

CHAPTER III

ENHANCEMENT OF n-3 PUFAs UTILIZATION FOR FUNCTIONAL MEAT PRODUCTION IN SLOW-GROWING KORAT CHICKEN: EVALUATION OF CHARACTERISTICS OF GLUCOSE TRANSPORTER-TARGETED LIPID NANOPARTICLES

3.1 Abstract

The aim of this research was to investigate the synthesis of suitable carrier of nanoparticles for improving the utilization of n-3 polyunsaturated fatty acids (n-3 PUFAs) source in chicken diets. Lipid nanoparticles were successfully prepared with two different n-3 oil sources, tuna and algal oils using hot and high-pressure homogenization method. Four preparations were defined as followed: non-targeting lipid nanoparticles containing tuna oil (TO_NPs), non-targeting lipid nanoparticles containing algal oil (AO_NPs), targeting lipid nanoparticles containing tuna oil (TO_TNPs) and targeting lipid nanoparticles containing algal oil (AO_TNPs). A second study was conducted for the targeting procedure, the treatments as followed: Control, TO_NPs and TO_TNPs. Thirty-three slow-growing chickens were examined during the post-administration kinetic at 2, 4, 8, 12 and 24 h. The physicochemical characteristics of lipid nanoparticles, storage stability and *in vivo* biodistribution were evaluated. The results showed that the particle diameters of TO_NPs and AO_NPs were 223.7 and 294.4 nm, whereas the particle diameters of TO_TNPs and AO_TNPs were 134.7 and 184.0 nm, respectively. The polydispersity index (PDI) and zeta-potential (ZP) of nanoparticles showed a good distribution and stability in colloid dispersions, respectively. Moreover, the nanoparticles of the TNPs groups were less susceptible to lipid oxidation than that of the NPs groups during a storage at 4°C. The study of the biodistribution based on the Nile red intensity and the determination of n-3 PUFAs composition in chicken meat confirmed the effectiveness of targeted lipid-based nanoparticles to transport directly fatty acids into the skeletal muscle cells of chicken.

Keywords: n-3 polyunsaturated fatty acids, Lipid-based nanoparticles, Physiochemical characteristics, In vitro storage stability, *In vivo* biodistribution, Slow-growing chicken.

3.2 Introduction

Oils containing high amounts of n-3 PUFAs are susceptible to oxidation process (Packer et al., 2001). Many studies have tried to improve the storage stability of these oil sources, and the encapsulation technology was the more efficient (Chang et al., 2020; Wang et al., 2022). Another possibility is to use the nanotechnology to improve the utilization of n-3 PUFA oil source (Kharat et al., 2019). This technology has a high potential and we investigated its use to enhance the accumulation of n-3 PUFAs in chicken muscles to produce functional meat.

Korat chicken, a slow-growing breed that is very popular in Thailand, has been developed as an alternative option catering to the preferences of Thai chicken producers (Kaewsatuan et al., 2022) and could be used to produce functional meat enriched with n-3 PUFAs. Previous studies showed that dietary 4% tuna oil supplementation increased n-3 PUFAs content reaching 19.03% of total fatty acids in Korat chicken meat (Hang et al., 2018).

However, n-3 PUFA sources are susceptible to lipid oxidation that can affect the feed quality (Sanguansri et al., 2013). The protection of these lipids through encapsulation alone is inadequate to enhance the utilization of n-3 PUFA sources, as a portion of these fatty acids will also be distributed among non-targeted organs such as liver.

Different studies showed that lipid nanoparticles could be synthesized by applying the edible oil such as fish oil (Zhu et al., 2015; Shahparast et al., 2019; Taha et al., 2023). However, various nanoparticle size, dispersion ability of particles, zeta-potential (the observation of a robust electric potential at the particle surface boundary demonstrates the existence of a potent repulsive force, which contributes significantly to enhancing the overall physical colloidal stability) and high amount of polyunsaturated fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) might increase the unstable form of particle size during storage (Shahparast et al., 2019) and plain lipid nanoparticles could be degraded by

digestive enzymes (Wang & Luo, 2019). Another important parameter is the ability of nanoparticle surface coating (Zhang et al., 2014). Modifying their surface with PEGylating helps to avoid enzyme activity in the digestive tract (Zhang et al., 2014; Li et al., 2016). Moreover, generating a surface with a specific substrate recognized with target transporter/receptor could allow the transport of beneficial substance directly to the target organs (Qin et al., 2016; Kou et al., 2018; Krishna et al., 2019; Tunki et al., 2019).

Therefore, we investigated the physicochemical characteristics of lipid nanoparticles and their susceptibility to lipid oxidation during storage and their ability to transfer the bioactive compounds (n-3 PUFAs) into the muscles. For feeding animals, the important point to consider is that lipid nanoparticles need to transfer perfectly to the target organ after the passage through the digestive tract and adsorptive cells that depends on the stability of lipid nanoparticles.

3.3 Objective

The aim of this study was to analyze the effects of n-3 PUFA source, targeting process, and storage temperature and duration on the physico-chemical characteristics of lipid nanoparticles, their oxidation susceptibility, and their ability to transfer n-3 PUFAs to chicken meat.

3.4 Materials and methods

3.4.1 Experimental design for in vitro study

For in vitro study, five treatments were considered with three replications in each including crude tuna oil (TO; feed grade, T. C. Union Agrotech Co. Ltd., Bangkok, Thailand and algal oil, AO (*Schizochytrium* sp.); Shanghai Wellboost Health Food Co., Ltd, Shanghai, China), and loaded non-targeted nanoparticles and targeted nanoparticles, respectively, including TO_NPs, AO_NPs, TO_TNPs and AO_TNPs. The physicochemical characteristics and in vitro storage stability of lipid nanoparticles were investigated.

3.4.2 Lipid nanoparticles synthesis

The n-3 PUFA loaded lipid-based nanoparticles were prepared by hot and high-pressure homogenization approach (Figure 3.1) according to Yostawonkul et al. (2017). In this method, the lipid phase consisting of glyceryl monostearate (GMS) (5 g), span 80 (3 g) (Croda, Bangkok, Thailand) and n-3 PUFA (20 g) (tuna oil or algal oil) was blended and melted above 70°C to form an organic phase. The fatty acid composition of crude tuna oil and algal oil is indicated in Table 3.1. Meanwhile, the aqueous phase was prepared by dispersing emulsifying agent, glycerol (2.5 g) Tween 20 (3 g), Poloxamer 188 (2 g) (Croda, Bangkok, Thailand) in distilled water. The aqueous phase was added drop wise to lipid phase at above 70°C with continuous agitation at 300 rpm for 3 min to get a primary emulsion. The pre-emulsion was then homogenized by using high-speed homogenizer (IKA, Altra-Turrac T25, Germany) at 8,000 rpm for 3 min. Then, it was sonicated (Qsonica sonicator, USA) at 30 A pulse on 30 sec and off 10 sec interval for 5 min to obtain n-3 PUFA loaded non-targeted lipid-based nanoparticles (NPs). For targeted lipid-based nanoparticles (TNPs), 2 g of alkyl polyglucoside (APG) was blended with organic phase preparation. Subsequently the dispersion was cooled in ice water bath. The prepared dispersion was stored in airtight container at 4°C.

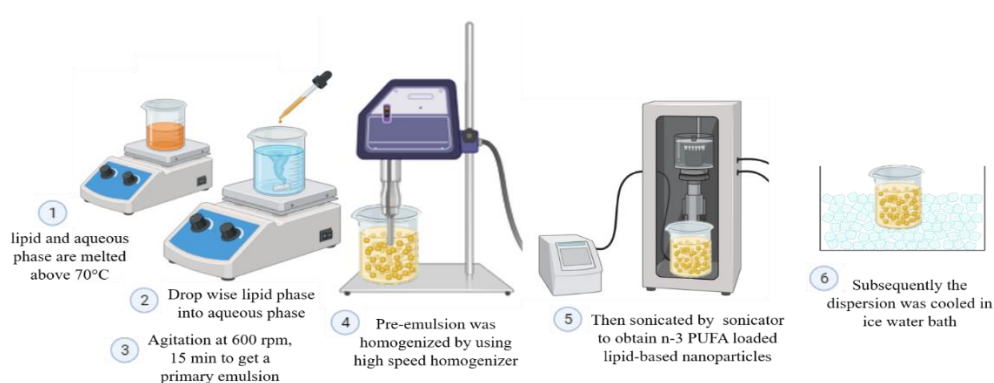


Figure 3.1 lipid nanoparticle preparation.

3.4.3 Physicochemical characteristic analysis

A Zetasizer 3000HS (Malvern Instruments, Malvern, UK) was employed to characterize NPs and TNPs for particle diameter, polydispersity index (PDI), and zeta-potential (ZP).

Table 3.1 Fatty acid composition of crude tuna oil and algal oil (% of total fatty acids).

Fatty acids	Crude tuna oil	Algal oil ¹	Tuna oil loaded in nanoparticles
Palmitic Acid (C16:0)	20.28	38.01	29.43
Stearic Acid (C18:0)	5.28	1.47	8.46
Oleic Acid (C18:1n-9)	14.16	-	22.64
Linoleic Acid (C18:2n-6)	2.84	0.23	7.69
α -Linolenic acid (C18:3n-3)	1.68	0.59	2.40
Arachidonic Acid (C20:4n-6)	2.52	0.49	4.30
EPA (C20:5n-3)	6.98	0.72	3.60
DHA (C22:6n-3)	20.03	37.63	9.24
SFA	39.15	52.91	43.95
MUFA	24.39	-	28.82
PUFA	36.45	46.96	27.23
n-3 PUFA	30.30	46.01	15.24
n-6 PUFA	6.11	8.02	11.99
n-6/n-3	0.20	0.17	0.79

¹ *Schizochytrium* sp.

3.4.4 Thermal and chemical properties of lipid nanoparticles

3.4.4.1 Differential scanning calorimetry (DSC)

DSC is a thermo-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. Usually, both the sample and reference are maintained at nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function of time. The reference sample should have a well-defined heat capacity over the range of temperatures to be scanned. DSC (Pyris 1 DSC, Perkin Elmer, USA) was performed on samples of nanoparticles with and without feed or oil ingredient components to determine whether there were any interactions with the core-shell components.

To determine the phase transition temperatures of the nanoparticles content n-3 PUFA and glyceryl monostearate (GMS), dried samples were placed in open aluminum pans. An empty pan was used as a reference pan compared with sample pan. Baseline correction and data treatment were conducted with OriginPro 9.85 (OriginLab, Northampton, Massachusetts, USA).

3.4.4.2 Fourier-transformed infrared spectroscopy (FTIR)

In order to evaluate potential interactions between functionalized ligands and excipients within the lipid nanoparticles, a FTIR spectrum was acquired. The samples, consisting of solid lipid, liquid lipid, glucose-functionalized ligand, surfactant, physical mixture, non-target lipid nanoparticles loaded with tuna oil and algal oil, as well as targeting lipid nanoparticles loaded with tuna oil and algal oil, were meticulously ground with potassium bromide (KBr) to generate an infrared transparent matrix. Subsequently, FTIR scanning was performed using a Shimadzu Corporation FTIR-8400S spectrometer, covering the spectral range of 4,000 to 500 cm^{-1} with a resolution of 1.0 cm^{-1} . Baseline correction and data treatment were conducted with OriginPro 9.85.

3.4.5 In vitro storage stability

The assessment of storage stability was conducted following the methodology outlined by Chang and Nickerson (2018) with some modifications. unencapsulated oil and oil synthesized within nanoparticles (n=3) were stored at 4°C in stability cabinets during 4 wks. The hydrodynamic diameter (HD) was measured at different time points by dynamic light scattering (DLS) using a Malvern Zetasizer at an angle of 90° at 25°C and the oxidative stability was investigated by measuring peroxide value (PV) and 2-thiobarbituric acid reactive substances (TBARS value).

The determination of the primary oxidation products was carried out using the procedure outlined by Salminen et al. (2013). In order to measure the peroxide value (PV), the sample was added to 9.8 mL of a chloroform: methanol mixture (2:1, v/v) and mixed for 5 sec on a vortex mixer. Subsequently, a solution of ammonium thiocyanate (50 μL) was introduced into the mixture, followed by the addition of iron (II) solution (50 μL). The iron (II) solution was prepared by combining BaCl_2 and FeSO_4 at a final concentration of 0.144 mol/L, resulting in individual concentrations of 0.132, respectively. After 5 min incubation period at room

temperature, the absorbance of the samples was measured at 500 nm against a blank (solution without any sample) by a Shimadzu-1800 UV-visible spectrophotometer (Shimadzu, Tokyo, Japan). The quantities of lipid hydroperoxides were determined by employing an external standard curve constructed with cumene hydroperoxide (purity 80%, sigma-Aldrich Co, USA) with concentrations ranging between 0 and 16 μ M.

The secondary oxidation products were monitored by quantification of the thiobarbituric acid (TBA) reactive substances (TBARS) as described by Wang et al. (2015). In brief, the samples were mixed with 1.8 mL of deionized water and 4.0 mL of TBA solution. TBA solution was prepared by dissolving 15 g of trichloroacetic acid (15% w/v) and 0.375 g of TBA (0.375%) in 100 mL of 0.25 mol/L HCl. In the following, the mixtures were heated in a boiling water bath for 15 min and then cooled to room temperature. Finally, the mixtures were centrifuged (2000 g for 15 min). The intensity of the color created as a result of the reaction between TBA with malondialdehyde (MDA), an important by-product of lipid peroxidation, was measured at 532 nm. The standard curve of 1,1,3,3-tetraethoxypropane was used to determine the MDA concentrations.

3.4.6 Experimental design for in vivo study

All procedures in the present study were approved by the Ethics Committee on Animal Use of the Suranaree University of technology, Nakhon Ratchasima, Thailand (SUT-IACUC-009/2021). The experiment was conducted at SUT farm. The experimental model employed a completely randomized design, comprising three treatments and three replicates. The treatments consisted of control, tuna oil-loaded non-targeted lipid nanoparticles and tuna oil-loaded targeted lipid nanoparticles. Thirty-three female chickens, aged 56 days with an average weight ranging from 750 to 800 g, were individually housed in cages for a period of 7 days as an adaptation phase prior beginning the experiment. The chickens received commercial diet and water ad libitum for the whole period and withdrawal feeding 12 h before investigation. At 63 days of age, the lipid nanoparticles (2 mL for each chicken) were administered through oral administration at once, utilizing a syringe affixed to a designated feeding tube. After administration, the chickens were euthanized by cervical dislocation at distinct time intervals after gavage, 2, 4, 8, 12, and 24 h (3 chickens per treatment and per time interval). At each time point, all the breast muscle of the

chickens was harvested and stored at -25°C for imaging purposes, as well as for the subsequent analysis of fatty acid composition.

3.4.7 *In vivo* imaging of tissue biodistribution

The investigation of nanoparticle distribution in muscle samples involved assessing the quantity of nanoparticles deposited in the target organ by utilizing the optical *in vivo* imaging system (IVIS), which captured the signal emitted by the fluorescent molecules. The fluorescence was observed within the appropriate wavelength range during the absorption (excitation) and emission processes. This methodology bears resemblance to a confocal laser-scanning microscope. However, the IVIS system is specifically designed for detecting signals from larger specimens. In this study, this instrument was employed to validate the presence of lipid nanoparticles within the target organ, the muscle.

The *in vivo* imaging of nanoparticle accumulation in birds was assessed following the methodology described by Qin et al. (2016). The birds were administered NR-NPs or NR-Glu-NPs orally at a dose of 1 mg/kg (equivalent to 3.14 μ M, 2 mL) via oral gavage. Samples were eliminate unwanted fluid before quantify the near-infrared fluorescence signal intensities by using an In Vivo Imaging System MS FX PRO (Carestream Health Inc., Rochester, NY, USA) with an excitation band pass filter at 540 nm and an emission at 600 nm. Images were processed using Bruker Molecular Imaging software version 7.1.3.20550 (Bruker, Billerica, MA, USA). Mean intensity was performed to analyze statistics. The Nile red concentration was equivalent in all experiments (n=3 birds per treatment).

3.4.8 Fatty acid composition

The lipids were extracted from approximately 5 g of each muscle sample using 90 mL of chloroform: methanol (2:1, v/v) (Folch et al., 1957). After that, the methylation was conducted with around 20 to 25 mg of extracted fat according to the method described by Metcalfe et al. (1966). The fatty acid methyl esters (FAME) were analyzed using gas chromatography (Hewlett-Packard 7890A; Agilent Technologies, Santa Clara, CA, USA) with a capillary column (SP 2560, Supelco Inc., Bellefonte, PA, USA, 100 m $\times$ 0.25 mm i.d., 0.20- μ m film thickness) and a flame ionization detector. The carrier gas was helium at a flow rate of 0.95 mL/min. The temperatures of the injector and detector were 260°C. The initial column temperature was 70°C. It raised to 175°C at a rate of 13°C/min, and then to 240°C at a rate of 4°C/min. Compound

were identified and quantified by using Masshunter software (v10.0.707.0, Agilent, USA). Coefficient of efficacy (%) was performed to evaluate the potential of lipid nanoparticles using the following formula. Where, FAn = Fatty acid amount:

$$\text{Coefficient of efficacy (\%)} = \left(\frac{\text{FAn in sample treated} - \text{FAn in untreated}}{\text{FAn in suspensions used}} \right) \times 100$$

3.4.9 Statistical analysis

Analysis of variance (ANOVA) will be performed using the GLM procedure for a completely randomized design in SPSS Version 18.0 (SPSS Inc., Chicago, IL, USA; University Edition) with the following statistical model:

$$Y_{ij} = \mu + \tau_i + \epsilon_{ij}$$

Where: Y_{ij} = the dependent variable, μ = the overall mean, τ_i = the treatment effect, and ϵ_{ij} = the random residual error. The means of the Nile red fluorescence intensity were assessed by t-test. Significant differences between treatment means were assessed by Tukey's multiple comparison tests after a significant F-test. The level of statistical significance will establish at $P < 0.05$. The values for physical characteristic of lipid nanoparticles were expressed as means $\pm$ standard error (SE) and the value for storage stability and fatty acid composition were expressed as means $\pm$ standard error of the mean (SEM), which represents the pooled SEM for the model.

3.5 Results

3.5.1 Physicochemical characteristics of lipid-based nanoparticles containing n-3 PUFA oil

The physical characterization of nanoparticles containing n-3 PUFA oils are presented in Table 3.2. The mean particle diameter of the NPs groups was higher than that of the TNPs groups ($P \leq 0.001$) whereas tuna oil in both nanoparticle forms had smaller particle diameters than algal oil ($P \leq 0.001$). The TO_TNPs group had the highest PDI value ($P < 0.001$) and the NPs groups had higher zeta-potential values than the TNPs groups ($P < 0.001$) and the value was highest in AO_NPs group.

Table 3.2 Physical characteristic of n-3 PUFA oils within different type of lipid-nanoparticles¹.

Treatment	Average diameter (nm)	Polydispersity index	Zeta-potential (mV)
TO_NPs	223.7 ± 1.01 ^c	0.315 ± 0.014 ^b	-42.6 ± 0.47 ^b
AO_NPs	294.4 ± 1.83 ^d	0.261 ± 0.007 ^a	-34.2 ± 0.46 ^c
TO_TNPs	134.7 ± 1.18 ^a	0.365 ± 0.008 ^c	-48.1 ± 0.68 ^a
AO_TNPs	184.0 ± 1.21 ^b	0.298 ± 0.010 ^{ab}	-49.4 ± 0.67 ^a
P-value	≤ 0.001	≤ 0.001	≤ 0.001

¹ Two different n-3 PUFA oil sources (tuna or algal, TO or AO) within different type of lipid targeted (TNPs) or not (NPs) nanoparticles.

Data are presented as mean ± SE (n = 3). ^{a-d} Values in the same column with different superscripts letters are significantly different (P < 0.05).

3.5.2 Thermal and chemical properties of lipid-based nanoparticles

The melting point of GMS a raw material used for nanoparticle synthesis was 76.6°C, whereas the nanoparticle suspension including TO_NPs, AO_NPs, TO_TNPs and AO_TNPs showed lower endothermic peaks at 46.8, 46.4, 45.3 and 45.0°C, respectively (Figure 3.2). Both the n-3 PUFA sources and nanoparticle types changed the melting point in nanoparticle form by lipid nanoparticles composite of algal oil and the targeting groups had the lowest melting points of lipid nanoparticles.

The FTIR analysis of nanomaterials and lipid-based nanoparticles is shown in Figure 3.3. The peak intensity of lipid nanoparticles was more flatten than individual spectra of each material. It can be concluded that tuna crude oil was packed tightly inside the nanoparticles. Due to functional group which is composite in the structure of lipid, triglyceride and fatty acid that could be found in tween 20, alkyl polyglucoside, GMS and tuna oil, the peak intensity decreased after the synthesis into lipid-based nanoparticles.

The FTIR spectra show in Figure 3.3 (a), The bands related to hydroxyl group (3499-3233 cm⁻¹, -OH stretching) (Banu et al., 2015) was found in tween 20, poloxamer, alkyl polyglucoside and GMS. For lipid (2921 - 2850 cm⁻¹, asymmetric and symmetric stretching vibration of CH₂ groups in lipid alkyl chain, **V**_{asym} CH₂ and **V**_{sym} CH₂) (Kiefer et al., 2010) and fatty acid (1641 cm⁻¹, C=C stretching, 1470 – 1460 cm⁻¹, -CH₃

bending and $1240 - 1207 \text{ cm}^{-1}$, $-\text{CH}_2$ bending) (Gholami et al., 2017; Raeisi et al., 2019; Takeungwongtrakul, 2020) were found in tween 20, alkyl polyglucoside, and tuna oil. The ester linkages ($1741 - 1734 \text{ cm}^{-1}$, ester carbonyl group ($-\text{C}=\text{O}$)) (Gholami et al., 2017) was found in GMS, tween 20 and triglyceride in tuna oil but not occurred in alkyl polyglucoside and poloxamer. The cyclic ether (1029 cm^{-1} , cyclic ether ($-\text{COC}$) and pyranose ring (1150 cm^{-1} , $-\text{COC}$ glycosidic bond) (Md Salim et al., 2021) were found in alkyl polyglucoside. The wavelength at 720 cm^{-1} indicated that this long chain carbon (Ng et al., 2018) was found in GMS, tween 20, alkyl polyglucoside, and tuna oil. In addition, the wavelength ranging at $2921 - 2850 \text{ cm}^{-1}$ ($-\text{CH}$ stretching) and $1500 - 500 \text{ cm}^{-1}$ ($-\text{CH}$ bending and long chain carbon) had a reduced intensity in physical mixture and synthesized of lipid nanoparticles.

The analysis suggests that the molecules in the mixture are undergoing physical interactions and rearrangements, likely driven by Van der Waals forces rather than the formation of new chemical bonds. The disappearance or weakening of peaks in the FTIR spectrum indicates that the molecular environment has changed, but without the creation of new covalent structures. This reinforces the idea that the synthesis primarily involves noncovalent interactions rather than chemical modifications.

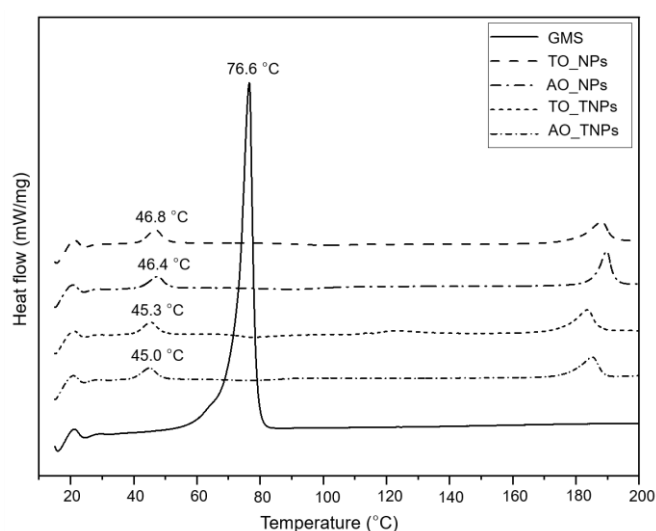


Figure 3.2 Differential scanning calorimetry (DSC) thermographs of nanomaterial including glyceryl monostearate (GMS), and lipid-based nanoparticles with tuna oil (TO) or algal oil (AO) and targeted (TNPs) or not (NPs).

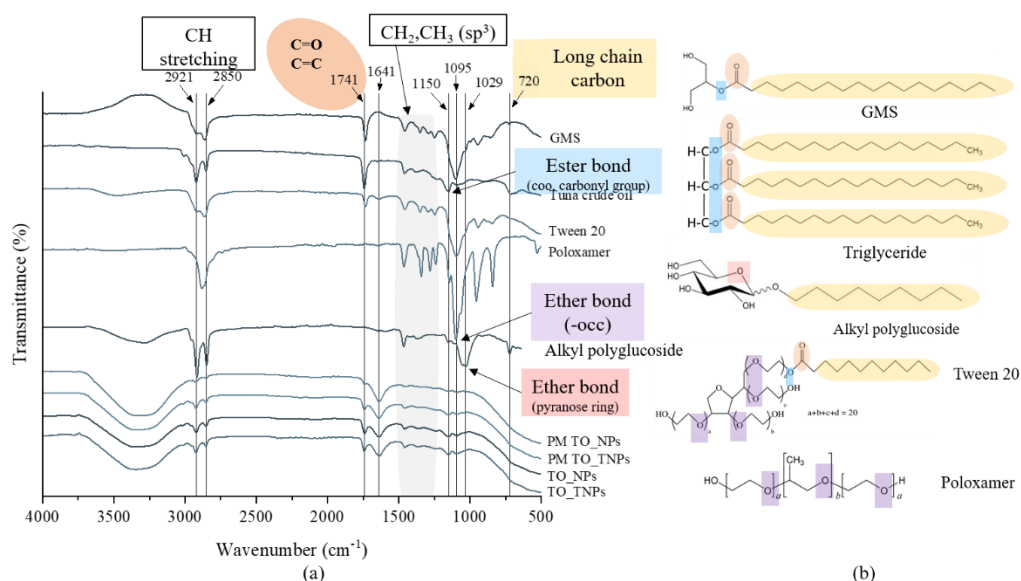


Figure 3.3 (a) FTIR spectra of nanomaterial, physical mixture (PM) and lipid-based nanoparticles (NPs and TNPs) and (b) chemical structure of nanomaterials (GMS, tuna crude oil, tween 20, poloxamer, alkyl polyglucoside (APG)).

3.5.3 In vitro storage stability of n-3 PUFA lipid-based nanoparticles

3.5.3.1 Particle size stability

The results of the storage stability on particle size of the n-3 PUFA lipid-based nanoparticles are shown in Table 3.3. The synthesis of n-3 PUFA source oils in the form of targeted-lipid nanoparticles groups (TO_TNPs and AO_TNPs) can help to maintain the size of the nanoparticles, but the storage conditions were better at low temperature storage (4°C) for 4 wks, while the particle size of the non-targeted lipid nanoparticles groups changed at a faster rate at all temperature storage (TO_NPs_4°C, TO_NPs_RT, AO_NPs_4°C and AO_NPs_RT).

However, during the storage, there were statistically significant changes in nanoparticle size for the AO_NPs_4°C ($P < 0.05$), AO_NPs_RT ($P < 0.01$), TO_TNPs_RT ($P < 0.05$), TO_TNPs_RT ($P < 0.001$) and AO_TNPs_RT ($P < 0.001$).

During the storage, the change in nanoparticle size was faster at room temperature than at 4°C. In addition, the use of algal oil resulted in changes particle size faster in lipid-based nanoparticles (AO_NPs_4°C, AO_NPs_RT, and AO_TNPs_RT).

3.5.3.2 Oxidative stability evaluated with peroxidative value

The peroxidative values are presented in the Table 3.4. The unencapsulated tuna oil (TO_4°C) and tuna oil within lipid nanoparticles were stored at 4°C (TO_NPs_4°C and TO_TNPs_4°C) and had reduced peroxidative values ($P < 0.001$) compared to the groups kept at room temperature except at the start of the experiment where the values were not different. Moreover, no statistically significant differences in peroxide production in unencapsulated algal oil were found between the two storage temperatures (AO_4°C and AO_RT) from wk 1 to wk 4.

In addition, algal oil within lipid nanoparticles (AO_NPs_4°C, AO_NPs_RT, AO_TNPs_4°C and AO_TNPs_RT at first day) showed a statistically highly significant ($P < 0.001$) increase in peroxide value after synthesis.

The synthesis of oil within nanoparticle form and storage at suitable temperature influenced the peroxidative value reaction. Pre-synthetic and post-synthetic oils in nanoparticle form kept at low temperature (4°C) showed a statistically significant ($P < 0.05$) slowdown of peroxidative activity by comparison with the storage at room temperature from wk 1 to wk 3.

Table 3.3 In vitro stability of n-3 PUFA (tuna oil or algal oil, TO or AO) lipid-based targeted (TNP) or not (NP) nanoparticles on particle diameter (nm) during storage at 4°C or room temperature (RT).

Treatments	Start	Wk 1	Wk 2	Wk 3	Wk 4	SEM	P-value
TO_NPs_4°C	225.23 ^{cB}	215.83 ^{cA}	233.37 ^{cB}	197.03 ^{bA}	230.02 ^{cB}	4.09	< 0.01
TO_NPs_RT	223.50 ^{cA}	233.83 ^{dA}	258.83 ^{cB}	258.8 ^{cB}	268.88 ^{dB}	6.02	< 0.05
AO_NPs_4°C	303.53 ^{dAB}	328.93 ^{eB}	316.87 ^{dA}	300.4 ^{dA}	320.03 ^{eA}	3.62	< 0.05
AO_NPs_RT	299.60 ^{dA}	332.17 ^{eB}	341.83 ^{dB}	358.53 ^{eB}	344.78 ^{fB}	5.93	< 0.01
TO_TNPs_4°C	136.50 ^a	137.9 ^a	139.67 ^a	142.73 ^a	139.4 ^a	1.39	0.853
TO_TNPs_RT	134.00 ^{aA}	137.47 ^{aA}	131.23 ^{aA}	136.5 ^{aA}	140.82 ^{aB}	1.04	< 0.05
AO_TNPs_4°C	176.7 ^b	183.7 ^b	177.57 ^b	178.8 ^b	183.51 ^b	1.21	0.312
AO_TNPs_RT	176.4 ^{bA}	187.53 ^{bB}	194.00 ^{bB}	192.13 ^{bB}	195.09 ^{bC}	1.92	< 0.001
SEM	12.92	14.89	15.40	15.43	15.23		
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		

For each attribute, different small letters indicate statistically significant ($P < 0.05$), highly significant differences ($P < 0.01$) and very highly significant differences ($P < 0.001$) between different samples at a same time of storage. Different capital letters in a same row indicate very highly significant differences ($P < 0.001$) over time.

Table 3.4 Stability of n-3 PUFA (tuna oil or algal oil, TO or AO) lipid-based targeted (TNP) or not (NP) nanoparticles evaluated with peroxidative value (meq of oxygen/kg fat) during a storage at room temperature (RT) or 4°C.

Treatment	Start	Wk 1	Wk 2	Wk 3	Wk 4	SEM	P-value
TO_4°C	0.106 ^{dB}	0.110 ^{cdB}	0.113 ^{cdB}	0.079 ^{cA}	0.184 ^{cC}	0.009	< 0.001
TO_RT	0.113 ^{dA}	0.160 ^{fB}	0.213 ^{iC}	0.153 ^{eB}	0.306 ^{dD}	0.018	< 0.001
AO_4°C	0.033 ^{aC}	0.022 ^{aA}	0.018 ^{aA}	0.026 ^{aB}	0.017 ^{aA}	0.002	< 0.001
AO_RT	0.032 ^{aA}	0.031 ^{aA}	0.032 ^{aA}	0.041 ^{abB}	0.035 ^{aAB}	0.001	< 0.001
TO_NPs_4°C	0.094 ^{cAB}	0.108 ^{cBC}	0.129 ^{deD}	0.119 ^{dCD}	0.085 ^{bA}	0.005	< 0.001
TO_NPs_RT	0.095 ^{cA}	0.130 ^{deB}	0.196 ^{hiC}	0.211 ^{fc}	0.112 ^{bAB}	0.013	< 0.001
AO_NPs_4°C	0.113 ^{dA}	0.153 ^{fB}	0.161 ^{fgB}	0.164 ^{eB}	0.161 ^{cB}	0.006	< 0.001
AO_NPs_RT	0.111 ^{dA}	0.210 ^{gCD}	0.181 ^{ghBC}	0.242 ^{gD}	0.159 ^{cB}	0.012	< 0.001
TO_TNPs_4°C	0.059 ^{bB}	0.064 ^{bB}	0.064 ^{cB}	0.062 ^{bcB}	0.039 ^{aA}	0.003	< 0.001
TO_TNPs_RT	0.063 ^{bA}	0.116 ^{cdC}	0.097 ^{cB}	0.107 ^{dBC}	0.102 ^{bBC}	0.005	< 0.001
AO_TNPs_4°C	0.107 ^{dA}	0.099 ^{cA}	0.127 ^{dB}	0.108 ^{dA}	0.103 ^{bA}	0.003	< 0.001
AO_TNPs_RT	0.108 ^{dA}	0.140 ^{efB}	0.149 ^{efBC}	0.164 ^{eC}	0.114 ^{bA}	0.006	< 0.001
SEM	0.005	0.009	0.01	0.011	0.013		
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		

For each attribute, different small letters indicate very highly significant over time ($P < 0.001$) between different samples at a same time of storage. Different capital letters in a same row indicate highly significant differences over time ($P < 0.001$).

3.5.3.3 Oxidative stability evaluated with TBARS value

The malondialdehyde values are presented on the Table 3.5. Synthesizing tuna oil into the targeted-lipid nanoparticle increased the oxidation stability as the amount of MDA was lower in the non-targeted lipid nanoparticles but

for algal oil, the reverse was observed. Moreover, storage at low temperature (4°C) slow down the production rate of MDA. The synthesized algal oil within lipid nanoparticles in the experimental groups (AO_NPs_4°C, AO_NPs_RT, AO_TNPs_4°C and AO_TNPs_RT) had a greater increase in MDA than the unsynthesized algal oil (AO_4°C and AO_RT).

Table 3.5 Stability of n-3 PUFA (tuna oil or algal oil, TO or AO) lipid-based targeted (TNP) or not (NP) nanoparticles evaluated with malondialdehyde value ($\mu\text{mol MDA/kg oil}$) during storage at room temperature (RT) or 4°C.

Treatment	Start	Wk 1	Wk 2	Wk 3	Wk 4	SEM	P-value
TO_4°C	14.01 ^{eC}	10.81 ^{dB}	7.95 ^{bcA}	14.89 ^{dC}	13.32 ^{deC}	0.69	< 0.001
TO_RT	13.76 ^{eB}	14.08 ^{efB}	17.97 ^{fC}	6.53 ^{cA}	17.9 ^{fC}	1.13	< 0.001
AO_4°C	1.23 ^{aB}	1.53 ^{aC}	1.25 ^{aB}	0.70 ^{aA}	2.15 ^{aD}	0.13	< 0.001
AO_RT	1.23 ^{aA}	2.36 ^{aC}	1.60 ^{aB}	1.40 ^{abAB}	2.82 ^{aD}	0.17	< 0.001
TO_NPs_4°C	10.61 ^{dA}	18.88 ^{gC}	9.41 ^{cA}	14.92 ^{dB}	15.65 ^{efB}	0.94	< 0.001
TO_NPs_RT	10.61 ^{dA}	22.42 ^{iD}	12.38 ^{dB}	15.72 ^{dC}	21.73 ^{gD}	1.29	< 0.001
AO_NPs_4°C	9.21 ^{cB}	15.38 ^{fC}	9.10 ^{cB}	6.75 ^{cA}	8.51 ^{cB}	0.79	< 0.001
AO_NPs_RT	8.88 ^{cA}	21.06 ^{hC}	21.65 ^{gC}	20.75 ^{eC}	13.55 ^{deB}	1.39	< 0.001
TO_TNPs_4°C	13.70 ^{eC}	13.03 ^{eC}	6.42 ^{ba}	8.58 ^{cB}	9.07 ^{cB}	0.76	< 0.001
TO_TNPs_RT	13.64 ^{eA}	14.58 ^{fA}	14.70 ^{eA}	21.74 ^{eB}	21.68 ^{gB}	1.03	< 0.001
AO_TNPs_4°C	4.54 ^{bB}	4.31 ^{bB}	2.92 ^{aA}	3.00 ^{ba}	5.64 ^{bc}	0.28	< 0.001
AO_TNPs_RT	4.54 ^{ba}	7.89 ^{cB}	8.15 ^{cB}	7.61 ^{cB}	11.4c ^{dC}	0.61	< 0.001
SEM	0.78	1.14	1.03	1.17	1.09		
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		

For each attribute, different small letters indicate very highly significant differences ($P < 0.001$) between different samples at a same time of storage. Different capital letters in a same row indicate very highly significant differences ($P < 0.001$) over time.

3.5.4 Transfer of lipid nanoparticles to the target organ

The Nile red distribution in *Pectoralis major* sample is shown in Figure 3.4. The Nile red distribution was increased within targeted-lipid nanoparticles (TNPs) at 8

and 12 h after oral gavage by comparison with non-targeted lipid nanoparticles (NPs). Then it decreased to the initial content at 24 h. The results demonstrated that the targeted lipid nanoparticles had the potential to be absorbed into the bloodstream and transferred to the target organ faster than other forms of nanoparticles.

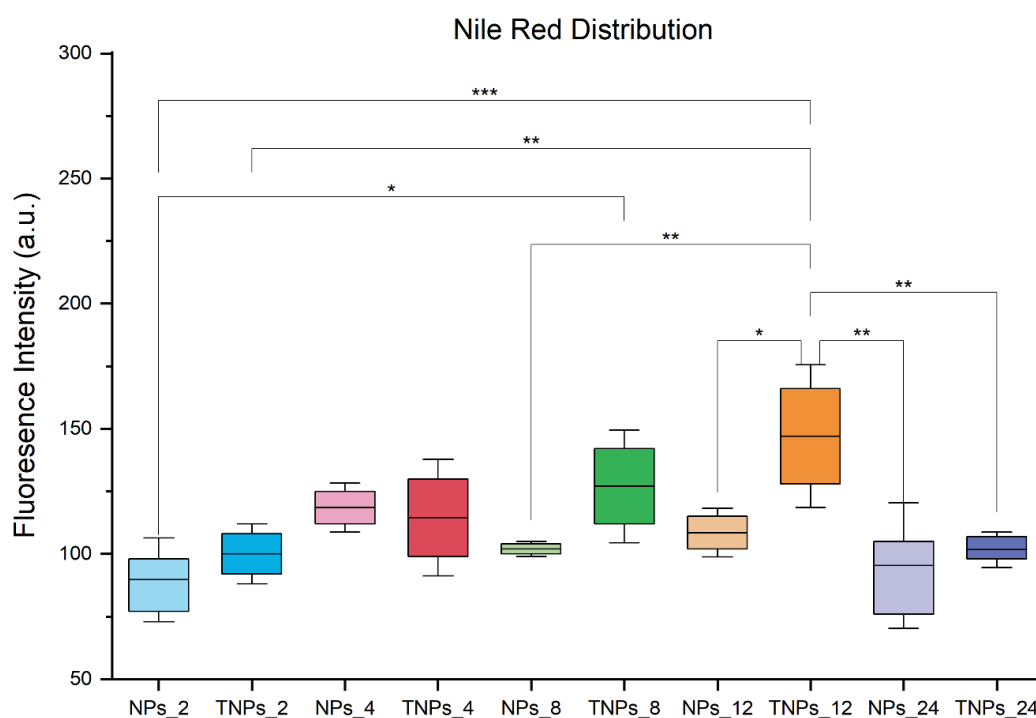


Figure 3.4 Nile Red distribution in Pectoralis major muscle of chicken imaging by IVIS of treatment group including non-targeted lipid nanoparticles (NPs) and targeted lipid nanoparticles (TNPs) at 2, 4, 8, 12 and 24 h after oral administration. The data are presented as bar graphs showing means $\pm$ SD (n=3). * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

3.5.5 Fatty acid composition of breast meat of Korat chicken

The fatty acid profile of breast meat of the chickens after oral gavage with the lipid-based nanoparticles is shown in Table 3.6. C18:3n-3 content in breast meat of TNPs treatment at 4 h was decreased ($P < 0.05$) than that in the NPs treatment. NPs treatment at 4, 8 and 12 equal to control ($P > 0.05$) whereas TNPs treatment at all time point lower than control ($P < 0.05$). The EPA content in TNPs group at 4 h was

higher ($P < 0.05$) than that in the NPs treatment. The DHA content in TNPs treatment at 4 h was higher ($P < 0.05$) than that in the NPs. By the way, the highest of DHA content was showed in the TNP group at 24 h. No significant different in EPA contents at 2, 8, 12 and 24 h, and DHA content at 2, 8 and 12 h compared between NPs and TNPs treatment. C18:2n-6 contents no different compared between NPs and TNPs at each time. C20:4n-6 contents in TNPs treatment at 24 h higher ($P < 0.05$) than that in NPs treatment. But at 2, 4, 8 and 12 h showed no difference ($P > 0.05$). C18:1n-9 contents in TNPs treatment at 24 h were lowest ($P < 0.05$) compared to that in NPs and control treatment.

Table 3.6 Fatty acid composition of TO oil within different type of lipid-nanoparticles¹ at 2, 4, 8, 12 and 24 h after oral administration.

Treatment	Fatty acid composition (% of total fatty acids)						
	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	C20:4n-6	EPA	DHA
Control	9.31	20.33 ^b	22.94 ^b	0.35 ^d	23.21 ^{ab}	0.21 ^{ab}	1.71 ^{ab}
NPs at 2h	9.13	20.23 ^b	21.01 ^{ab}	0.22 ^{abc}	26.11 ^{ab}	0.20 ^{ab}	1.63 ^{ab}
TNPs at 2h	9.76	19.52 ^b	21.69 ^b	0.24 ^{bc}	24.65 ^{ab}	0.27 ^{ab}	1.85 ^{abc}
NPs at 4h	9.84	21.75 ^b	21.09 ^{ab}	0.32 ^{cd}	22.47 ^{ab}	0.16 ^a	1.57 ^a
TNPs at 4h	9.87	18.60 ^b	20.18 ^{ab}	0.20 ^{ab}	26.60 ^{ab}	0.28 ^b	2.46 ^d
NPs at 8h	9.63	20.64 ^b	22.35 ^b	0.29 ^{bcd}	22.02 ^{ab}	0.21 ^{ab}	1.82 ^{abc}
TNPs at 8h	8.96	20.16 ^b	20.83 ^{ab}	0.22 ^{abc}	25.23 ^{ab}	0.25 ^{ab}	2.40 ^{cd}
NPs at 12h	8.55	25.89 ^{bc}	22.13 ^b	0.27 ^{bcd}	18.75 ^a	0.20 ^{ab}	1.78 ^{abc}
TNPs at 12h	8.38	23.09 ^{bc}	21.85 ^b	0.23 ^{abc}	22.38 ^{ab}	0.23 ^{ab}	2.22 ^{bcd}
NPs at 24h	9.24	19.75 ^b	20.21 ^{ab}	0.20 ^{ab}	27.77 ^b	0.23 ^{ab}	2.55 ^d
TNPs at 24h	9.15	14.49 ^a	17.50 ^a	0.12 ^a	35.62 ^c	0.29 ^b	3.43 ^e
SEM	0.12	0.53	0.32	0.01	0.84	0.01	0.10
P-value	0.058	< 0.001	< 0.01	< 0.001	< 0.001	< 0.05	< 0.001

¹ Tuna oil (TO) within different type of lipid targeted (TNPs) or not (NPs) nanoparticles. Statistical analysis is based on F-test. For each attribute, different small letters indicate statistically significant ($P < 0.05$), highly significant differences ($P < 0.01$) and very highly significant differences ($P < 0.001$).

3.5.6 Coefficient of efficacy of lipid-nanoparticles

The coefficient of efficacy of targeted and non-targeted lipid nanoparticles are showed in Table 3.7. TNPs had higher potential to transfer EPA and DHA into the chicken skeletal muscle than the NPs groups. The coefficient of efficacy of TNPs was higher than that of the NPs treatment in each time. At 2 h both NPs and TNPs treatments were able to transfer the n-3 PUFA but after that the TNPs treatment showed higher potential to transfer the n-3 PUFA at 4 and 24 h in TNPs treatment. The n-3 PUFA amount at 8 and 12 h after oral administration was decreased. In contrast, the n-3 PUFA content in NPs treatment was reduced at 4 h then increased slightly until 24 h.

Table 3.7 Coefficient of transfer efficacy of TO within different type of lipid-nanoparticles¹ at 2, 4, 8, 12 and 24 h after oral administration relative to control.

Treatment	Coefficient of efficacy (%)	
	EPA	DHA
Control	0.002	0.013
NPs at 2h	0.62	0.76
TNPs at 2h	2.32	2.27
NPs at 4h	-1.41	-1.85
TNPs at 4h	2.55	9.65
NPs at 8h	0.06	0.64
TNPs at 8h	0.80	4.93
NPs at 12h	0.55	2.85
TNPs at 12h	1.65	6.52
NPs at 24h	1.07	9.70
TNPs at 24h	2.38	16.98

¹ Tuna oil (TO) within different type of lipid targeted (TNPs) or not (NPs) nanoparticles.

3.6 Discussion

3.6.1 Effect of n-3 PUFA source on the physicochemical characteristics of lipid-based nanoparticles

By comparison with AO nanoparticles, TO nanoparticles had lower diameter, higher PDI and lower ZP. AO contains a high amount of long-chain unsaturated fatty acids such as DHA. The fatty acid can move to the surface of the nanoparticles, resulting in an enlarged particle size (Caldas et al., 2021). It seems to be indicated the n-3 PUFA oil can change the uniformity of the nanoparticle diameter. Moreover, the PD index involved their particle size population. As is obviously in AO nanoparticles lower PD index than TO nanoparticles. A larger PD index value indicated a broad particle size distribution of the formulation whereas a low value of the PD index indicated monodisperse samples (Mudalige et al., 2019). This implies that AO can maintain the uniformity of particle size better than TO. In reference to the quality of TO contains phospholipids (Šimat et al., 2019), this compound had potential to lesser particle size (Sreedhar et al., 2019) meanwhile can increase zeta-potential value with their negative charged (Álvarez-Benedicto et al., 2022).

TO and AO was applied in this present study is unrefined oil and refined oil, respectively. Unrefined TO contains impurities such as phospholipid, free fatty acid, aldehyde, ketones and pigment (Sathivel, 2005) that rapidly activated by pro-oxidant effect more than refined AO result to higher oxidized of TO at the beginning of experiment whereas algal oil could be better potent lipid peroxidation by greater quality, lower oxidative rate and might be prevented lipid oxidation by the carotenoids activity (Shi et al., 2020; Chang et al., 2020; Sehl et al., 2022). Thus, the lipid oxidative products were higher for TO than the AO at start of experiment.

On the reverse, TO_NPs had lower peroxidative values than AO-NPs, and TO_TNPs had lower peroxidative values than AO-TNPs. In contrast, the MDA content of TO_NPs and TO_TNPs was higher than that of AO-NPs and AO-TNPs. The possibility of the increase of PV value due to the sources had high free fatty acid induce lipid peroxidation stage (Tengku Mohamad & Birch, 2013). AO had higher PV after lipid nanoparticle synthesis indicated that the hydroperoxides was produced continuously with higher rate from these free fatty acids, especially the oxidation of n-3 PUFA (Gawel et al., 2004) whereas TO had lower oxidized rate which change into the MDA slowly

than that AO. By the way, the lower of the MDA content in AO because the changed of the n-3 PUFA oxidized into the secondary product (Nogueira et al., 2018). Moreover, the products higher in oxidation may have a low PV when the primary oxidation metabolites are largely metabolized to secondary oxidation metabolites (Ismail et al., 2016). Therefore, the MDA for AO in lipid nanoparticles may rapidly shifted to another compound quicker than TO in lipid nanoparticles. By the way, the further study needs to invested the volatile compound of n-3 PUFA oxidized to confirm the phenomenon. Therefore, tuna oil loaded in lipid nanoparticles was selected to further investigated the potential of the targeting process on the *in vivo* biodistribution, fatty acid profile, and coefficient of efficacy.

The lower melting point of lipid nanoparticles mixtures than that of GMS due to the lipid matrix within the nanoparticles. Oils containing n-3 PUFA (tuna oil and algal oil) have liquid lipid properties leading to convert crystalline state to an amorphous state due to the disordered arrangement of molecules (Zhu et al., 2015; Shahparast et al., 2019).

More than that, tuna oil in lipid nanoparticles has higher endothermic peak than that algal oil due to undesirable compounds of tuna oil. Unrefined oil or triglyceride are more complex than those of the fatty acids and required more thermal energy (Yin, 2011). Moreover, the melting point was affected by the high content in unsaturated fatty acids in the formulation (de Barros et al., 2021). The polyunsaturated fatty acids implying a larger space to be accommodated into the polymeric matrix indicate induced a more amorphous structure of the lipid carrier (Lacatusu et al., 2016). Thus, there are reasonable that the endothermic peak in TO treatment was shifted at higher temperature than that AO treatment. The DSC is an effective tool to investigate the melting behavior and crystalline state of nanocarriers and their impact on some properties such as stability of lipid nanoparticles, which is useful for further use in animal feed processing.

3.6.2 Effect of temperature and storage duration on the physicochemical characteristics of lipid-based nanoparticles

During the storage at room temperature (RT), the diameter of nanoparticles was higher than that of nanoparticles stored at 4°C particularly after 3 and 4 wks of storage particularly for NPs treatment whereas there was no effect of

temperature storage for TNPs treatment. The diameter of nanoparticles increased with the storage duration. Similarly with literature researches indicated the diameter of lipid nanoparticle was enlarged by storage for long time particularly unmodified-surface nanoparticles (Nejadmansouri et al., 2016; Sreedhar et al., 2019; Zaky et al., 2023). The reason is the aggregation of surfactants between nanoparticle (Khosa et al., 2018) or the interaction of reactive oxygen species (ROS) with surface of nanoparticle (Terada et al., 2021). In contrast, the modified-surface nanoparticles can resolve the conflict and enhance oxidative stability by preventing pre-oxidation on the surface of the nanoparticle boundary (Meulewaeter et al., 2023; Tang et al., 2023). Furthermore, the reduce particle size during storage due to the major compounds such as n-3 PUFA in the matrix were descended by high temperature (Shah et al., 2016; Meulewaeter et al., 2023).

The peroxidative value and the MDA level were higher during the storage at RT by comparison with the treatments stored at 4°C. The high temperature can increased the kinetic energy contact of oil with oxygen (Fadda et al., 2022). The higher of temperature had increase the rate of hydroperoxide decomposition. The reactivity of redox reactions had greater rates (Musakhanian et al., 2022). The amphiphilic lipid was reacted with the pro-oxidation at the interface and hydroperoxide was produced. The free radicals penetrate through the surface layer and stimulated the oxidation with n-3 PUFA (Wang et al., 2020). Thus, the study of storage stability with different temperature indicated that the lipid oxidation continuously occurred during of storage particularly under high temperature for unmodified-surface nanoparticles. This should be noted that the storage of n-3 PUFA in the lipid nanoparticle should be given priority for improving the sustainable quality prolonged enough for further procedures such as the drying process.

3.6.3 Effect of targeting process on the physicochemical characteristics of lipid nanoparticles

The smaller nanoparticles formability in TNPs treatments might caused by the alkyl polyglucoside that was not present in the NPs groups. The nanoparticles synthesis with targeting had greater ability to perform lipid nanoparticles (Ling et al., 2009; Kou et al., 2017; Tian et al., 2017a; Tian et al., 2017b).

The stable of particles size in TNPs groups were indicated that the alkyl polyglucoside which use as the targeting lipid nanoparticles, have protective property

to maintain the physical structure during storage. The change of particle size was faster in algal oil group possibly due to high amount of free fatty acids that have a carboxylic group with anionic, and are able to transfer into the surface of nanoparticles (Guillen and Cabo, 1997). However, the treatment AO-TNPs_4°C was different because the low temperature condition slowdown the breakdown of triglycerides to free fatty acids, particularly, n-3 PUFAs such as DHA that can affect the particle size (Caldas et al., 2021). It is possible that the algal oil group had higher amount of free fatty acids inducing an increase in the nanoparticle size.

Although in the previous studies demonstrated that the storage conditions no effect on particle size of nanoparticles (Averina et al., 2011; Zhu et al., 2015; Pornputtapitak et al., 2019; Saedi et al., 2019) contrast with present study shown kept at 4°C and RT are effect diameter of the unmodified surface nanoparticle. This indicated that NPs is unstable form due to may interact with ROS present at the interface or surrounding aqueous phase (Lúcio et al., 2023) such as impurities in crude oil which act as pro-oxidants (Cameron-Smith et al., 2015; Šimat et al., 2019).

In addition, the PDI values describe the width or spread of the particles size distribution of the nanoparticles ranged between 0.1 and 0.3 indicating a good stability of the emulsion (Awad et al., 2009). However, the TO_NPs group had the highest PDI value, possibly due to attractive hydrophobic interactions between the particles (Helgason et al., 2008). The use of liquid oils such as fish oil can make the nanoparticles more physically stable by reducing the crystalline structure. Therefore, it prevents the incorporation of nanoparticles (Awad et al., 2009) and increases PDI value. Moreover, the lower PDI value in AO nanoparticles indicated that is more dispersion in suspension than that TO nanoparticles. The PD Index is based on size of nanoparticles (Mudalige et al., 2019). The higher of the multiple particle size population show more PDI value while higher uniformity is lower polydisperse result to lower the PD Index (Lúcio et al., 2023).

Moreover, zeta-potential found in all groups were lower than -30 mV. The NPs groups had lower values than the TNPs groups, ($P < 0.001$). The synthesis including tween 20, poloxamer and alkyl polyglucoside, resulted in a decrease in the electric potential difference of the nanoparticles. This observation was previously reported by Surassmo et al. (2013).

For the NPs groups, the n-3 PUFA oils source influenced the zeta-potential value. This is due to the movement of carboxylic fatty acids in the region near the outer layer of the nanoparticles. This resulted in more negative charge conversion (Caldas et al., 2021). The TNPs groups contained alkyl polyglucoside. When this raw material is used in combination with a poloxamer, it results in a lower voltage difference than that observed for the NPs groups. Moreover, unrefined tuna oil contained phospholipids which are anionic groups (Oomah, 2001), and may have contributed to the high zeta-potential in TO_NPs group.

The melting temperature was higher for NPs nanoparticles than for TNPs nanoparticles because of targeting process effect on decreasing particle size. The size of particle became smaller resulting in decreased melting point (Akhoond Zardini et al., 2018). By the way, the surface-modification of lipid nanoparticles could be effect on increase the particle size of nanoparticles (Qin et al., 2016; Tunki et al., 2019) whereas the particle size was decreased in the present study due to the ratio of targeting surfactant in formulation (Luo et al., 2018).

It has been shown that the active substances and raw materials have similar properties, namely the lipid molecules, which affect the form of nanoparticles. The hydroxyl group ($3499\text{--}3233\text{ cm}^{-1}$, -OH stretching) was found in raw materials with properties as an emulsifier, including tween 20 (Banu et al., 2015), poloxamer (Kannan, 2013), alkyl polyglucoside (Pajić et al., 2017) and GMS (Weiseng et al., 2018). However, after the synthesis, the nanoparticles containing tuna oil had no effect on triglyceride degradation as it did not convert to free fatty acids, which should appear at wavelength of 1711 cm^{-1} (Guillen and Cabo, 1997).

The wavelength at 1095 cm^{-1} (-OCC, ether bond) provided by tween 20 and poloxamer implicated in the properties of emulsifier hydrophilic part of the molecule. The parts of raw materials, alkyl polyglucoside occur the wavelength at 1029 cm^{-1} indicated the vibration of functional group of cyclic ether (-COC) and Pyranose ring and 1150 cm^{-1} (-COC glycosidic bond) (Md Salim et al., 2021).

The peak wavelength was changed towards a lower intensity in the physical mixture and lipid nanoparticles. It indicated that the raw material was integrated into the system before the nanoparticles would be formed. Moreover, the peak that disappeared at wavelength below 1095 cm^{-1} in both mixtures was due to

the structure of the molecules (including CH-stretching, long chain carbon) that bind together very densely. The nanomaterial changed their position from its own specific properties. The groups that had the hydrophobic properties occurred together inside the particles, while the hydrophilic groups occurred around the periphery of the particles. This phenomenon presented the similar wavelength peak of -OH stretching a functional group found in the surfactant family of tween 20, alkyl polyglucoside, and poloxamer. Moreover, the apparent wavelength in the spectrum at $1741\text{--}1734\text{ cm}^{-1}$, 1641 cm^{-1} and 1150 cm^{-1} showed that the nanoparticles contained oil, such as tuna oil, inside the mixtures.

The wavelength ranging at $2921\text{--}2850\text{ cm}^{-1}$ (-CH stretching) and $1500\text{--}500\text{ cm}^{-1}$ (-CH bending and long chain carbon) disappeared or had a reduced intensity, indicating that the molecules in the mixture are moving according to the molecular properties mentioned above. These functional groups can be found in fat-containing raw materials, which have nonpolar molecules. This property is more prone to interactions together by noncovalent bonds such as van der Waals binding within particles (Aryani et al., 2021). Furthermore, the absence of new peaks in both mixtures showed that during the synthesis, there was no reaction that formed intermolecular covalent bonds (Pezeshki et al., 2014; Pornputtapitak et al., 2019).

The targeted-lipid nanoparticles were able to slow down the peroxidative reaction by comparison with the oil within non-targeted lipid nanoparticles because the surfactants act as protectors avoiding the inside oil to react with outside oxidants. The peroxidative value was significantly different in weekly storage of each treatment. This means that the initial lipid oxidation reaction was dynamic over the time storage and this constantly occurring leads to increase rate of the reaction (Palmquist and Jenkins, 2003). In addition, the amount of peroxide in some week was found to be low. This could mean that the substance has already converted to another molecule, or it may not have happened yet and because of the product of initial stage was quickly oxidized into the next stage.

However, the industry recommended peroxidative value for fish oil should not exceed 18 meq active O_2/kg after 1 month storage (Chang and Nickerson, 2018). So, the n-3 PUFA lipid nanoparticles can be kept more than one month because their peroxidative values are lower than the recommended value. By the ways, further

study should carry out the long-term storage period to confirm our data on storage stability for commercial products.

The MDA content were higher for NPs nanoparticles than for TNPs nanoparticles suggesting a higher susceptibility to oxidation for NPs nanoparticles. May be, the triglycerides were hydrolyzed to free fatty acids with negatively charged carboxylic groups causing deposition in the outer layer of the nanoparticles. The resulting negative ions induced lipid oxidation (Komaiko et al., 2016). During the synthesis, lipids were heated may be favoring fatty acid oxidation.

3.6.4 Effect of targeting process on the efficiency of dietary n-3 PUFA deposition in muscles

The measurement of lipid-based nanoparticles by in vivo study indicated that the targeted lipid-based nanoparticles showed the potential to transfer the fatty acids directly into skeletal muscle cells of Korat chickens.

Nile red staining showed that FA deposition in muscles was more efficient by using TNPs compared to NPs nanoparticles at 8 h and 12 h after oral gavage with lipid-based nanoparticles. The lipid nanoparticles seem to act as a target carrier specific to the receptors at the microvilli. Specific-transporters such as SGLT1 and GLUT 2, 5, 7, etc., serve to accept single molecules such as glucose and galactose into enterocytes (Cohen et al., 2014; Poulsen et al., 2015; Byers et al., 2017). For EPA deposition, the amount deposited 4 h after oral gavage was higher with TNPs nanoparticles compared with NPs nanoparticles. There was no difference between treatments for the other time points for EPA deposition. For DHA deposition, the amount deposited between 4 h and 24 h post-gavage was higher with TNPs nanoparticles compared with NPs nanoparticles. TNPs treatment was more efficient than NPs treatment and the efficacy of deposition was higher for DHA than for EPA that could be explained by a higher amount of DHA in nanoparticles compared to the EPA content.

The results revealed the potential for both the receptors to function as individual molecules within the cell and to bind to those same molecules when attached to the nanoparticles and introduced into the cell. For example, nanoparticle uptake by microvilli receptor-specific monomolecular such as galactose (Siu et al.,

2018), L-carnitine (Kou et al., 2017) and taurocholic acid (Tian et al., 2017b) has been studied in digestive tract and muscle cells (Yeh et al., 2014).

The utilization of receptor-specific molecules enhances the uptake of essential substances into the bloodstream and subsequent delivery to the targeted organs (Qin et al., 2016; Tunki et al., 2019). There are indications that intestinal cells possess mechanisms capable of responding to the absorption of active substances in the nanoparticle form, wherein these nanoparticles are transported into the bloodstream through penetration into intestinal cells and subsequently directed towards target cells with specific transporter including GLUT via the endocytosis pathways (Grefner et al., 2015).

However, intracellular transport is divided into several pathways that affects the delivery of nanoparticles into the bloodstream differently (Settembre and Ballabio, 2014). Consequently, the comprehension and elucidation of the nanoparticle delivery pathway in functional intestinal cells at the molecular level will enable the identification of the underlying processes involved in nanoparticle transmission to the target organs. This understanding can further facilitate the development of appropriate models utilizing lipid nanoparticles.

The coefficient of efficacy in TNP groups indicated that the targeting process had potential to transfer the n-3 PUFA into target organ. By the way, the slightly decrease of n-3 PUFA amount in this group might due to the entering process of targeting-lipid nanoparticles in the cell via receptor-mediated endocytosis and sorting into lysosome, endocytosis with low pH in endosome make the ligand dissociated from their receptor (Ling et al., 2009). Then, the endosome vesical combined with lysosome for degradation and the cargos were degraded by hydrolases to be single molecules (Settembre & Ballabio, 2014). Moreover, plain lipid nanoparticles are discriminated and weakened and could be recognized by the reticuloendothelial system (Zhang et al., 2019). On the other hand, targeting lipid nanoparticles escape the phagocytosis, resulting in a longer circulation time (Chen et al., 2012) and might enhanced the n-3 PUFA accumulation. Further study should be conducted in hematology and serum biochemistry to determine the response of lipid nanoparticles in chicken.

3.7 Conclusion

This study provides conclusive evidence that nanotechnology has the potential to enhance the utilization of n-3 PUFA oil resources through nanoparticle synthesis. However, it is crucial to consider the source of the oil used for synthesis, as it can significantly affects parameters such as nanoparticle size, nanoparticle dispersion, and zeta-potential. Based on the outcomes of thermal and chemical analysis, it is established that n-3 PUFA oils were successfully encapsulated within lipid nanoparticles along with solid lipid, glyceryl monostearate. This was evidenced by the observed reduction in melting points as indicated by DSC analysis. Furthermore, the utilization of n-3 PUFA oil in nanoparticle form exhibited the ability to hinder lipid oxidation, with the rate of oxidation being contingent upon the storage temperature. Finally, the *in vivo* distribution findings substantiated the efficacy of targeted lipid-based nanoparticles to deposit long-chain n-3 PUFA in the chicken muscles.

3.8 References

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