparticle, which can stabilize a Pickering emulsion by adsorbing at the interface of two immiscible liquid phases, such as rosemary oil and water. The optimal composition of E2-LC2-stabilized Pickering emulsion is determined to be rosemary oil/water ratio 0.11 (v/v) with protein mass fraction 0.35 (wt %). The emulsion shows excellent stability in neutral to basic pH's, ionic concentrations up to 250 mM, and storage temperatures up to 50 °C. The optimized Pickering emulsion is a pH-switchable colloidal system (at pH's from 4 ± 0.2 to 8 ± 0.2), and the process is reversible for at least five cycles. At a higher rosemary oil/water ratio, the emulsion forms a gel-like structure showing viscoelastic properties.

Chapter 5

Structural integrity of E2 protein nanocage at liquid-liquid interface

5.1. Introduction

Amphiphilic proteins, containing both hydrophilic and hydrophobic residues, are highly surface active molecule which adsorbs on the liquid-liquid interface and stabilizes emulsion [181, 199]. The adsorption of proteins at the interface of two immiscible liquid phases is associated with the change of potential and free energy landscape of its native structure [200]. The adsorbed protein molecules undergo conformational changes to ensure that both hydrophobic and hydrophilic patches are in favorable conditions in the process of adsorption. Since protein functionality is closely related to its three-dimensional structure [201], the change of conformation during an adsorption process often leads to compromised functionality. Therefore, the investigation of the extent of conformational changes or structural integrity of protein is of utmost importance in any condition.

In recent years, extensive studies have been carried out to understand the mechanism and kinetics of adsorption of the globular proteins on interfaces [202-205]. Zhai et al. investigated the conformational changes of globular proteins, α -lactoglobulin [206] and β -lactoglobulin [207, 208], at different interfaces of different hydrophobicities. The structure of β -casein [209], whey protein isolate

[210] as well as peptides [203] and antibodies [204], adsorbed on the different hydrophobic interface, have also been studied recently. Although a significant number of studies have been carried out on globular protein, there is no extensive study on the adsorption kinetics and the molecular assembly of any cage-like protein at the interface.

In Chapter 4, we have demonstrated, E2 protein nanocage as the first non-viral protein nanocage as a Pickering emulsifier [211]. E2 protein is part of the pyruvate dehydrogenase multienzyme complex found in thermophilic bacterium *Geobacillus stearothermophilus* [26] which has very well-defined size and shape in bulk solution. In this chapter, we report an investigation on the extent of conformational changes of E2 protein nanocage adsorbed at the oil/water interface. A genetically modified version of E2 protein cage, referred to as E2-LFtn, has been used in this part of the study. E2-LFtn has unique cargo loading capacity that will be useful for its future application as a nanocarrier of foreign molecules.

Adsorption kinetics and properties of the adsorbed layer of E2 have been studied by quartz crystal microbalance and dissipation (QCMD) technique on the model hydrophobic surface. The thickness of adsorbed protein layer has been estimated to predict the molecular assembly of adsorbed protein at the interface by using Kelvin-Voigt model [212]. To investigate the molecular assembly of E2 at the liquid-liquid interface, tryptophan fluorescence (TF) and circular dichroism (CD) analyses have been performed. The fluorescence of tryptophan (Trp) is sensitive to the polarity of its local environment [213, 214]. Therefore, TF has been exploited in studying protein folding/unfolding and the tertiary structure extensively [209, 215]. Similarly, CD has also been used as a basic

characterization tool in studying the structure of globular proteins adsorbed at the interface [207, 216]. To visualize the structure of E2 at the interfacial conditions, the microstructure of the oil/water interface has been investigated by transmission electron microscopy (TEM). Alongside investigation of a structural trace of E2 adsorbed on a liquid-liquid interface, we have studied the structural information of E2 under different stimuli (e.g. pH and temperature) and in the presence of the denaturing agent (e.g. GuHCl). Subsequently, the structural information under the different conditions is compared with the adsorbed E2 at the interface to understand the extent of conformational changes. Finally, an experimental investigation using molecular dynamic simulation has been carried out to identify the active forces that stabilize the cage structure of E2 in the bulk solution and at the interface. Subsequently, the total active stabilizing force has been compared with tangential surface force to determine the structural integrity.

This is the first study on the adsorption kinetics and molecular structure of any cage-like protein, adsorbed on the liquid-liquid interface. This study will provide an insight into the properties of the adsorbed layer of protein nanocages on the hydrophobic interface and the integrity of molecular assembly at the liquid-liquid interface. The understanding of the structural integrity of E2 upon adsorption on a hydrophobic surface can be applied in designing delivery platform in the formulation of functional food, nanomedicines, and personal care products.

5.2. Results and discussions

Purified E2 protein nanocage was characterized by investigating size, shape and molecular weight. **Figure 5-1A** shows the 5-fold crystallographic structure of E2. The correct assembly of purified E2 is studied by measuring

volume-based particle size distribution shown in **Figure 5-1B**. The hydrodynamic diameter of the nanocage is ~ 26 nm with a polydispersity index (PDI) 0.230 which is similar to the previously reported value[150]. The assembled protein cages are homogeneous as shown by TEM image (**Figure 5-1C**)

Figure 5-1: (A) 5-fold crystallographic representation of E2-WT protein nanocage where modification sites are marked as red; (B) Volume-based particle size distribution of purified E2-LFtn; (C) Transmission electron microscopy (TEM) image of dodecahedral hollow cage structure of the E2-LFtn (Inset: box chart shows the average size of E2 Protein nanocage)

5.2.1. Adsorption kinetics of E2 at hydrophobic surface

To investigate the adsorption kinetics and thickness of the adsorbed layer of E2 on a hydrophobic surface, quartz crystal microbalance and dissipation technique has been applied. A self-assembled monolayer of 1-dodecanethiol has been formed on an AT-cut quartz crystal sensor to prepare a hydrophobic surface which will partially mimic the interface of an oil droplet in the emulsion. The contact angle of water droplet on the 1-dodecanethiol-modified AT-cut quartz crystal sensors is measured to be $106 \pm 1^\circ$ which reflects the hydrophobicity of the sensor surface. The bare crystal surface, without modification, is hydrophilic in nature upon which the contact angle of the water droplet is measured to be $70 \pm 2^\circ$

(Figure B-3).

E2 protein cages are adsorbed on the model hydrophobic surface of quartz crystals while the real-time frequency and dissipation shift are monitored as a function of time. **Figure 5-2 A** represents the frequency and dissipation shift of quartz crystal surface with the adsorption of E2 on the hydrophobic surface. Upon introduction of E2 to the sensor, a rapid drop of the frequency signal is observed while the dissipation increases in parallel. The shift of frequency and dissipation signal confirm the adsorption of E2 on the hydrophobic surface. The rate of adsorption reduces with time as the surface becomes saturated. The adsorption continues until an equilibrium occurred between adsorption and desorption of protein which is represented by the plateau in the frequency and dissipation shift signal. The loosely bound or unbound cages were washed off by flowing solvent over the sensor surface. The corresponding increase in the frequency signal and a decrease in dissipation signal ensure the complete removal of unbound from the sensor surface. The remaining cages were irreversibly bound to the hydrophobic quartz surface that forms a layer on the crystal surface.

Figure 5-2: (A) Frequency and dissipation shift versus time at 3rd overtone for the adsorption of E2-LFtn on the 1-dodecanethiol-modified hydrophobic quartz

crystal surface; (B) The Kevin-Voigt thickness of the adsorbed layer of E2-LFtn protein nanocage.

A large dissipation shift ($\Delta D > 2.5 \times 10^{-6}$) with the adsorption of E2-LFtn at 3rd overtone is observed (**Figure 5-2A**) alongside frequency shift during the adsorption of E2 on the crystal surface. The energy dissipation (ΔD) can be described as the ratio of energy loss and energy stored in the adsorbed layer during oscillation which represents the viscoelastic properties of the adsorbed layer. The normalized dissipation shifts at different overtones e.g. 3rd, 5th and 7th (**Figure B-4**) do not overlap with each other which indicates the viscoelastic properties of the adsorbed film [217]. For rigid layer where the loss of energy is not significant, the Sauerbrey model is commonly applied to describe the linear relationship between frequency shift and adsorbed mass. However, many polymer/biomolecule entraps water between molecules leading to damping of crystal oscillation. As the damping of the energy increases, the Sauerbrey model is no longer remain valid [218]. A more complex mathematical model, Kevin-Voigt model describes the propagation and damping of acoustic waves in a single undifferentiated viscoelastic adsorbed film in contact with a Newtonian bulk liquid[212]. The frequency and dissipation shift data are fitted with a Kevin-Voigt model to estimate the thickness of the E2 layer, adsorbed on sensor surface (**Figure 5-2B**). The thickness is estimated to be 26 nm which is equal to the diameter of E2 (**Figure 5-1B**) suggesting that the adsorbed E2 retains its cage-like structure.

5.2.2. Tertiary structure analysis of adsorbed E2

Tryptophan shows different quantum yield depending on solvent hydrophobicity. In a hydrophobic environment or less polar solvent tryptophan

shows high quantum yield resulting in high fluorescence intensity. On the contrary, in the hydrophilic environment or polar solvent the quantum yield of tryptophan decrease which leads to lower fluorescence. Alongside the intensity, large stoke shift can occur depending on the solvent property. Therefore, a shift in maximum emission wavelength will be observed. Exposure to the hydrophilic environment will show red-shift and exposure to the hydrophobic environment will show blue-shift in maximum emission wavelength.

E2-Lftn protein has one Trp residue (W355) in each subunit which makes total 60 residues per nanocage. All these residues are buried inside the cage structure (**Figure B-5**). Therefore, the residues are not solvent accessible in native condition. Denaturation of the native structure by any imposed condition will expose the Trp residues to a hydrophilic environment results in a red-shift of the maximum emission wavelength. Similarly, conformational rearrangement at the oil/water interface and is expected to expose the Trp residue either in hydrophilic or in a hydrophobic environment and shift the position of the maximum fluorescence wavelength in either direction.

The fluorescence spectra of E2-LFtn in 100 mM HEPES buffer solution in ambient condition and E2-LFtn adsorbed on the oil/water interface are illustrated in Figure 5 3 in native condition and different denaturing conditions, such as temperature, pH, and in the presence of GuHCl. A red-shift of λ_{max} has been observed at a higher temperature, lower pHs and in the presence of GuHCl which are the well-known denaturation conditions of E2. Red-shifting of λ_{max} indicates the exposure of Trp residues to more hydrophilic environment and unfolding of the native tertiary structure. The results are consistent with previous reports on little or no stability of E2 in acidic conditions (<4), at high temperature

(>90°C), and in the presence of GuHCl (>1.5mM). [219, 220]

Figure 5-3: Shift of emission spectra of tryptophan fluorescence at different (A) incubation temperatures, (B) pHs, and (C) concentrations of GuHCl.

In native condition (i.e. at room temperature, at pH 8.7, and in the absence of GuHCl), E2-LFtn shows a maximum fluorescence, λ_{max} , at 340nm (**Figure 5-4**). No obvious shift of λ_{max} has been observed for the E2-LFtn adsorbed on the oil/water interface which indicates the Trp residues are not being exposed to either hydrophilic or hydrophobic environment after adsorption at the interface.

Figure 5-4: Comparison of tryptophan emission spectrum of protein nanocage before and after adsorption at the oil/water interface.

The absence of blue-shift of λ_{max} for E2-LFtn either in denaturation conditions or at the interface is similar to previous reports on several globular proteins.[215] The intensity of fluorescence is not comparable because of the turbidity of the emulsion sample. The incident and emitted electromagnetic waves interact with the emulsion droplet and scatter extensively which changes the intensity of radiation reaching the fluorescence detector.[214]

5.2.3. Secondary structural analysis of adsorbed E2

Circular dichroism is becoming an increasingly distinguished technique and an excellent tool to estimate the secondary structural contents of proteins and peptides. In this study, CD has been used as the primary detection technique to determine the secondary structure content of E2 at different denaturation conditions as mentioned in the previous Section (**Figure 5-5**). Subsequently, the secondary structural content in denaturation pathway is compared with the E2-LFtn adsorbed at oil/water interface.

The temperature-induced denaturation (**Figure 5-5A**) of E2-LFtn is associated with the reduction of both negatives (at 208nm and 222nm) and positive (at 190nm) ellipticity. Reduction of α -helicity and the increase of β -sheet content at high-temperature ($>85^{\circ}\text{C}$) can be postulated from the reduction of ellipticity. A similar trend has been observed for pH-induced (**Figure 5-5B**) and GuHCl-induced (**Figure 5-5C**) denaturation of E2-LFtn. The reduction of α -helicity of the native structure during denaturation in different conditions indicates the structural arrangement of E2. These findings are consistent with the tryptophan fluorescence analysis.

Figure 5-5: Far-UV CD spectra of E2-LFtn at different (A) incubation temperatures, (B) pHs, and (C) concentrations of GuHCl. The secondary structural proportion of E2-LFtn at different (D) incubation temperatures, (E) pHs, and (F) concentrations of GuHCl.

Figure 5-6 illustrates a comparison between far-UV CD spectrum of E2-LFtn in 100 mM HEPES and E2-LFtn after adsorption at oil/water interface. Both spectra show a positive ellipticity at 193 nm and a negative ellipticity at 210 nm. However, the second negative peak has shifted from 221 nm for E2-LFtn in solution to 224 nm for E2-LFtn adsorbed at the oil/water interface. The relative intensity has increased in each peak position in terms of molar ellipticity which could have resulted from the polarity of a hydrophobic surface on which the nanocages are adsorbed.[219] The shift of the negative peak of E2-LFtn after adsorption reflects in the secondary structure analysis which shows the significant increase in α -helix proportion and decrease in β -strand proportion, displayed in **Table 5-1**. The relative proportions of the secondary structure of E2-

LFtn in solution are measured to be 40.18% α -helix and 10.65% β -strand which is comparable to the previously reported value by Peng et al.[220] The secondary structural content of E2-LFtn adsorbed at the oil/water interface is measured to be 56.3% α -helix and 5.7% β -strand.

Figure 5-6: Comparison of far-UV CD spectra of E2-LFtn in solution (100mM HEPES) and of adsorbed E2-LFtn on oil/water interface.

The domination of α -helicity in protein structure after adsorbing on the oil/water interface has been reported previously in several studies.[202, 221] Zhai et al. reported that the conformational rearrangement of α -lactoglobulin, β -lactoglobulin, β -casein, and deamidated wheat gliadins at oil/water interface result in the increase of α -helix content in structure.[202] Day et al. reported the decrease of α -helical content and increase in β -sheet in the secondary structure of adsorbed lysozyme, bovine serum albumin, and myoglobin on different oil/water interface[215]. The dominance of α -helical content was observed in overall secondary structure content of these proteins as well. Consequently, Zhai et al.[202] hypothesized that α -helix is the preferred secondary structure at the

interfacial condition. Both Zhai et al. and Day et al. analyzed the secondary content by using CD as a primary detection technique. On the contrary, a few studies have been performed using Fourier Transform Infrared spectroscopy (FTIR) which illustrates the β -sheet as a preferred secondary structural content at the oil/water interface for α -lactoglobulin and β -lactoglobulin. Zhai et al. argued that the reason for these contradictory findings could be the experimental techniques used to study the secondary structure of proteins. [202] Circular dichroism and Fourier transform IR spectroscopy have limitations in sensitivity and experiment conditions.[202]

Levinthal et al. discussed the conformational changes of the protein upon adsorption at the interface where it was mentioned that the conformational changes of protein at the interface is driven by kinetic rather than thermodynamics which is also known as Levinthal paradox. [222] Matsumura et al. confirmed the paradox by proving that the conformation of α -lactoglobulin at the interface is different in different conditions. [223] Levinthal et al. argued that tendency of protein structure to adopt more α -helix structure than β -sheet at the interface upon adsorption as the α -helix is the simple to form among these two amphiphilic moieties. A similar trend of conformation has been observing in transmembrane proteins which have a very high α -helical content in the transmembrane domain. [224] Several simulation-based studies reported the formation of α -helix structure of unstructured peptide upon adsorption on a lipid membrane. [225, 226] As the ratio of ellipticity at 222nm and 208nm, $[\theta_{222}/\theta_{208}] = >1$, we hypothesized that the conformational changes into α -helicity has been accompanied by formation of coiled-coil ($[\theta_{222}/\theta_{208}] = 0.85-0.95$ for single α -helix strand) [227]

The α -helix domain of any protein provides high resilience to large deformation. The domain allows proteins to tolerate large displacements (strain% >150%) before the fracture of protein backbone occurs and to dissipate large energy. On the contrary, β -sheets are characterized by strength, providing great resistance when loaded in shear, even though at relatively small displacements.[228] We hypothesize that at the interfacial condition, as protein cages experience the effect of surface forces, the molecular arrangement is taking place in such a way that the structure compensates the change by increasing its α -helix content. Moreover, the α -helix content consists of both hydrophilic and hydrophobic residues which make it amphiphilic in nature. Therefore, the α -helix has been proposed as the favored structure in stabilizing oil/water interface. [229]

Table 5-1: Comparison between secondary structure proportions of E2-LFtn in 100 mM HEPES and E2-LFtn at oil/water interface.

	E2-LFtn	
	In solution (100mM HEPES)	at oil/water interface
α -helix	40.9	56.3
β -strand	10.7	5.7

Figure 5-7: Transmission electron microscopy (TEM) image of E2-LFtn at different incubation temperatures (A and E), at different pHs (B and F), at different concentrations of GuHCl (C and G) and before and after adsorption on oil/water interface (D and H).

5.2.4. Microstructure analysis of Pickering emulsion

The microstructure of E2-stabilized Pickering emulsion has been analyzed to investigate the molecular assembly of E2 around the oil droplets. This technique has been utilized to study assembly of E2-LFtn at different denaturation conditions as described earlier. The structure of E2-LFtn in solution is also compared with the structure of E2-LFtn after adsorption on oil/water interface. **Figure 5-7A-C** and **6E-G** represent the molecular assembly of E2-LFtn under native and denaturing conditions, respectively. At 75°C, at pH ~7 and in absence of GuHCl, native structure of E2 protein nanocage is visible while at denatured state, the structure of E2-LFtn is not detectable under microscopy. Previous studies showed that E2 protein nanocage breaks down to its trimeric state during denaturation by disturbing its tertiary structure.[230] **Figure 5-7D** and **6H** represents the molecular assembly of E2-LFtn in solution and after

adsorption on oil/water interface. **Figure 5-7H** shows the oil droplets (in yellow circles) is covered by E2-LFtn (in red circles). The assembled cage-like structure is clearly visible at the oil/water interface which confirms the findings of integrity of tertiary structure from tryptophan fluorescence analysis.

Figure 5-8: (A) Inter-trimer electrostatic interaction between cationic residues (K239, K240) and anionic residues (D419, E421); (B) Crystallographic structure of E2 showing different trimers interacting at the inter-trimer interface; (C) hydrophobic interactions between amino acids (#313PIF#315 and #424LM#425) located at the trimer-trimer interface.

5.2.5. Theoretical approach to predict structural integrity of E2 upon adsorption

Three-dimensional structure of any protein is stabilized by both covalent and non-covalent interactions between amino acid residues. Covalent interaction mainly consists of the formation of disulfide bonds between intra-molecules or inter-molecules cysteine residues while the non-covalent interactions are consist of hydrophobic interactions, electrostatic interactions, hydrogen bonding and van der Waal's forces. E2 subunit contains only a single cysteine amino acid (C343) at the inner core of the cage structure which makes 60 cysteine residues in overall cage structure. The long distance between intra-trimer and inter-trimer cysteines eliminate the presence of disulfide bond and the effect of covalent bond in

stabilizing cage structure of E2. Therefore, the overall 60-mer structure of E2 consists of 20 trimers, bound with each other by non-covalent interactions located at the inter-trimer interface (**Figure 5-8**)

Figure 5-9: Distances between charged residues (R238, K239, K240, D419, and E421) of the neighboring monomers as a function of time. A snapshot of interactions between neighboring monomers was created using Visual Molecular Dynamics. [231]

The main inter-trimer non-covalent interactions acting at the trimer-trimer interface of E2 are hydrophobic interactions and electrostatic interactions. To understand these interactions between trimers of E2, molecular dynamics (MD) simulations of two neighboring monomers were performed for 200 ns. Distances between side chains of neighboring monomers were calculated to quantify the strengths of specific electrostatic and hydrophobic interactions. **Figure 5-9** compares distances between anionic and cationic residues of the neighboring monomers, showing that the distances for K239-D419, K239-E421, and K240-E421 are less than 1 nm. This indicates that K239 interacts with both D419 and E421, while K240 interacts mostly with E421, as also observed from the crystal

structure in PYMOL. K+E and K+D has moderate free energy of electrostatic interactions in formation of salt bridges. Lysine (K) appears to be suited for non-specific interactions, therefore, it interacts with both Glutamic acid (E) and Aspartate (D) with similar affinity. [232] Distances between hydrophobic residues were also calculated (**Figure 5-10**), showing relatively short distances between P313/I314/F315 and L424/M425. These simulation findings indicate strong electrostatic interactions of K239-D419/E421 and K240-E421, and strong hydrophobic interactions of P313/I314/F315-L424/M425, which stabilize the cage structure of E2. Estimation of relative hydrophobicity [153] and electrostatic interaction forces [233, 234] have shown the domination of hydrophobic interaction to stabilize the cage structure of E2.

Figure 5-10: Distances between hydrophobic residues (P313, I314, F315, A316, L317, A318, L422, L423, L424, and M425) of the neighboring monomers as a function of time.

Besides, the surface free energy, experienced by a spherical particle straddling a perfectly flat liquid interface, can be estimated by the equation derived by Rahmani et al.[235, 236] We hypothesize that the tangential surface force is responsible for the conformational changes of E2 adsorbed on the interface. However, the combined force of non-covalent interactions is several times higher than the tangential force acting on E2. Therefore, we hypothesize the integrity of E2 will be maintained with minimum conformational changes.

Distribution of hydrophilic and hydrophobic residues on the surface of the protein and oppositely charged amino acid residues are two important factors that play a vital role in maintaining the structural integrity of protein at the interface. Even distribution of amino acids of opposite properties ensures the stiffness of the structure while the uneven distribution ensures the flexibility of the structure in different conditions. [237] The cage structure of E2 is highly symmetric. Therefore, the distribution of hydrophobic, hydrophilic and oppositely charged amino acids is even throughout the structure (**Figure 2-9**) which might be effective in maintaining the integrity of the cage structure upon adsorption.

5.3. Conclusion

A genetically modified version of E2-WT, E2-LFtn, was employed to adsorb on a model hydrophobic surface to investigate the adsorption kinetics and thickness of the adsorbed layer. The results confirm the formation of a monolayer of E2 with a thickness of 26 nm on a hydrophobic model surface. The molecular arrangement of E2-LFtn was investigated thoroughly by comparing the structure of proteins in native condition and after adsorption at oil/water interface. The structural rearrangement at the interface also compared with the denaturation

pathway of E2-LFtn observed in different denaturing conditions i.e. incubation temperature, pHs, and in the presence of guanidine hydrochloride. CD spectrum for E2-LFtn adsorbed on interface shows a slight increase in α -helicity in the secondary structure. However, the tertiary or cage-like structure remained intact upon adsorption as confirmed by the fluorescence emission of tryptophan which shows no shift in the peak position of the emission wavelength. Microstructure visualization under TEM further confirms the integrity of E2-LFtn adsorbed on an oil/water interface. A theoretical analysis by molecular simulation suggests that the hydrophobic forces at the trimer-trimer interfaces dominates over tangential force at the oil/water interface and may play an important role in maintaining the structural integrity of the caged structure.

Chapter 6

Protein-based nanocarrier for micronutrient delivery in functional food design

6.1. Introduction

The advancement in nanotechnology has opened new opportunities for understanding and designing nanostructured delivery system of active ingredients in food technology.[238-240] The nanostructured delivery system in functional food has great potential to combat different global ailments like diabetes, obesity and cardiovascular diseases.[173, 238] Incorporation of micronutrients into food matrix in their pure form have difficulties due to the biological and physiological constraints such as poor solubility, undesired sensory attributes, sensitivity to physical, chemical or enzymatic degradation. Therefore, there has been significant interest in developing a food grade colloidal delivery system to incorporate, protect, and release nutrient molecules in specific delivery sites.[173, 241]

Applications of nanotechnology have widened the opportunity of developing and designing of the nanoparticle-based colloidal delivery system in food industries.[242] A wide range of food-grade particles (e.g. proteins, surfactants, lipids, polysaccharides, minerals) has been used in developing nanoparticles for delivery purpose.[243] Surfactant-based nanoparticles, e.g. micelle and microemulsion [244, 245], liposome, nanoliposomes[246-248],

lipid-based nanoparticles [242, 249], solid-lipid nanoparticles [250], molecular inclusion [251], biopolymer-based nanoparticles [252] are some of the reported nanoparticles used as the carrier of active ingredients. The selection of carrier nanoparticle to deliver any active molecules depends on the properties of both the active molecule as well as the properties of the food matrix[173]. The physicochemical properties (i.e. density, polarity, refractive index, rheology), particle properties (i.e. size, shape, charge), encapsulation properties (i.e. encapsulation capacity, encapsulation efficiency), and release properties (i.e. trigger rate, and extent) are crucial factors for considering nanoparticles as carriers in food systems.[173]

In this chapter, we are introducing E2 protein nanocage as a potential nanocarrier of active molecules in the emulsion-based functional food matrix. In earlier studies, E2 has been engineered as a nanocarrier of various foreign molecules e.g. iron, AF488, and doxorubicin. [149, 150, 211] The physiochemical, particle, encapsulation and release properties of E2 allow the protein cage to encapsulate and protect foreign molecules in its inner cavity. In Chapters 4 and 5, E2 has been established as a Pickering emulsifier where the structure of the protein cage remain intact at the interface after adsorption. In this study, a couple of model micronutrient molecules (e.g. calcium and iron) have been encapsulated in E2 to quantify the encapsulation capacity and encapsulation efficiency and therefore, the potential of E2 as a carrier of micronutrients. Encapsulation of micronutrients is followed by simulation of digestion of micronutrient-loaded E2 in the gastrointestinal tract to study the digestion profile and release of micronutrients from protein cage.

Simulation of gastrointestinal digestion is a widely employed technique

in the field of food and nutrition science to investigate the efficacy of functional food in carrying and delivering active molecules in specific sites. Simulation of the gastrointestinal tract (GIT) determines the endpoints, such as bioaccessibility of delivered active micronutrient or non-nutrient molecules[253] and digestibility of macronutrients e.g. proteins[254, 255], lipids[256, 257], carbohydrates[258]. Though human studies are considered as “gold standard” in addressing diet-related issues, *in-vitro* simulation method has certain advantages over human studies, such as less expensive, rapid screening, and free from ethical disputes.

A variety of analytical techniques has been used to characterize the fate of protein and emulsion as they pass through the simulated gastric conditions. The most important and commonly used techniques include analyses of enzymatic activities, molecular weight, and mass spectrophotometry, particle size, zeta potential, and microstructure of the emulsion. Rheological analysis and bioaccessibility of loaded cargo are also used in analyzing the progress of digestion of protein and emulsion.

We simulate the gastric phase digestion to compare the digestibility of micronutrients loaded E2 and micronutrients loaded E2-stabilized Pickering emulsion followed by the estimation of bioaccessibility of micronutrients. The pepsin enzymatic activity in digesting E2 and the E2-stabilized emulsion is estimated and the size and microstructure of E2-stabilized Pickering emulsion.

This study is introducing novel material, a non-viral protein nanocage, as a nanocarrier of active micronutrients in the design of functional food matrix. We also develop a spectrophotometric assay for screening *in-vitro* digestion process by measuring the enzymatic activity. This development will pave the way to design and rapid screening of protein-based functional food models.

6.2. Results and discussions

E2-LFtn, a modified version of E2-WT has been used to carry out the experiments in this chapter. The details on the site-directed mutagenesis of E2-WT to form E2-LFtn has been discussed in Section 5.2 in Chapter 1. The basic characterization of purified E2-LFtn has been shown in the previous Chapter in **Figure 5-1**.

6.2.1. Mineralization of E2 protein nanocage

6.2.1.1. Mineralization

E2 protein nanocage has been mineralized with model micronutrient molecules (e.g. calcium and iron) and followed by the evaluation of the potential of E2 as a nanocarrier of micronutrients by estimation of encapsulation capacity and encapsulation efficiency. Calcium plays a significant role in bone health, blood clotting, nerve conduction, weight loss, muscle contraction and enzyme regulation. [259, 260] However, calcium has to be soluble in the gastrointestinal tract to increase its bioavailability. Several dietary factors affect the solubility and bioavailability of calcium at the absorptive surface of intestinal cells. Therefore, an *in-vitro* method is useful in investigating the bioaccessibility and bioavailability of different salts of calcium that are contained in food intakes or in supplements. [261] Iron is another micronutrient that ensures the development of a healthy immune system and normal red blood cells. Iron deficiency causes disability and even death of people. Therefore, it is considered as a prime risk factor worldwide, affecting an estimated two billion people. [262] Iron deficiency has substantial health and economic consequences in developing countries. These consequences include decreased productivity, impaired school performance, poor pregnancy outcome. [261] Therefore, calcium and iron have been selected to be

encapsulated in E2 protein nanocage. Mineralization of E2 has been aided by diffusion of mineral ions into the inner cavity of the E2 followed by the formation of an ionic bond with charged amino residues. The modified version of E2-WT, E2-LFtn, introduces iron binding sites, similar to ferritin—an iron storage protein—in the inner cavity of E2. The substitution of loop-forming six amino acids in the inner cavity of E2 with Frog M-ferritin-mimicking peptide (QEHEDE) has introduced a negatively charged core with high affinity towards iron. [150] This peptide contains acidic functionalities leading to a maximum negative charged state which is the primary binding sites for calcium ion as well. [263] Glutamic and aspartic acid residues are responsible for the metal coordination in the first level of binding. Ca^{2+} ion becomes attached to an in-chain negatively charged carboxylic group, after which it attacks the hydroxyl group of a threonine or serine residue which leads to a formation of very stable ionic bidentate structure.[264] The introduced peptide forms a ferroxidase sites that oxidize Fe^{2+} to form Fe^{3+} . Aspartic acid, together with glutamic acid demonstrate to be an essential residues to promote the iron oxidation at the ferroxidase site. [265]

The encapsulation of iron and calcium were confirmed by the co-elution of E2-LFtn and the minerals (**Figure C-1B**). Mineralized E2 was purified to remove unbound minerals and characterized by measuring size and shape using DLS and TEM techniques, respectively.

Figure 6-1: Volume-based particle size distribution of (A) Ca-loaded and (B) Fe-loaded E2-LFtn.

6.2.1.2. Characterization of mineralized E2

E2 loaded with the different amount of calcium show similar hydrodynamic diameters (**Figure 6-1A**). The correlation coefficient of measurement shown in **Figure C-2** confirms the quality of the samples in terms of non-randomness in measurement. An investigation by TEM microscopy confirms that Ca-loaded E2 has the native molecular assembly after mineralization irrespective of the amount of encapsulated mineral (**Figure 6-2**).

Figure 6-2: Microstructure of E2-LFtn encapsulated with different amount of calcium; (A) No Calcium loading; (B) (Ca 50)₃₂₀₀ E2; (C) (Ca 150)₄₈₀₀E2; all scale bars are 50 nm. [Legend as (Ca (amount loaded per cage) initial amount added)]

Mineralization of E2 with iron shows a slight change in hydrodynamic diameter of nanocage structure. **Figure 6-1B** shows the volume-based particle size distribution of iron-loaded E2 which demonstrates a slight increase in the diameter. However, the investigation of molecular assembly of E2-LFtn, loaded with iron, using TEM microscopy (**Figure 6-3**) confirms the native molecular assembly of E2 at different loading amount of iron.

The oxidized state of iron (Fe^{3+}) is more stable compared to calcium, an alkali earth metal. Therefore, the higher binding affinity of iron with E2 protein nanocage elevate the loading amount of iron compared to calcium. The higher influx of iron in the cavity of E2 protein nanocage results in the slight increase of the hydrodynamic diameter of iron-loaded E2. The randomness of measurement also confirms the increase of the hydrodynamic diameter of iron-loaded E2.

Figure 6-3: Microstructure of E2-LFtn encapsulated with different amount of iron; (A) $(\text{Fe}2300)_{2400}\text{E}2$, (B) $(\text{Fe}3200)_{3200}\text{E}2$, and (C) $(\text{Fe}3200)_{3200}\text{E}2$ (unstained). [Legend as (Fe (amount loaded per cage) initial amount added)]

6.2.1.3. Estimation of encapsulation parameters

Encapsulation parameters express the efficiency of mineralization and

effectiveness of the E2 as a nanocarrier. **Table 6-1** shows the encapsulation capacity and encapsulation efficiency of E2-LFtn for loading of calcium and iron. The capacity of calcium encapsulation in E2-LFtn, achieved in our experiment, is ~1.5–3 wt% where the efficiency of encapsulation is ~20% (**Table 6-1**). The maximum encapsulation capacity achieved for encapsulation of iron in E2-LFtn is ~11% showing the encapsulation efficiency up to 100%. The obtained values are comparable with previously reported data of encapsulation of doxorubicin (1–4 wt. %)[149], AF488 (1–3 wt. %)[211] and iron (~8 wt. %)[150]. The higher affinity of iron to bind with E2-LFtn can be explained from the origin of modification[150] as discussed earlier.

Table 6-1: Encapsulation capacity and encapsulation efficiency of calcium and iron encapsulated E2-LFtn

Calcium			Iron		
Initial loading	Encapsulation capacity	Encapsulation efficiency	Initial loading	Encapsulation capacity	Encapsulation efficiency
3200	1.50±0.11	19.93±1.46	1600	4.70±0.21	89.81±3.93
4800	1.73±0.35	15.30±3.13	2400	7.49±0.43	95.35±5.44
6400	2.95±0.52	19.61±3.45	3200	10.48±0.50	100.01±4.76

6.2.2. Gastric phase digestion: *In-vitro* study

Simulation of digestion in gastrointestinal tract typically consists of a simulation of oral, gastric and small intestine phase digestion. Oral phase digestion is usually mimicked by the artificial saliva (contains ~99.5% water), minerals (<1%), various proteins (0.1–0.2%), enzymes and antibacterial compounds.[40, 218] Simulation of oral phase digestion has been omitted for this

study as the retention time of liquid food in oral phase is very short [174, 266] and the negatively charged emulsion has no significant change in presence of saliva [267]. Gastric phase digestion starts with mixing the meal in the lower part of stomach, antrum, and digested with enzymes and hydrochloric acid and grounding by antral movements and finally emptying into the duodenum. Though the retention time of liquid or semisolid food in gastric phase is highly dependent on the composition of micronutrition (carbohydrates, lipid, and proteins), [268] half emptying time, 2 hours, has been considered to cover moderately nutritious and semi-solid food for the *in-vitro* simulation study. The optimal pH for simulation is selected as 3, considering the activity of gastric phase enzymes—pepsin and lipase. However, due to commercial unavailability of gastric phase lipase of human and alternatives with similar characteristics,[269] lipase in the simulation of gastric phase has been omitted.[174]

The digestion profile of mineralized E2 in bulk solution and E2-stabilized Pickering emulsion have been analyzed by gastric phase simulation. Characterization of digested chyme is studied using different techniques e.g. size distribution of E2 and droplets of Pickering emulsion at the different stage of the digestion and spectrophotometric analysis was carried out to estimate the digestion kinetics in terms of the enzymatic activity of pepsin.

6.2.2.1. Particle size distribution of digested chyme

The digested chyme of both E2 in bulk solution and E2-stabilized emulsion are analyzed by measuring the change of the hydrodynamic diameter of E2 and droplet size of emulsion respectively with the progression of digestion. The particle size distribution of digested chyme, shown in **Figure 6-4**, demonstrates the denaturation of E2 as the digestion progresses. In the absence

of pepsin, a single peak in volume-based particle size distribution for E2 confirms the homogeneity of protein size distribution before digestion. Within 5 sec of digestion, a secondary peak started to appear around <10nm range which confirms the breakdown of cage structure in presence of pepsin. As the digestion progresses the peak of particle size distribution shift towards the left side which confirms the proteolysis of E2-LFtn. Another peak started to appear around ~300nm which could be the result of the aggregation of broken peptides. A parallel reduction of peak intensity at 25 nm is observed with the progression of digestion which confirms the reduction of undigested or intact protein nanocages. A significant portion of E2 was digested within 15 min of simulation period—showing two peaks of similar volume percentage in particle size distribution. On the other hand, in the digestion of E2-stabilised emulsion, the droplet size distribution of digested emulsion shows an increase in the droplet size, dispersed in the digested chyme. **(Figure 6-4 B)** A simultaneous increase in a polydispersity of the droplet size and the randomness of the sample have also been observed with the progression of the digestion **(Figure C-3)**.

Figure 6-4: (A) Volume-based particle size distribution of E2-LFtn, secondary peak respective to aggregation of digested peptides (inset); (B) Volume-based droplet size distribution of E2-LFtn-stabilized Pickering emulsion at different time points of digestion.

We attempt to explain the unexpected increase of the hydrodynamic diameter of E2-stabilized Pickering emulsion upon digestion. Our previous studies show that the droplets flocculate at acidic pHs (Chapter 4, Section 4.2.4, Appendix A-**Figure A-5**). Therefore, the occurrence of flocculation in the acidic condition of gastric phase digestion could lead to an increase in droplet size and randomness in measurements. The microstructure analysis in confocal microscopy confirms the occurrence of flocculation with the progression of digestion shown in **Figure 6-5**.

The alternate explanation for the increasing droplet size could be the coalescence of droplets with the degradation of E2 from the liquid-liquid interface in the process of digestion. Ostwald ripening could be another pathway of increasing droplet size as the surface coverage of droplet with E2 will be decreased. The reduction of surface coverage could facilitate the fusion of adjacent droplets. Similar observation of reversible flocculation and irreversible coalescence are found by Golding et al. [270] and Sarker et al. [267, 271, 272] for WPI and β -lactoglobulin-stabilized emulsion respectively.

Figure 6-5: Confocal micrographs of E2-LFtn-stabilized Pickering emulsion at different time points of simulation of gastric phase digestion: *In-vitro*; (A) 0min, (B) 5 min, (C) 30 min, and (D) 60 min.

6.2.2.2. Percentage of hydrolysis of E2

The digestion kinetics were studied by spectrophotometric analysis by measuring absorption kinetics of protein-coomassie blue complex at 595 nm over the simulation period of digestion. Charge neutral Coomassie blue (green) has both hydrophobic interactions (via Phe, Trp, etc) and electrostatic interaction with the protein. Negatively charged dissociated sulfonic group and positively charged guanidino group of Arg show electrostatic interactions, primarily (and secondarily with Lys and His). Upon the formation of protein-dye complex, Coomassie blue behaves spectrally as a pseudo-anionic species.[273] With the progression of enzymatic reaction of pepsin with E2, the breakdown of protein cages reduces the formation of protein-CBB dye complex. Therefore, the

absorption of protein-CBB dye decreases with the progression of the reaction. The amount of protein nanocage digested can be estimated from the absorption of the protein-CBB complex. (Figure C-4) The percentage of digested E2 has been estimated, shown in Figure 6-6. The fitting of the digestion percentage curve has been shown in Appendix C in Figure C-5. The digestion of E2 in bulk solution shows a higher percentage of digestion in gastric phase. The initial rate of digestion of both E2 in bulk solution and adsorbed at the liquid-liquid interface are similar. The phenomena can be explained by the highly accessible pepsin cleavage sites on the surface of the protein nanocages. The loosely adsorbed or unabsorbed protein cages react first with pepsin followed by the E2 adsorbed at the interface.

Figure 6-6: Percentages of hydrolysis of E2-LFtn with the progression of gastric phase digestion.

6.2.3. Bioaccessibility of encapsulated minerals in gastric phase

The bioaccessibility is defined as the fraction of a loaded molecule available for absorption after releasing in the process of digestion. Digestion of

mineralized-E2 protein nanocage in gastric phase releases the encapsulated minerals in the gastric chyme. Released minerals will be available for absorption by epithelial cells. Comparison of concentrations of released minerals in digested chyme of both E2 and E2-stabilized emulsion showed that the bioaccessibility of minerals from the emulsion is lower than bioaccessibility of minerals from E2 in bulk solution. As the percentage of hydrolysis of E2 in the E2-stabilized emulsion is lower than the hydrolysis of E2 in bulk solution, the lower bioaccessibility of mineral from the emulsion is expected. The bioaccessibility of calcium is higher than iron which can be explained by the higher binding affinity of iron with the E2 compared to calcium. Therefore, bioaccessibility of the different loaded molecule may have different release profile depending on their binding affinity as well as binding mechanism.

Figure 6-7: Bioaccessibility of encapsulated minerals in gastric phase after digestion at a different state.

6.3. Conclusion

E2 has been successfully mineralized by encapsulation of calcium and iron in the cavity of the nanocage. The efficiency of encapsulation shows the E2

can be used as an effective nanocarrier of micronutrients or foreign active molecule. The *in-vitro* simulation study reveals the higher digestibility of E2 in bulk solution compared to adsorb at the liquid-liquid interface of Pickering emulsion. Bioaccessibility of loaded cargo in the gastric simulated fluid is lower when the cage is adsorbed at the liquid-liquid interface. Application of E2-stabilized Pickering emulsion in the design of functional food, pharmaceuticals or nutraceuticals for delivery of micronutrients and other active ingredients will ensure at least 50% release of the loaded cargo in stomach phase.

Chapter 7

Conclusion and future work

7.1. Conclusion

The long-term objective of the project is to use of E2 protein nanocage as an effective carrier of active molecules in emulsion-based delivery media for different field of applications such as food, pharmaceuticals, and cosmetics. The overall project was divided into three specific goals. The works on these three main goals have been discussed in Chapters 4, 1, and 1, respectively.

The amphiphilic nature of the surface of E2 makes it a highly surface active macromolecule. Surface activity of E2 has been exploited to stabilize Pickering emulsion. The stability of E2-stabilized emulsion depends on compositions of oil, water, and protein. The composition is optimized to achieve a stable Pickering emulsion using oil-water ratio and protein mass fraction as two independent variables. Stability of E2-stabilized Pickering emulsion has been investigated in different conditions such as pHs, temperatures, and ionic strengths. The pH-responsiveness of Pickering emulsion is an attractive feature to trigger the delivery of loaded cargo in food, pharmaceuticals, and cosmetic industries. Moreover, the stability of Pickering emulsion at high temperature and ionic strength makes the colloidal system more robust. The rheological analysis revealed the condition of formation of a gel-like structure which has long-term stability and high mechanical strength.

Structural integrity is an important factor for protein to retain its functionality in any condition. Unlike other biomacromolecules, E2 Protein nanocage retains its structural integrity at the liquid-liquid interface. The integrity of the tertiary structure is preserved while the secondary structure of E2 shows an increase in alpha helicity at interface conditions. Theoretical analysis of forces acting on a protein nanocage adsorbed at the liquid-liquid interface suggests that hydrophobic interaction is the dominant force in maintaining the integrity of the caged structure. The integrity of cage structure at the interface has opened a new opportunity to design a delivery system where protein cage will be loaded with deliverable cargo.

The scope of this thesis is limited to demonstrate the functionality of only one structure of protein nanocage E2. Nevertheless, it can be hypothesized that other non-viral protein nanocage (e.g. Ferritin[96]) and viral protein cage (e.g. TMV[20], Turnip yellow mosaic virus[22]), which has been reported earlier for their high surface activity, will show comparable behavior at the liquid-liquid interface. The behavior and structural integrity of protein will be affected by several factors such as presence and location of disulfide bonds, distribution of polar/nonpolar amino acids and also the distribution of hydrophilic/hydrophobic amino acids. These factors ensure the flexibility or rigidity of protein structure at the interface. Interfacial tension between two immiscible liquids also plays a significant role. In fact, the higher interfacial tension between air-liquid compared to the liquid-liquid interface is a significant barrier to translate the findings from this current study into stabilization of air-water interface in the formulation of foam. Protein structure with moderate wettability, high contact angle (near 90°), contact angle hysteresis, and a higher concentration of proteins

will certainly increase the higher stabilization of emulsion system. Furthermore, some other non-quantifiable factors such as aspect ratio, surface roughness, and surface properties will affect the stabilization.

In order to exploit the potential of designing food matrix using E2 as a carrier of active molecules, E2 protein nanocage has been biomineralized by encapsulating micronutrients, e.g. calcium and iron, in the inner cavity of E2. The *in-vitro* simulation of digestion of E2-stabilized Pickering emulsion shows a slow rate of digestion in gastric phase compared to the digestion of free E2 dissolved in bulk solution and the bioaccessibility of minerals in gastric phase confirms the release of at least 50% of loaded minerals.

The formation of E2-stabilized Pickering emulsion, confirmation of structural integrity at the liquid-liquid interface and the encapsulation/release of model micronutrients have created an opportunity to apply E2-stabilized Pickering emulsion for designing emulsion-based delivery platform. Depending on the requirement of the rate of release or the properties of active molecules, cargo can be loaded either in E2 or in lipid phase as well.

7.2. Future works

The establishment of E2 as the first non-viral Pickering emulsifier has opened new opportunities to develop organic emulsion which serves a dual purpose which acts as a surfactant and simultaneously as a carrier of active cargos. The pH-switchability of the E2-stabilized Pickering emulsion can be used as a triggering option to release the loaded cargo from the cavity of E2. Development of emulsion-based delivery product in the field of food, pharmaceuticals, and cosmetic has the most potential for immediate application.

Application of E2-stabilised emulsion in delivering micronutrients has been explored in the thesis. In the future, this platform can be useful in designing a compact delivery media to deliver hydrophilic and hydrophobic molecules simultaneously. The investigation of delivery of different nutrition molecules, measurement of bioaccessibility and bioavailability is important for the design of functional foods. This study will open the opportunity to design functional food for delivery of loaded cargo. Investigation of the synergistic and antagonistic effect of micronutrients in the gastrointestinal tract is also an interesting area to explore to design effective functional food.

The application of E2-stabilised emulsion in developing skincare products is another potential area to explore in the future. The encapsulation capacity of E2 protein nanocage enables the loading of active ingredients inside the inner cavity. The enzymatic activity or change of pH can trigger the delivery of active ingredients in the skin cells.

Alongside exploring the applications of the E2-stabilized emulsion, investigation of the kinetics of conformational changes upon adsorption and the effect of external conditions on the adsorption of E2 on a liquid-liquid interface can be a potential area of research in future. This study can be extended in the more adverse interface like air-water interface which will aid the understanding of the mechanism of conformational changes of E2.

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A. Appendix A

A.1.Purification and characterization of E2

Figure A-1: Purification of E2 protein nanocage by ion-exchange chromatography in fast protein liquid chromatography (FPLC) system.

Figure A-1 shows the chromatogram of purification of E2 protein nanocage by ion-exchange chromatography. Absorbance at 280 nm confirms the presence of protein in solution. Flow through fraction contains the purified E2 where the elution fraction contains the impurities eluted at the presence of strong negatively charged Cl^- ion. Flow-through fractions e.g. X1, X2, and X3 were collected and ran for SDS-PAGE analysis to confirm the purity of E2. **Figure A-2** shows a single band around 27kDa, confirms the purity of E2.

The isoelectric point of E2 protein nanocage has also been identified by measuring the zeta potential of E2 at various pHs. The isoelectric point of E2 is measured to be ~ 3.73 (**Figure A-2**).

Figure A-2: (A) SDS-PAGE analysis of E2 protein nanocage; (B) Zeta potential of E2 protein nanocage at different pHs

A.2. Optimization of Pickering emulsion compositions

The composition of E2-stabilized emulsion has been optimized considering oil-water ratio and protein mass fraction as two independent variables. The ranges of variables used in optimization process have been shown in

Table A-1. **Figure A-3** shows the kinetic stability of E2-stabilized emulsion (A) at constant oil/water ratio and (B) at a constant protein mass fraction. At constant oil/water ratio, the stability increases with the increase of protein mass fraction which can be explained by the droplet size of corresponding emulsions. The emulsions with low protein mass fraction result in forming of droplet size in the micrometer range. Therefore, the kinetic stability of emulsion reduces as the drag force dominates and emulsion separates by creaming effect. Similar phenomena have been observed at higher oil/water ratio at a constant protein mass fraction. Higher oil fraction in the formulation of emulsion associated with the formation of the higher surface area which was unable to cover by the protein. Therefore, the optimized composition is a trade-off between the droplet size and minimum surface coverage required to form a stable

emulsion by the available proteins.

Table A-1: Compositions of formulated Pickering Emulsions of different rosemary oil/water ratio (v/v) and E2-LC2 mass fraction (wt %)

Sample No.	Oil/ H ₂ O ratio (v/v)	Protein mass % (wt %)	Set No.	Oil/ H ₂ O ratio (v/v)	Protein mass % (wt %)	Set No.	Oil/ H ₂ O ratio (v/v)	Protein mass % (wt %)
01	0.11	0.05	13	0.25	0.05	25	0.43	0.05
02		0.15	14		0.15	26		0.15
03		0.20	15		0.20	27		0.20
04		0.25	16		0.25	28		0.25
05		0.30	17		0.30	29		0.30
06		0.35	18		0.35	30		0.35
07	0.18	0.05	19	0.33	0.05	31	0.67	0.05
08		0.15	20		0.15	32		0.15
09		0.20	21		0.20	33		0.20
10		0.25	22		0.25	34		0.25
11		0.30	23		0.30	35		0.30
12		0.35	24		0.35	36		0.35

Figure A-3: (A) Effect of protein mass fraction (PMF) on emulsion kinetic stability (visual) at constant Oil/water ratio (B) Effect of Oil/water ratio (OWR) on emulsion stability (visual) at a constant protein mass fraction. Visual images were taken after 10 days of shelf-life at ambient conditions

A.3. Effect of pH on Pickering emulsion

Figure A-4: Effect of pH on the stability of Pickering emulsion containing oil-water ratio 0.11 and protein mass fraction 0.35% after 10 days of storage at ambient condition.

The visual image of E2-stabilized Pickering emulsion at different pHs has shown in **Figure A-4**. The kinetic stability of the emulsion is higher at neutral to basic pHs where pH near isoelectric point (pH~3.73) results in separation of emulsion phase from serum phase by depletion flocculation.

A.4.pH-switchability of Pickering emulsion

Figure A-5: The microstructure of E2-stabilised Pickering emulsion at different pHs (A) at pH= 8 and (B) at pH= 4

Figure A-5 shows the microstructure of E2-stabilized Pickering emulsion during the stabilization (pH 8)-destabilization (pH 4) state respectively. At pH 8, the droplets of Pickering emulsions are well dispersed in the continuous phase which can be explained by the zeta potential which is measured to be -49mV. Larger zeta potential ($> 30\text{mV}$) ensures the stability of the colloidal system. On the contrary, the microstructure of emulsion at pH 4 shows extensive flocculation results in separation of emulsion and serum phase.

A.5.Rheology analysis of Pickering emulsion

Figure A-6: Viscosity of emulsion and emulsion-gel structure with the change of shear rate

B. Appendix B

B.1. Formulation of Pickering emulsion and characterization:

Pickering emulsion was formulated using rosemary oil as the oil phase, Milli-Q water as the water phase and E2-LFtn in 100mM HEPES buffer at pH 8.0 as Pickering emulsifier. The Pickering emulsion was formulated by ultrasonication and characterized by DLS following the composition and protocol described in Section 3.2.2 (**Figure B-1**).

Figure B-1: (A) Physical appearance of Pickering emulsion of rosemary oil/water system; (B) Z-Average oil droplet diameter of Pickering emulsion stabilized by E2 and Zeta potential of oil droplets (inset).

The droplet size of E2-stabilized Pickering emulsions was measured by DLS in zetasizer nano zs (Malvern Instruments). Droplet size of Pickering emulsion was measured after diluting the emulsion 100 times to reduce the probability of multiple scattering. All measurements were performed in triplicate and average values are reported.

The physical appearance of E2-LFtn stabilized Pickering emulsion is shown in **Figure B-1A**. The size of dispersed phase oil droplet has been measured by DLS which shows the size of droplets $\sim 400\text{nm}$ with PDI value less than 0.6. Zeta potential of the droplet of the emulsion has been shown in **Figure B-1** (Inset) which is measured to be -49mV indicating the stability of the colloidal system.

B.2. Characterization of SDS-stabilised emulsion

Figure B-2: Volume-based particle size distribution of droplet size of SDS-stabilized emulsion and E2-LFtn stabilized the emulsion.

B.3. Sessile drop analysis to investigate surface hydrophobicity

Figure B-3: Sessile drop analysis of gold coated AT-cut piezoelectric quartz crystals (A) before and (B) after thiol modification with 2mM 1-dodecanethiol for 16hrs.

B.4. QCMD analysis: Dissipation shift of different overtones

Figure B-4: Normalized dissipation shift versus time at different overtones for the adsorption of E2-LFtn on the 1-dodecanethiol-modified hydrophobic quartz crystal surface.

B.5. PYMOL analysis of surface properties of E2

Figure B-5: 3-fold crystallographic image of E2 protein nanocage showing the tryptophan (Trp) residues; (A) PYMOL generated cartoon view where Trp residues are shown in green sphere, (B) PYMOL generated surface view where the Trp residues (green sphere) are not visible either from outer or inner surface as the residues are buried inside the nanocage.

B.6. Theoretical analysis of adsorption of E2 on oil/water interface

For adsorption on a hydrophobic interface, usually, a group of 6–8 nonpolar amino acids is required to anchor on the interface.[237] From the structural analysis in PYMOL, we hypothesize that the amino acid residues #300–#306 (GLLVPI) is the anchoring site for E2 on the hydrophobic interface which is the longest hydrophobic sequence, located on the surface of E2.

B.6.1. Tangential surface force at oil droplet

Oil/water interfacial tension, $\gamma = 0.0006 \text{ N/m} = 0.6 \text{ mN/m}$

Radius of the protein nanocage, $R = 12.5 \text{ nm} = 12.5 \times 10^{-9} \text{ m}$

Radius of the oil droplet, $r = 200 \text{ nm} = 200 \times 10^{-9} \text{ m}$

The surface free energy of a spherical particle of radius R straddling a

perfectly flat liquid interface located at $z = 0$ can be expressed as,

$$E_s = \gamma\pi(Z - Z_E)^2 + 2\pi\tau\sqrt{R^2 - Z^2}$$

(Assuming, $\theta = 90^\circ$ and line tension, $\tau = 1000$ pN)

$$= 2.95 \times 10^{-19} + 7.85 \times 10^{-17} \text{ N.m}$$

$$= 7.88 \times 10^{-17} \text{ N.m}$$

Tangential force acting on adsorbed particle, $F_s = -\frac{E_s}{2\pi r}$

$$= -\frac{7.88 \times 10^{-17} \text{ Nm}}{2 \times 3.1416 \times 200 \times 10^{-9} \text{ m}}$$

$$= -6.27 \times 10^{-11} \text{ N}$$

$$= -63 \text{ pN}$$

[Negative sign for outward direction]

B.6.2. Electrostatic force acting at trimer interface:

Force acting between arginine (K239) and glutamic acid (D419) molecule,

$$= \frac{K q_1 q_2}{\epsilon d^2} = \frac{9 \times 10^9 \times (1.6 \times 10^{-19})^2}{20 \times (6.5 \times 10^{-10})^2} \text{ N} = 2.048 \times 10^{-11} \text{ N}$$

Force acting between lysine (K239) and aspartic acid (E421) molecule,

$$= \frac{K q_1 q_2}{\epsilon d^2} = \frac{9 \times 10^9 \times (1.6 \times 10^{-19})^2}{20 \times (4.9 \times 10^{-10})^2} \text{ N} = 4.79 \times 10^{-11} \text{ N}$$

Force acting between lysine (K240) and aspartic acid (E421) molecule,

$$= \frac{K q_1 q_2}{\epsilon d^2} = \frac{9 \times 10^9 \times (1.6 \times 10^{-19})^2}{20 \times (6.5 \times 10^{-10})^2} \text{ N} = 2.72 \times 10^{-11} \text{ N}$$

Total electrostatic force = $(2.048 \times 10^{-11} + 4.79 \times 10^{-11} + 2.72 \times 10^{-11}) \times 2$

$$= 1.9 \times 10^{-10} \text{ N}$$

$$= 190 \text{ pN}$$

B.6.3. The hydrophobic force acting at trimer interface

Table B-1: Relative hydrophobicity of residues located at intra-trimer interface based on the assigned value reported by Matthews et al. [153]

Residues position	Residues	Hydrophobicity (KJ/mol)	Hydrophobicity (J/atom)
313	P	3.01	5.0×10^{-21}
314	I	7.53	1.25×10^{-20}
315	F	7.49	1.24×10^{-20}
424	L	7.11	1.18×10^{-20}
425	M	5.14	8.53×10^{-21}
Total hydrophobicity			5.02×10^{-20}

Van Der Waals radius, $R_v = 3.5 \times 10^{-10}$

$$\text{Total hydrophobic force, } = \frac{5.02 \times 10^{-20} \times 2}{3.5 \times 10^{-10}} \text{ N} = \mathbf{2.87 \times 10^{-10} \text{ N}}$$

$$= \mathbf{287 \text{ pN}}$$

The total noncovalent force that acts to retain the integrity of the nanocage structure is $\sim (190+287) \text{ pN} = 478 \text{ pN}$. On the other hand, the tangential force, experienced by a nanocage due to adsorption on a liquid-liquid interface, is $\sim 63 \text{ pN}$ which is at least 7.5 times lower than the stabilizing force. Therefore, we hypothesize that the adsorbed E2 on a liquid-liquid interface will retain its native structure at the interface.

C. Appendix-C

C.1.Desalting of mineralized E2

Figure C-1: Flow through a fraction of desalting column contains the protein of interest; (A) The blue peak at 280 nm represents the elution of Ca-loaded E2-LFtn; (B) absorbance at 280 nm represents the E2-LFtn where the absorbance at 350 nm shows the presence of iron. The co-occurrence of both peaks at 280 and 350 nm confirms the coexistence or encapsulation of iron in cage structure.

C.2.Characterization of mineralized E2

Figure C-2: Correlation coefficient of particle size distribution of (A) Ca-encapsulated and (B) Fe-encapsulated E2-LFtn.

C.3.Droplet size of E2-stabilized emulsion in digested chyme

Figure C-3: Correlation coefficient of particle size distribution of (A) E2 and (B) E2-stabilized Pickering emulsion in the digested chyme.

C.4.Digestion kinetics of E2 in bulk solution and E2-stabilized emulsion

Figure C-4: Amount of E2-LFtn digested with time, the initial concentration of E2-LFtn was 3.5mg/ml in both bulk solution and Pickering emulsion.

Figure C-5: Fitting of digestion kinetics curve from raw data (A) E2-LFtn in bulk solution, (B) E2-LFtn in Pickering emulsion.