Solid Lipid Microparticles as leaching free, slow-release encapsulation system for Methionine in aquaculture

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Abstract

The enrichment of the fish feeds with the essential amino acids like Methionine is a common strategy in Aquaculture. Leaching of the amino acids in the water environment and rapid absorption from the Gastro-Intestinal tract are some issues with the free amino acids supplementation strategy. The study aimed to investigate the use of Solid Lipid Microparticles as microencapsulation system for an essential amino acid, Methionine. Solid Lipid Microparticles (SLMs) of two lipids, Dynasan 114 and Dynasan 118 were fabricated using a melt emulsification method to encapsulate Methionine and compared for their leaching protection and release characteristics *in vitro* and *in vivo*. Leaching study performed in the simulated water environment showed that the microparticles were leaching free. In simulated Gastro-Intestinal medias, release was minimal in the gastric media for both systems, while Dynasan 114 based microparticles showed better release in the simulated intestinal media than Dynasan 118 based microparticles. Oral delivery study in Tilapia by mixing SLMs with the feed showed that the encapsulated Methionine can provide slow release compared to the free Methionine. Among the two lipids, Dynasan 114 based SLMs were found to be better digested by the fish. The encapsulation of Methionine in lipids can be a suitable alternative for traditional free supplementation strategy for preventing leaching losses and better absorption characteristics for enhanced utilization by the fish.

Keywords: Amino acids, Methionine, Solid Lipid Microparticles, Leaching, Aquaculture, Tilapia

Abbreviations: SLMs: Solid Lipid Microparticles, D114: Dynasan 114, D118: Dynasan 118

1 Introduction

Aquaculture is one of the fastest growing food industries, with total fish production estimated to grow by 37% reaching a total of 109 million tonnes in 2030 as compared to 80 tonnes in 2016 (FAO, 2018). Generally in a fish feed, fish meal – meal from pelagic fishes, is the major source of essential amino acids like methionine, taurine, and lysine. However, with increasing prices, limited availability of pelagic fishes through overfishing, these have raised concerns over the sustainable use of fish meal (R. L. Olsen & Hasan, 2012). Plant proteins, although a suitable choice for the replacement of the fish meal protein, require supplementation with essential amino acids like methionine, lysine, and taurine to maintain a well-balanced amino acid profile (Li, Mai, Trushenski, & Wu, 2009). While improved growth and other physiological functions have been reported via supplementation (Figueiredo-Silva, Lemme, Sangsue, & Kiriratnikom, 2015), several reports have reported the leaching losses of these supplemented amino acids as well as its inefficient utilization compared to protein-bound amino acids. Amino acid being a small, highly water-soluble molecule is prone to leaching in an aqueous environment of aquaculture tanks or farms. Reports have shown that about 60% are leached within 40 minutes of immersion before the fish can ingest the feed (Watson, Barrows, & Place, 2015). Furthermore, even after ingestion, the free amino acids are readily available for absorption compared to the protein-bound amino acids and this difference in uptake can cause elevated levels of the supplemented amino acids resulting in excessive catabolism (Ambardekar, Reigh, & Williams, 2009; Zarate & Lovell, 1997). Microencapsulation of the amino acids can thus be a viable method for protection against leaching, especially in an aquatic environment. In addition, the encapsulation also provides the means to achieve sustained release in the Gastro-Intestinal (GI) tract for better absorption and utilization (Nunes, Sá, Browdy, & Vazquez-Anon, 2014).

Methionine, an essential amino acid, is one of the major limiting amino acids in plant protein-based fish feeds. It is a sulphur-containing amino acid with important roles in protein synthesis and as a precursor for S- Adenosylmethionine (SAM), a Methyl donor for methylation processes (Brosnan & Brosnan, 2006). Methionine deficient fish feeds have been shown to affect the growth and lower feed efficiency (Elesho et al., 2021; Wang et al., 2021). Supplementation of feeds with free methionine is, therefore, a common strategy for methionine deficient plant protein-based feeds. However, leaching of Methionine due to its water solubility, rapid uptake compared to protein bound amino acids can lead to issues of suboptimal diets and increased catabolism (Simon, Truong, Habilay, & Hines, 2021). A microencapsulation technique for encapsulation of Methionine whereby, the microparticles are able to protect the Methionine from leaching and eventually, releasing in the fish's gastrointestinal tract (GI) in a slow and sustained manner, can be an effective alternative to traditional free methionine supplementation (Seyedehsara, Noemi, Andrea, & Ali). Commonly used polysaccharides or protein-based encapsulation materials systems suffer rapid release of nutrient due to their porous structure (Masoomi Dezfooli, Gutierrez-Maddox, Alfaro, & Seyfoddin, 2019) and are not suitable for encapsulation of hydrophilic, small molecules like amino acids. Solid microparticles of hydrophobic lipids are considered better suited for encapsulation of hydrophilic nutrients for protection as well as sustained release application (Aditya, Espinosa, & Norton, 2017).

Solid lipid microparticles (SLMs) are micron sized particles of solid lipids that can encapsulate drugs and nutrients. SLMs are usually fabricated from triglycerides, fatty acids, waxes, and fully hydrogenated oils that remain solid in room temperature and melt at a certain higher temperature. These lipids are nature derived, low cost and can be fabricated using solvent free technique like melt emulsification which make these lipids suitable for oral consumption (Jaspart, Piel, Delattre, & Evrard, 2005). Vitamins like ascorbic acid, riboflavin, proteins like lysozyme are some examples of water-soluble molecules that has been encapsulated in SLMs made up of lipids like fully hydrogenated palm oil, trimyristin, and tristearin (Carvalho, Oriani, de Oliveira, & Hubinger, 2019; Christophersen et al., 2013; Onal & Langdon, 2004). In an oral administration, the lipid-based system undergoes hydrolysis induced degradation due to the lipase enzyme which involves breaking down the lipid into free fatty acids in the stomach and the intestine (Albertini, Bertoni, Perissutti, & Passerini, 2019). These properties make the solid lipid microparticles, a suitable system for encapsulation of nutrients in an aquaculture setting. However, detailed investigations on the use of the solid lipid microparticles for encapsulation amino acids and its suitability in application *in vivo* are scant.

This study proposes the use of SLMs as a carrier for encapsulation of the essential amino acid, Methionine. Two of the commonly used lipids for SLMs i.e., Dynasan 114 and Dynasan 118, were used to fabricate the Methionine loaded SLMs. Trimyristin (Dynasan 114) is a 14-carbon-chain triglyceride of myristic acid and Tristearin (Dynasan 118) is an 18 carbon-chain triglyceride of stearic acid (Bertoni, Albertini, Dolci, & Passerini, 2018). *In vitro* studies were performed to study the protection ability of the SLMs against leaching of the amino acids in water environment. Similarly, the microparticles were subjected to the simulated GI tract media to investigate the release characteristics of the SLMs under the effect of different enzymes simulating an in-vivo conditions of a fish's GI tract. The fatty acid carbon chain length of the lipid is known to show varying lipolysis susceptibility and can affect the release of the encapsulated cargo (Benito-Gallo et al., 2015; Bertoni et al., 2018). Therefore, in this study, two different carbon chain length lipids were investigated to identify a suitable lipid material for encapsulation of Methionine and use in an aquaculture setting. Furthermore, the SLMs were investigated for *in vivo* absorption of the Methionine by quantifying the blood level after feeding of the SLMs containing feed to a commercially important fish, Tilapia.

2 Material and Methods

2.1 Materials

Lipids (Dynasan 114 and Dyansan 118) were obtained as a gift from IOI Oleochemical GmbH, Germany. DL Methionine, Poly Vinyl Alcohol (PVA) were purchased from Sigma Aldrich, Singapore. Chemicals for dissolution media preparation like Pancreatin, Bile salts, Pepsin, Calcium Chloride were purchased from Sigma Aldrich, Singapore. High Performance Liquid Chromatography (HPLC) grade ultrapure water and Acetonitrile was used for chromatographic detection.

2.2 Fabrication of SLMs

The SLMs were fabricated using Solid in Oil in Water (S/O/W) based melt emulsification method as described previously with minor modifications (Reithmeier, Herrmann, & Göpferich, 2001). Briefly, 1 gram of Dyansan 114 and Dynasan 118 was heated at 70^{o}C and 85^{o}C respectively, which is higher than the melting points of the lipids (Melting point~59^{o}C for Dynasan 114, Melting point ~73^{o}C for Dynasan 118). Methionine was powdered and sieved through a 106 µm sieve. It was added to the melted lipids at 9.09% (w/w) and stirred for 1 hour to disperse the methionine in the lipids creating a solid in oil (S/O) dispersion. The S/O dispersion was then emulsified into an equivalent temperature 50ml 1% PVA aqueous phase (W) using an overhead stirrer operating at 600rpm. Immediately, 0.1% cold PVA (~4^{o}C) was poured into the S/O/W emulsion mixture to solidify the lipids. The obtained SLMs were filtered, washed two times, and freeze dried. The dried SLMs were stored at -20^{o}C.

2.3 Physio-chemical Characterizations

The morphology of the microparticles were observed using the JOEL 6360 Scanning Electron Microscopy (SEM) operated at 5KV accelerating voltage. The SLMs were attached to a stub with carbon tape and coated with gold prior to imaging. The sizes of the SLMs were measured using Image-J software using a low magnification image of the SLMs. For samples that were incubated with simulated intestinal media, the particles were passed through a sieve of 20µm and freeze dried before imaging. Attenuated Total Reflectance/Fourier Transform Infrared Spectroscopy (FT-IR) was performed using Perkin Elmer Frontier using a spectrum range of 4000-600 cm^{-1} and air as control. Differential Scanning Calorimetry (DSC) analysis was performed using TA Q10 DSC system. The samples were placed in a hermetic aluminium pan and heated from -10^{o}C to 300^{o}C with an empty pan as reference under nitrogen gas flow of 50ml/min.

2.4 Encapsulation and Loading Efficiency

To measure the encapsulation efficiency (EE) and loading efficiency (LE), 10mg of SLMs (n=3) were dissolved in 2ml of Dichloromethane (DCM) to dissolve the SLMs and 3ml of water was added to extract methionine into the aqueous phase under vortexing and sonication. The mixture was centrifuged at 4000rpm for 4 mins and the supernatant was collected for quantification of methionine. A Liquid Chromatography-Mass Spectrometry (LC-MS) method was used for the quantitative detection of Methionine using Agilent 1290/6120 LC-MS instrument. A Phenomenex Luna NH_2 column was used with two mobile phases in gradient mode. Mobile phase A contained 90% Acetonitrile (ACN) + 10% 50mM Ammonium Acetate (pH 5.8) and mobile phase B contained 10% 50mM Ammonium Acetate (pH 5.8) in water. The gradient method involved 0-2 mins (100% of A), 2-9 mins (100 to 80% of A), 9.10 to 20 mins (100% of A). Standards were prepared from 0.39 ug/ml to 500 ug/ml with a sample injection of 5µl. The mass spectrometer was operated under the Positive Electrospray Ionization (ESI) mode. The electrospray chamber conditions included: capillary voltage of 3kV, drying gas flow of 10l/min, nebulizer pressure of 30

psig, drying gas temperature of 350°C. Selected Ion Monitoring (SIM) mode was used for detection of Methionine using mass to charge (*m/z*) of 150.

The EE and LE was calculated as follows:

Encapsulation Efficiency (EE%) = Observed Methionine content in 10 mg of SLMs/ Theoretical Methionine content in 10mg of SLMs *100 (1)

Loading Efficiency (LE%) = Methionine content in 10mg of SLMs/ Weight of SLMs (10mg) *100 (2)

2.5 *In vitro* release in simulated dissolution medias

2.5.1 Leaching Test

The leaching test was performed in DI water at room temperature. Firstly, 10mg of microparticles (n=3) were added to 1ml of DI water in a 2ml tube and placed under a rotary shaker. At different timepoints, 200µl of the water was drawn out i.e., 10 mins, 30 mins, 1hour, 2 hours, 4 hours, and 6 hours. A same volume of media was replenished. The sample preparation was done as described in Section 2.5.3. The quantification was done using LCMS.

2.5.2 Release in Simulated GI media

Simulated gastric (SGF) and Intestinal fluid (SIF) were prepared to investigate the release of the methionine from SLMs in media. Variations are found in the GI conditions of the fish among species. However, in general, the GI conditions like enzymes, pH and salts are like other vertebrates. For ease of comparison with similar encapsulation systems, well established human based dissolution media models were used. Previously described SGF and SIF standard media were used with some modifications (Jantratid, Janssen, Reppas, & Dressman, 2008; Minekus et al., 2014). The SGF composed of 0.2% NaCl, 0.32% Pepsin, and 0.1% Tween 20 adjusted to pH of 1.2 using HCL. The SIF was composed of 125.5mM NaCl, 55.02mM Maleic Acid, 2mM Soy Lecithin, 0.3% (w/v) Bile Salts, 81.65 mM NaOH, 5mM Calcium chloride and Pancreatin at 1% with a final pH adjusted to 5.8.

For dissolution, 10mg microparticles (n=3) were added to a tube containing 1ml of the simulated media and placed in a rotary shaker. At timepoints of 10 mins, 30 mins, 1hour, 2 hours, 4 hours, and 6 hours, the 200µl of the media was taken out for quantification by LC-MS and replenished with same volume of media. The sample preparation was done as described in Section 2.5.3.

2.5.3 Sample Preparation for LC-MS

For methionine samples in water, 50µl of the sample was mixed with 950µl of the Mobile phase A and filtered using a 0.22µm filter before loading into the LC-MS.

For methionine samples in SGF and SIF, 50µl of the sample was added to 250µl of ACN to precipitate the proteins. The sample was then centrifuged at 13.3K rpm for 3 mins and the supernatant diluted to 1ml using ACN and Ammonium Acetate, and filtered before loading into the LC-MS.

2.6 *In vivo* absorption of Methionine from SLMs

An in-vivo study was conducted to investigate the suitability of the SLMs in delivering the Methionine via an oral delivery route. The experiment was performed at Singapore Food Agency's Marine Aquaculture Centre. Fish feeds containing Dynasan 114 and Dynasan 118 SLMs were prepared by in-feed mixing of the SLMs using a grounded commercial feed as the basal component. The mixture was then pelletized manually using a pasta maker to produce 5mm diameter feeds. The total Methionine content of the feed was 1% (w/w) of the dry ingredients. Starch (10%) was used as a binder to improve the stability of the feed in the water.

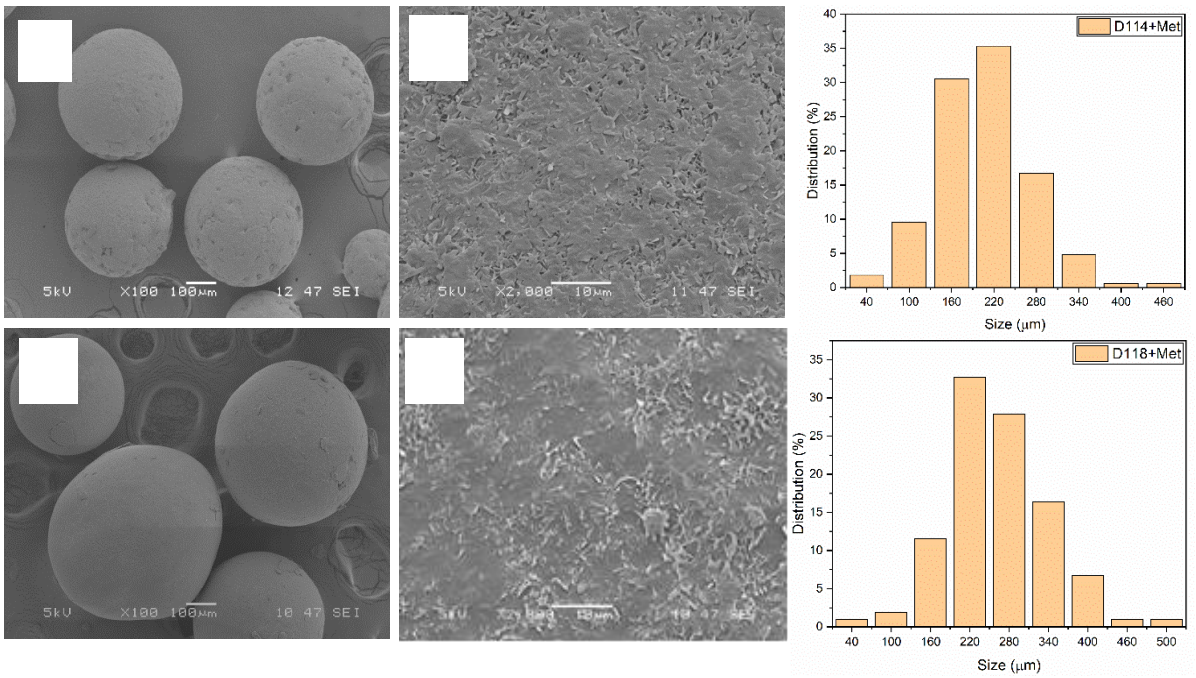
The fish trial was carried out in strict accordance with the protocol approved by Singapore Food Agency (SFA) Institutional Animal Care and Use Committee (Proposal Number: SFA-MAC-2021-02). Red Tilapia fishes (n=12) were placed individually in 12 tanks of 300L each. The fishes were divided into 4 groups namely, Dynasan 114 SLMs, Dynasan 118 SLMs, Free-Methionine and Control Commercial feed with average body weight of 436.93± 17.014gm, 461.26 ± 9.96gm, 436.4± 13.042gm and 425.73±5.88gm respectively. The salinity was maintained at 4-5 ppt and the fishes acclimatized for 1 week before the experiment. Feeding was withdrawn 48 hours prior to the start of the feed trial. After that the fishes in the experimental groups were fed 0.5% of its body weight to deliver a Methionine dosage of 0.05 mg/ gm of the fish. The amount of the control feed was equal to the amount of the basal feed the fish consumes in the experimental groups. Each fish was fed individually, one feed a time to ensure that all the feed was consumed.

At different timepoints i.e. 1hr, 3hr, 5hr, 7hr, and 24 hr after feeding, the fish were anesthetized using AQUI-S anaesthetic agent for few minutes. Immediately, 0.4ml of blood was drawn from the fish's caudal vein of the fish. The collected fish blood was centrifuged at 2000g for 15 mins to separate out the plasma. The plasma sample was prepared for the LC-MS detection of Methionine. Briefly, 50 µl of the plasma was mixed with 250 µl of ACN to precipitate the proteins, which was then centrifuged and diluted to 1ml before loading into the LC-MS.

3 Results

3.1 Morphology of SLMs

The SEM images and the size distribution of the Dynasan 114 and Dynasan 118 SLMs encapsulating Methionine are shown in Figure 1. The obtained SLMs were of spherical morphology. D114 SLMs showed a mean size of 196±66 µm, whereas Dynasan 118 SLMs had a mean size of 225±76 µm. The slightly larger D118 microparticles might be due to the higher viscosity of the Dynasan 118 melt. (Bertoni et al., 2020). The surface of the D114 appeared flakier than the D118 which might be due to the inherent solidification property of the lipids.



3.2 FTIR and Thermal Analysis

The incorporation of the methionine into the microparticles was also studied using ATR-FTIR as shown in Figure 2(a). In the methionine spectra, characteristic NH3+ bands at 1654 and 1610, and 1513 are due to the asymmetric and symmetric bending (Ramachandran & Natarajan, 2006). In the empty microparticles of D114 and D118, the presence of these bands cannot be detected while these bands are present in the Methionine loaded SLMs. This confirms the successful encapsulation of the Methionine. As Methionine is not soluble in the lipids used and present as a solid dispersion in the lipid matrix, no interaction between

the lipids and the Methionine is expected. Shifts in peak positions are, therefore, not evident in the FTIR plot for the Methionine encapsulated SLMs.

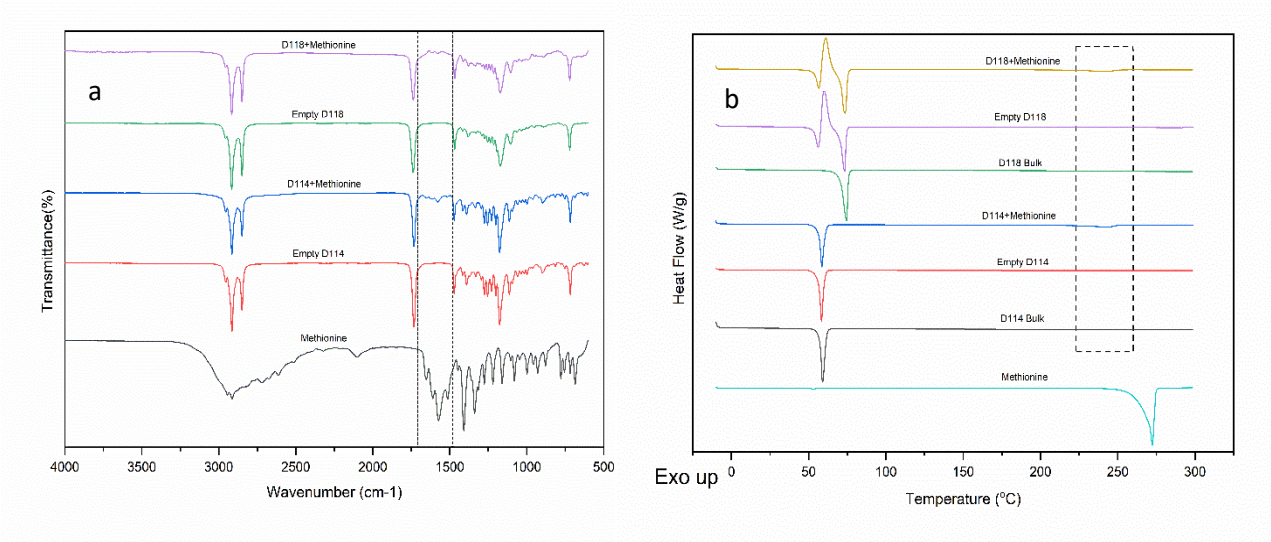


Figure 2: ATR-FTIR spectra (a) Dynasan 114 and Dynasan 118 SLMs with or without Methionine vs pure Methionine. Characteristic peaks of Methionine in the ATR-FTIR spectra at 1654, 1609, 1572 and 1513 cm^{-1} enclosed in the rectangular area. DSC curves (b) for Dynasan 114 and Dynasan 118 SLMs with or without Methionine vs pure Methionine. The rectangular area shows the decomposition peak of the Methionine.

Differential Scanning Calorimetry (DSC) analysis was used to study the polymorphic states of the lipids after melt-solidification process, and the graphs are shown in the Figure 2 (b). The DSC curve of Methionine shows a sharp endothermic peak at around 270.06°C which is related to the decomposition of the Methionine. The raw lipids show sharp endothermic melting peaks at 58.97°C for Dynasan 114 and 73.62°C for Dynasan 118 corresponding to their melting points. After the formation of the microparticles, no major shifts in the melting point of the Dyansan 114 was observed, however, the D118 microparticles with or without Methionine show multiple endothermic peaks. This is expected as melting-solidification process can lead to the formation of multiple polymorphs in the triglycerides (Rogers, Tang, Ahmadi, & Marangoni, 2008). The endothermic peak at 72.80°C corresponds to the melting of the β-form of D118 while an additional peak at around 56°C can be attributed to the formation of an α metastable form of D118 (Bertoni, Albertini, Facchini, Prata, & Passerini, 2019). Over time, a more arranged packaging of the crystals in the lipids lead to the formation of a stable β form (Sato). In the samples with methionine, endothermic depression can be observed at around 240°C indicating the decomposition of methionine, which indicates the successful encapsulation of Methionine in the SLMs.

3.3 Encapsulation Efficiency and Loading Efficiency

The encapsulation efficiency (EE) of both SLMs were found to be about similar at 58.20% and 55.42% for Dynasan 114 and Dynasan 118 SLMs respectively as shown in Table 1. While the initial loading of Methionine was 9.09% in the formulation for both the SLMs, experimental loading efficiencies (LE) of 5.29% and 5.03% was observed for Dynasan 114 and Dynasan 118 SLMs respectively. During the formation

of the S/O/W emulsion in the aqueous PVA phase, the methionine crystals dispersed in the oil emulsions could diffuse out into the aqueous phase resulting in loss of water-soluble molecules like methionine (Ramazani et al., 2016).

Table 1: Encapsulation and Loading efficiencies of Methionine in SLMs of Dynasan 114 and Dyansan 118

Sample	*Methionine (%w/w)*	*Encapsulation Efficiency (%)*	*Loading Efficiency (%)*
D114+Met	9.09	58.20±2.06	5.29±0.18
D118+Met	9.09	55.42±1.09	5.03±0.09

Data represented as Mean±SD for n=3

3.4 *In vitro* release tests in simulated dissolution medias

Simulated dissolution medias included the different aquaculture medias i.e. water environment, gastric fluid and intestinal fluid. DI water was used as a representation of the water environment and used to study the leaching protection abilities of the microparticles.

3.4.1 Leaching in Water

SLMs of Dynasan 114 and Dynasan 118 lipids were both effective in preventing the leaching of the encapsulated methionine with 0.57% vs 1.36% leached over a period of 6 hours in water environment for Dynasan 114 and Dynasan 118 respectively as shown in Figure 3. The complete release profile for 6 hours is shown in supplementary information. At the end of 6 hours, the media was removed, and the particles were dissolved for the detection of the remaining Methionine. It was observed that about 85% of the methionine was intact in the in Dynasan 114 SLMs and 87% in Dynasan 118 SLMs. This represents the Methionine that will be available for ingestion by the aquaculture species.

3.4.2 Release in Simulated Gastric Fluid (SGF)

The release of the methionine from the SLMs was also performed in the simulated gastric fluid and the results are shown in Figure 3. The release of methionine in the simulated gastric fluid was similar to release in the DI water environment. Complete release profile over 6 hours is shown in the supplementary information. Minimal release i.e., 2.05% and 2.34% was observed for Dynasan 114 and Dynasan 118 SLMs respectively after 6 hours of immersion in the gastric fluid as shown in Figure 3. The SLMs were tested for the residual methionine after 6 hours and most of the methionine (~71-76%) was detected inside the SLMs.

This means that most of the encapsulated methionine would remain intact in the microparticles as they reach the small intestine.

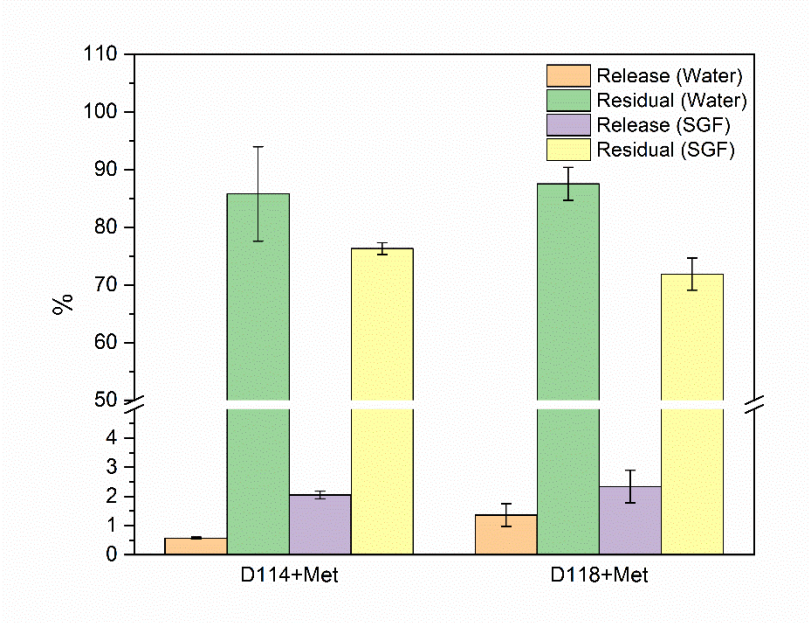


Figure 3: Released and Residual Methionine from Dynasan 114 and Dynasan 118 SLMs after 6 hours in Water and Simulated Gastric Fluid (SGF). Data represented as Mean±SD for n=3.

3.4.3 Release in Simulated Intestinal Fluid (SIF)

The SLMs were subjected to a simulated intestinal fluid consisting of the bile salts and lipase. As seen in Figure 4, it was observed that the release of methionine from Dynasan 114 reached about 71% in 6 hours. During the same period, the release of methionine from the Dynasan 118 was minimal (<3%). The release from the D114 showed a biphasic release behaviour with a slow release in the first hour with only 4.7% release and a steep increase in release over the next 5 hours. The release curve shows that the release increased with increased lipase induced degradation.

To confirm the lipase degradation induced release, the particles subjected to the SIF were imaged under SEM at 2 hours and 6 hours timings. As seen in the Figure 5 for Dynasan 114, the lipid degradation level is higher than that for Dynasan 118 SLMs at both the 2 hours and the 6 hours period. For Dynasan 114, at 2 hours timepoint, the breakdown of the Dynasan 114 lipid matrix due to the lipase can be clearly seen. At 6 hours, it can be observed that the SLMs have suffered major erosion. The release of the methionine from the Dynasan 114 is comparable to the lipolysis induced degradation of the SLMs with most of the methionine releasing out within 6 hours. However, the lipase induced degradation of the Dynasan 118 is negligible at both 2 hours and 6 hours. Even after 6 hours, visible degradation cannot be observed on the particle surface. Thus, minimal release of the methionine from Dynasan 118 SLMs is observed.

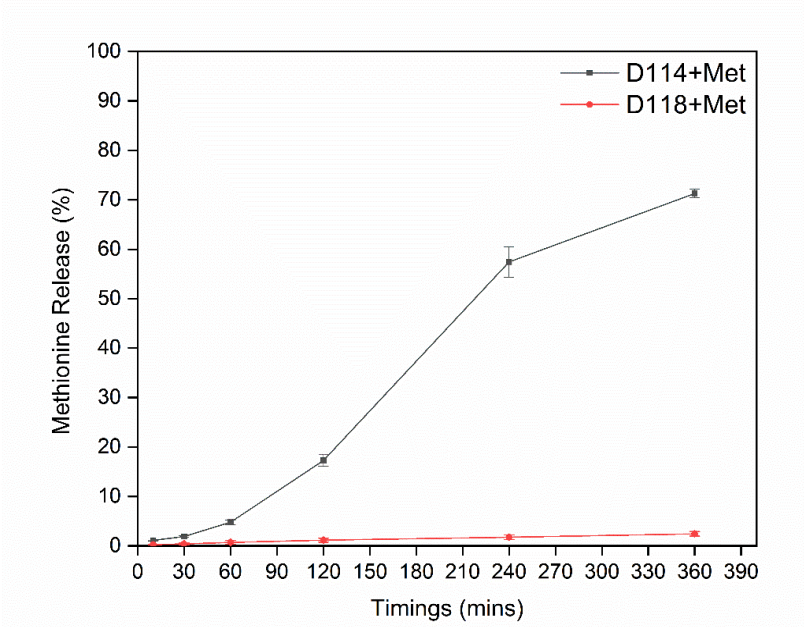


Figure 4: Release of Methionine from Dynasan 114 and Dyansan 118 SLMs in Simulated Intestinal Fluid (SIF) at 10mins, 30mins, 1hr, 2hrs, 4hr and 6hrs. Data represented as Mean±SD for n=3.

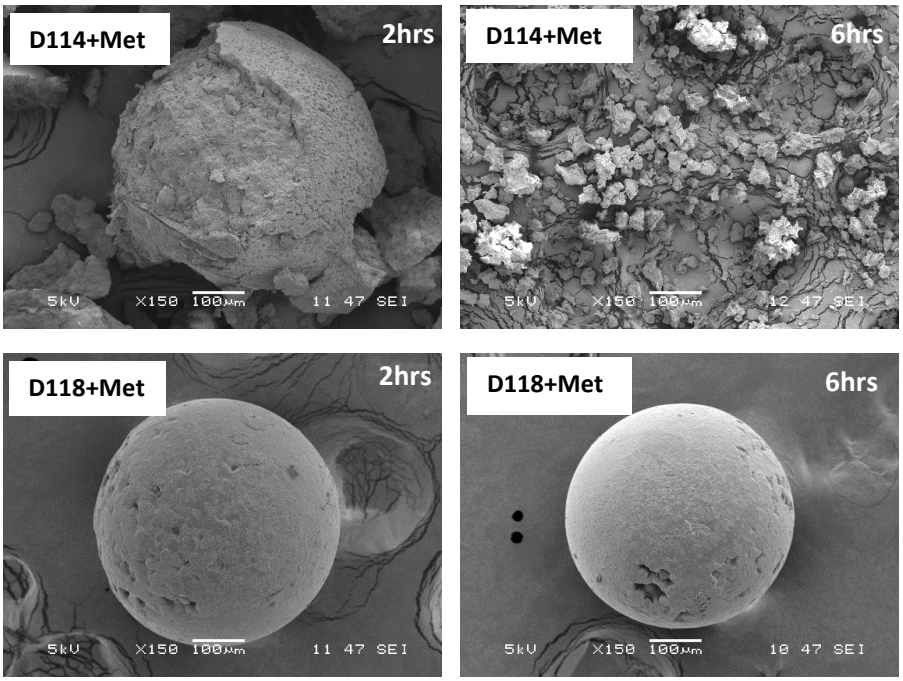
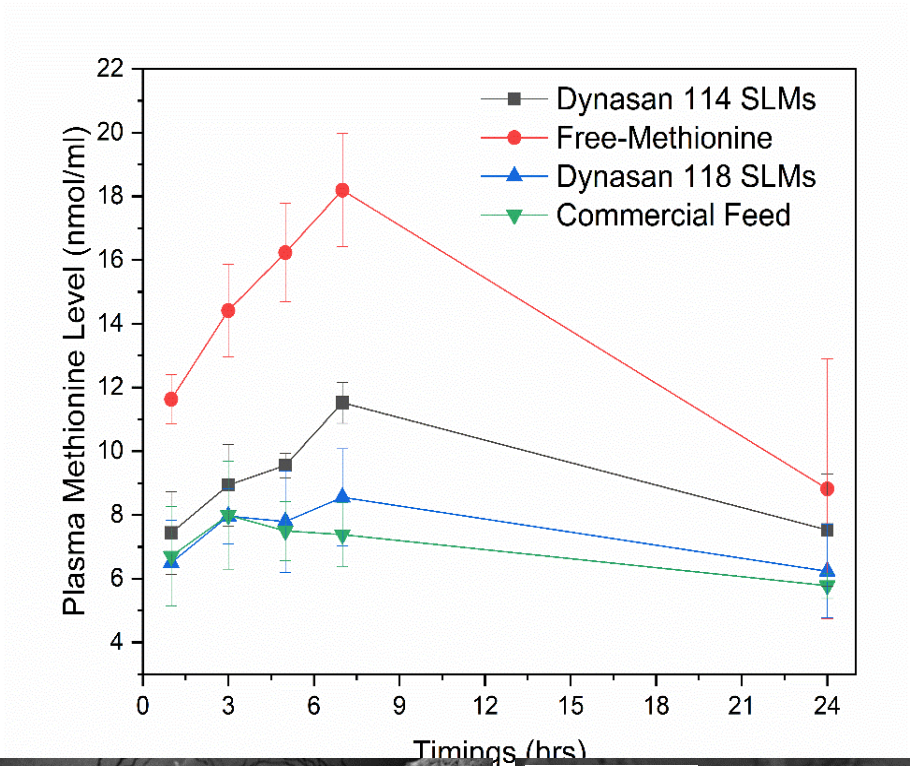


Figure 5: SEM images of Lipolysis of Methionine Loaded Dynasan 114 and Dynasan 118 SLMs after incubation in 2hrs and 6 hrs in Simulated Intestinal Fluid (SIF).

3.5 *In vivo* absorption of Methionine from SLMs

The plasma methionine level after one time feeding of the SLMs containing feed was studied over a period of 24 hours. The plasma profile for the Methionine from the different experimental groups post single feeding is shown in the Figure 6. The Free- Methionine supplemented group showed a rapid increase in levels starting from the first hour with Methionine level reaching 11.63 nmol/ml. The SLMs, on the other hand, showed low Methionine level in the blood in the first hour i.e., 7.43 for D114 and 6.49 nmol/ml for D118. Overtime, the steeper rise in Methionine levels for the Dynasan 114 group was observed from 5 to 7 hours reaching a maximum of 11.52 nmol/ml. This might be related to the degradation of the particles once they reach the small intestine. However, the level of Methionine in the Dynasan 118 group did not show similar rise reaching about 8.55 nmol/ml in 7 hours. In the same duration, the Methionine level in the fish's blood for the Free-Methionine group showed steep increase to reach 18.19 nmol/ml in 7 hours. Comparing the levels of Methionine for the control group and the D114 SLMs at 7 hours, the higher plasma value of Methionine for D114 confirms that the digestion and release from the SLMs.

At the end of the experiment, some undigested particles were observed in the faeces collected at 24 hours. The SEM images of the undigested particles seen in the fish's faeces in Figure 7 also show that Dyansan 114 was more susceptible to GI fluids components as evident by the coating of the microparticles with GI components on the surface of the microparticle. This confirms that the digestion of the D114 SLMs comparatively better than the D118 SLMs. This helps to explain the low plasma levels of Methionine for the D118 SLMs.



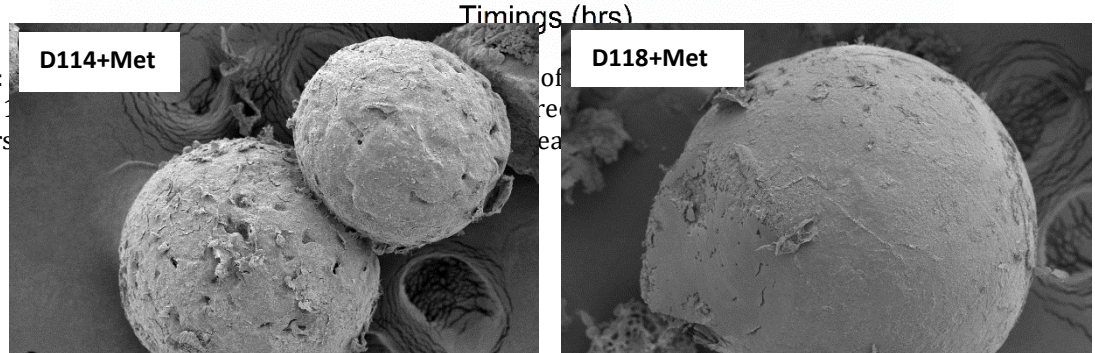


Figure 6: of
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Figure 7: SEM images of the undigested SLMs obtained from the Fish's faeces after 24 hours of the in vivo absorption study. Scale bar is 100μm.

4 Discussion

Dynasan 114 and Dynasan 118 are two of the popularly used lipids for designing micro and nano lipid encapsulation systems with different fatty acid carbon chain length (Bertoni et al., 2018; Bertoni et al., 2019). The amino acid, Methionine was incorporated into the solid lipid microparticles of Dyansan 114 and Dynasan 118 lipids using a Solid-Oil-Water (S/O/W) melt emulsification method. Solid in Oil (S/O) emulsion method is known to be useful for encapsulation of hydrophilic compounds with potential advantages of high encapsulation, low burst release and better stability of the compounds compared to Water-Oil-Water (W/O/W) based methods (Sawant, Kamath, Kg, & Kulyadi, 2021). Moreover, the S/O/W method for SLMs fabrication can be adapted for scale up production using methods like Spray Drying and Spray Congealing, making this approach a viable option for fabrication at an industrial scale. A solvent-free fabrication approach was used to ensure better compliance for oral delivery application in aquaculture.

The SLMs were hypothesized to provide protection against leaching of Methionine in an aqueous environment. In aquaculture, hydrophilic nutrients, like amino acids and vitamins, are reported to leach at rapid rates from feeds into the water (M. S. Alam, Teshima, Koshio, & Ishikawa, 2004; Soliman, Jauncey, & Roberts, 1987; Watson et al., 2015). Such leaching affects the amount of nutrients that aquatic species can consume, thus affecting their growth. For instance, leaching of amino acids was reported to cause inefficient protein synthesis and growth in shrimps (M. S. Alam et al., 2004; Teshima, Ishikawa, Shah Alam, Koshio, & Raafat Michael, 2004). Encapsulation of hydrophilic amino acids previously using polysaccharides and protein materials have been reported to show rapid leaching of 80-90% within twominutes (López-Alvarado, Langdon, Teshima, & Kanazawa, 1994). A hydrophobic lipid carrier is thus, a more suitable material for encapsulation hydrophilic nutrients like methionine. Due to the lipophilic nature of lipids, the SLMs fabricated in this study were able to mitigate leaching with <1.5% leached over six hours. The results here concur with previous reports that uses solid lipid systems to protect water-soluble nutrients like riboflavin (Onal & Langdon, 2004; Önal & Langdon, 2004). In this study, protection against leaching was found to be effective for at least six hours, sufficient for aquaculture applications in general. In addition, long-term protection of methionine in SLMs may also be applicable to slow and other bottom feeding aquatic species like Japanese Flounders or Shrimps (Md Shah Alam, Teshima, Koshio, & Ishikawa, 2002; Teshima et al., 2004).

While the protection against leaching is one of the key considerations, the microencapsulation system should also be able to release the encapsulated Methionine when digested in the GIT of the fish. To test the release of Methionine from the SLMs, simulated gastric and intestinal fluids were used. as the gastrointestinal transit time for Tilapia has been reported to be about 7.15-8 hours (Riche, Haley, Oetker, Garbrecht, & Garling, 2004; Uscanga, Moyano, & Alvarez, 2010), these timings were used to simulate release of Methionine along the GIT. The simulated gastric mimicked a low pH fluid with pepsin as the enzyme. , and a pH of 1.2 was used (Hlophe, Moyo, & Ncube, 2014). The results showed that release was also retarded whereby the major gastric enzyme, i.e. pepsin, was unable to degrade the lipid matrix (Figure 3).

The release of the encapsulated nutrients from SLMs was observed to be primarily dependent on lipase degradation of the lipid matrix in the small intestine (Bertoni et al., 2020; Christophersen et al., 2013). Like other mammals, pancreas in fishes is the major source of the lipase enzyme along with intestinal walls and some bacteria in the intestine (R. Olsen, 1997). The SIF media used in this work is a standardized media commonly used for lipid-based systems and closely resembles the complexity of the intestinal media (Jantratid et al., 2008). Dynasan 114 is a 14-carbon chain lipid and was observed to be more susceptible to lipase degradation as compared to the 18-carbon chain Dynasan 118. Contributing factors for increased susceptibility of short chain lipid include better affinity to lipase, and higher chain mobility at the digestion interface. (Benito-Gallo et al., 2015; Bertoni et al., 2018). This effect can be clearly observed in the release profile of Methionine between D114 and D118 SLMs. SEM images of the SLMs after incubation in SIF media further confirmed the observation. An initial slower release from D114 SLMs was due to the slower degradation of these SLMs in the initial hours (Figure 5). Microencapsulation systems based on proteins degrade in the stomach due to the effect of the protease enzymes, while some polysaccharides-based systems degrade in the intestine due to their swelling effects (McClements, 2018). For lipids, the release is triggered by lipolysis, which is dependent on lipid chain length (Acquistapace et al., 2019; Bertoni et al., 2018), making Dyansan 114 more suitable for oral delivery in aquaculture. Therefore, a proper selection of lipid material ensures that sufficient release can occur in oral delivery of microencapsulation systems.

To further validate the release of Methionine, pharmacokinetics study was conducted whereby encapsulated Methionine in SLMs was compared against free Methionine when added to feeds and fed to Tilapia. Optimal Methionine requirement for growth of Tilapia has been reported to be about 0.75-0.99% (w/w) of the feed (He et al., 2017; Liebert, 2009; Liebert & Benkendorff, 2007; Santiago & Lovell, 1988). In this *in vivo* study, a similar Methionine amount (1%) was thus used. The plasma level of Methionine for the Free Methionine group increased rapidly in the first hour after feeding and remained high over the experiment period. It is expected, as the free Methionine is readily available for absorption and is quickly absorbed. On the other hand, plasma level of Methionine for the Dynasan 114 SLMs group showed a slower increase. Similarly, the plasma levels of Methionine for the Dynasan 118 were comparable to the control feed, indicating that Dynasan 118 microparticles were not digested.. These *in vivo* results further validate that shorter chain Dynasan 114 showed better release properties in fish. The slow release of the Methionine from the SLMs provides a sustained absorption and prevents excessive level of Methionine in early digestion. This can prevent catabolism and improve its utilization for protein synthesis (Ambardekar et al., 2009). The presence of undigested particles in the faeces, however showed that some microparticles were

not completely digested. This could be due to a possible lipolysis suppression by the feed matrix. In addition, proteins, and starch present in the feed are known to affect the lipolysis (Heredia, Asensio-Grau, Calvo-Lerma, & Andrés, 2021). Further studies may therefore be required to optimize the amount of SLMs in feed to ensure complete utilization of Methionine.

5 .Conclusions

Microencapsulation of Methionine using lipid carriers can be an effective strategy to prevent premature leaching and achieve controlled-release of amino acids in fish. This can overcome the rapid leaching of hydrophilic nutrients, such as amino acids, in an aqueous environment, and improve the delivery of amino acids into the intestine of the fish. Methionine encapsulated into short-chain Dynasan 114 was observed to be released in simulated intestinal fluids under the action of lipase enzyme and detected in pharmacokinetics studies. Future studies should focus on optimizing the amount of lipids, size of SLMs using Dynasan 114 and the bioactivity of Methionine for the supplementation of encapsulated amino acids into aquaculture feeds.

Authors contributions

Mahotra Manish: conceptualization, methodology, investigation, formal analysis, writing, Hong Yu: methodology, investigation, writing, Xu Qunying: investigation, methodology, resources, Woei Chang Liew: investigation, methodology, resources, supervision, Kharel Sharad: methodology, formal analysis, writing-review and editing, Tan Lydia Shun En: investigation, resources, Say Chye Joachim Loo: conceptualization, formal analysis, resources, writing- review and editing.

Conflicts of interest

There are no conflicts of interest to declare.

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