Significant impacts of different types of alginate on the encapsulation stability of fish oil

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Master Thesis

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Declaration

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

By Zhaolin He

Abbreviations

cm	centimetre
EE	encapsulation efficiency
g	gram
kDa	kilodalton
kV	kilovolt
mA	milliampere
ml	milliliter
mM	millimolar
mm	millimeter
mol	mole
mPa/s	millipascal per second
nm	nanometer
rpm	revolutions per minute
UV	Ultra-Violet
^{o}C	Celsius scale
%	percent

Abstract

Fish oil exerts remarkable benefits for human health, especially the growth of infants due to its high concentration of omega-3 fatty acids. Sodium alginates have been widely used for as fish oil encapsulation. Recently, the depolymerization technology has shown the ability to reduce the molecular weight and viscosity of alginates to a controllable degree. However, the influence of depolymerized alginate on the encapsulation efficiency and stability of fish oil has not been investigated. Moreover, the sources and types of alginate may also influence the fish oil encapsulation efficiency and stability. The aims of this project were to determine the influence of different types of alginates on the encapsulation efficiency, stability, and beads size distribution of fish oil.

In this project, three different sodium alginates, including chemical synthesised alginate, seaweed sourced alginate, and depolymerized alginate from seaweed sourced alginate were investigated in fish oil encapsulation. The SEM results showed that fish oil was still encapsulated by calcium alginate gel bead and provide a more plump and spherical structure than the control group beads prepared without fish oil which had lost the basic bead structure. For the fish oil loading beads, the seaweed sourced alginate had more narrow diameter size distribution than the chemical synthesised alginate and depolymerized alginate from seaweed sourced alginate. The encapsulation efficiency of beads prepared with three alginates showed similar encapsulation efficiency around 97% ~ 98%. After 7 days incubation in petri dish under room temperature, the stability analysis showed that the beads formed by chemical synthesised alginate with higher G (α-L-guluronic acid) residues content had 13.71% oil leakage while the oil leakage of the beads prepared using seaweed sourced alginate was 58.33%. Using the

depolymerized alginate from seaweed sourced alginate, oil leakage was decreased to 28.21%, indicating that 30% oil leakage was reduced.

Results indicated that the chemical synthesised alginate with higher G residues formed gel beads with higher encapsulation stability for fish oil than the seaweed sourced alginate with lower G residues. The depolymerized alginate for seaweed sourced alginate with lower molecular and viscosity could improve the bead stability, achieving significant reduction in oil leakage. The main limitation of this study is the limited range of alginates available for the project to establish a full understanding of the impact of different alginate properties including G (α-L-guluronic acid)/M (β-D-mannuronic acid) ratio, molecular weight, and viscosity on the encapsulation of fish oil.

Thus, the further research should focus on the parameters of alginate with a range of properties by depolymerization and modification to satisfy the diverse applications in food industry.

CHAPTER 1: Introduction

1.1 Fish oil

It has been a long history for human to use marine organisms as food resource. In recent decades, bioactive compounds extracted form marine organisms have shown potentials for human benefits such as, antitumor (Nagle et al., 2004), antibiotic (Martins et al., 2014), and antioxidant (Park and Pezzuto, 2013) making them become the research hotspots. Actually, marine organisms, including animals, plants, and microorganisms have become the main source of natural bioactive compounds in food industry and pharmaceutical industry (Suleria et al., 2016). Large number of bioactive compounds have been isolated form marine organism including polysaccharides (Laurienzo, 2010), polyunsaturated fatty acids (PUFA) (Vaughan et al., 2013), vitamins (Škrovánková, 2011), antioxidants (De Souza et al., 2007), and enzymes (Trincone, 2011)

Fish oil is one of the most popular marine bioactive compounds in the market that has attracted lots of interest, for its high content of human beneficial constituents, omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Shahidi, 2015) as shown in Figure.1.1. Human body can only synthesize limited EPA and DHA from the conversion pathway of essential fatty acids, so it is necessary to get the omega-3 fatty acids form the daily diet (Brenna et al., 2009)

Figure 1.1 Chemical structure of EPA and DHA (Mozaffarian and Wu, 2012)

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It reported that EPA and DHA can reduce the risk of coronary heart disease (Cnnor, 2000) (and immune response disorders (Kelley, 2001). Several studies have indicated that EPA and DHA are involved with the promotion of development of brain and visual function of infants (Simopoulos, 1999). Food industries such as dairy products industry has added omega-3 fatty acids rich oils into the production of milk and yogurt for their

high nutrients (Kadam and Prabhasankar, 2010). More than half of the fish production in the world are harvested from marine circumstances (Kim and Mendis, 2006). The annual fish oil production is more than one million tons (Pike and Jackson, 2010).

For food industry, the problem for developing marine products is their production and storage because marine bioactive compounds generally have high risk of deterioration caused by unsaturated fatty acids (Huber et al., 2009). Especially for fish oil, its chemical instability and free radicals from oxidation in omega-3 fatty acids, as well as the fishy odor and taste cause negative effects for fish oil on shelf-life and acceptability as food products (Chang and Nickerson, 2018). The oxidation of oils will also cause the degradation of EPA and DHA (Barrow et al., 2007). Moreover, the directly oral intake pathway may cause side effects on the chemical structure and bioactivity of bioactive compounds during the intestinal and first-pass metabolism (Đorđević et al., 2015).

1.2 Encapsulation technology

Therefore, the need arises for an efficient technology to improve the stability and shelf life period of fish oil and other sensitive desired bioactive components. Microencapsulation technology has been widely used in food industry that can form small packaging called microcapsules (Comunian and Favaro-Trindade, 2016). In this process, sensitive core materials are entrapped into wall materials for protection and improvement of delivery (Zhang et al., 2018). It can protect the sensitive compounds from the influence of oxidation, heat, and moisture (Jafari et al., 2008). The release rate of core materials are controllable in the certain conditions (Tylkowski et al., 2017).

Figure 1.2 The schematic representation of microcapsules structure (Tylkowski et al., 2017)

In addition, for the compounds like fish oil, encapsulation can also enhance the taste and odor to an edible friendly form (Rivas et al., 2017). Moreover, encapsulation process can convert oil-based component from liquid form into solid form that improves both handling property and flow property (Fernandes et al., 2013). Recent studies have found that the encapsulation technology not only improves the stability of sensitive compounds, but also enhances their solubility (Wen et al., 2016).

1.3 Seaweed polysaccharides

The polysaccharides extracted from seaweeds considered to be the potential source of wall material in encapsulation process, some seaweeds polysaccharides such as agar has been widely used as gelling agent (Cosenza et al., 2017). Seaweeds, also known as marine macroalgae, have thousands of years' history as food resource to feeding livestock (Makkar et al., 2016). Generally, seaweeds can be classified into 3 groups: brown seaweed (Phaeophyceae), red seaweed (Rhodophyceae), and green seaweed (Chlorophyceae), respectively (Makkar et al., 2016).

Seaweeds have an advantage of high growth rate and photosynthetic efficiency (Van Hal et al., 2014) that makes their huge cultivation around the world (Loureiro et al., 2015). In 2008, almost 16 million tons aquatic plants were produced that created $7.4 billion USD commercial value to aquaculture industry and more than 90% of these aquatic plants were seaweeds (Ghadiryanfar et al., 2016). Numerous bioactive phytochemicals can be extracted from seaweeds, including carotenoids (Pangestuti and

Siahaan, 2018), fatty acids (Susanto et al., 2016), vitamins (Hamid et al., 2015), phycobilins (Guihéneuf et al., 2018), and polysaccharides (Kadam and Prabhasankar, 2010).

Seaweed have high percentage of polysaccharides that usually function for cell structure and storage (Kraan, 2012). Therefore, seaweeds have been considered as the most abundant source of natural polysaccharides (Venkatesan et al., 2015b) including, alginate (Fertah et al., 2017), carrageenan (Rhein-Knudsen et al., 2015), agarose (Efendi et al., 2015), ulvan (Cunha and Grenha, 2016), and fucoidan (Ivanov et al., 2016). In some species such as *Ascophyllum* and *Porphyra*, the total amount of polysaccharides can achieve even more than 70% dry weight (Holdt and Kraan, 2011).

1.4 Alginate gel bead

The unbranched polysaccharide alginate is one of the most commonly applicated biological material that is mainly extracted form brown seaweed (Goh et al., 2012, Axpe and Oyen, 2016). Alginate is one member of linear copolymer family and consists of blocks of β-D-mannuronic acid (M) and α-L-guluronic acid (G) which is its C-5 epimer 1,4 linked in the chemical structure (Pawar and Edgar, 2012). Alginate is composed of consecutive G residues, M residues, and alternating G and M residues, such as GGGGGGGG, MMMMMMMM, and GMGMGMGM (Fig.1.3). The alginates extracted from different sources would show the various contents in G and M residues and length of blocks. For this reason, more than 200 kinds of alginates have been produced up to now (Pawar and Edgar, 2012, Lee and Mooney, 2012).

Figure 1.3 The polymer segment structure for alginate. G and M represent α-L-guluronic acid and β-D-mannuronic acid (Yang et al., 2011)

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Alginate has been widely applicated in food industry as stabilizers and thickeners (Ching et al., 2017). It has also been employed in tablet production process for its ability to enhance the bioadhesive property (Goh et al., 2012). Alginate can be classed as a dietary fibre that has inhibition effects on digestive enzymes, which makes alginate beneficial in the treatment of obesity (Houghton et al., 2015).

Every year, there are around 30,000 metric tons of alginate produced and more than 90% is from natural source comparing with the biosynthesis pathway (Pawar and Edgar, 2012). As a kind of natural polymer, it has been proved that alginate is non-toxic, non-immunogenic, and biocompatible, and it can be biodegradable (Yang et al., 2011). For this reason, the alginate gel has been widely used for wound healing, bone tissue engineering (Ching et al., 2017, Venkatesan et al., 2015a), and encapsulation technology for food industry and pharmaceutical industry (Ching et al., 2017). One of most important advantages of alginate is the cheaper and simpler use of alginates comparing with other polymers (Martinsen et al., 1989). Moreover, alginate can be converted to hydrogels which give it the potential as the 3D scaffolding material (Sun and Tan, 2013). The hydrogel conversion of alginate is caused by the gel forming mechanism of alginate, known as ionic crosslinking (Ching et al., 2017).

1.4.1 Calcium alginate gel bead

Calcium alginate gel beads has been employed for fish oil encapsulation in previous researches (Bannikova et al., 2018). As shown in Figure 1.4, it utilizes the cross-linking

mechanism between Ca^{2+} and alginates, which looks like an egg-box (Ching et al., 2017). The Ca^{2+} and other multivalent cations can convert the sodium alginates into a reversible gel from a water-soluble state (Azevedo et al., 2014).

Figure 1.4 Gelling mechanism of calcium alginate gel (Ching et al., 2017)

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The composition of alginate is one important factor that influences the gel property. It has been identified that the gel forming capacity is based on poly-G blocks because only the G residues are involved in the process of cross-linking with divalent cations, while the flexibility is controlled by the poly-M and poly-MG blocks (Wu et al., 2017, Lee and Mooney, 2012). Every four G residues from alginates molecule bind with one cation in the egg-box structure that forms a three-3 dimensional network (Ching et al., 2017). It has been pointed out that for calcium alginate cross-link, the number of adjacent G residues should be around 8 to 20 for stable junction formation (Rehm, 2009). Moreover, it has been found that structure and molecular weight can be different of alginates because of the species of source (Pawar and Edgar, 2012). It means that the calcium alginate gel beads property will be quite different when the source of alginate changed.

Studies have proved that hydrogels formed by ionically cross-linking usually exhibit a better swelling property than covalently crosslinked products when the pH condition changed (Feng et al., 2017). Although other cations such as Cu^{2+} and Sr^{2+} can also form gels with alginate with a higher affinity, these gels have a potential toxic effect that makes them rarely applicable comparing with calcium alginate gel (Simó et al., 2017). The hydrogel formed by alginate and Ca^{2+} has been widely used in the microencapsulation technology for drug molecules, cells, and bioactive compounds

(Pasparakis and Bouropoulos, 2006). For the oily base component, it has been indicated that the encapsulation efficiency of palm oil using calcium alginate gel bead was more than 90%, while over 85 % weight was provided by oil component for these beads (Chan, 2011).

1.4.2 Drawback of calcium alginate gel bead

The weak stability and controllable release of core materials are the main challenges encountered in this technology (Matricardi et al., 2008). In fact, researchers pointed out that the hydrogel beads formed by calcium alginate generally had microporous structure that might cause negative effect on its encapsulation efficiency (Kim et al., 2008) and stability on mechanical and chemical aspect (Simó et al., 2017). For fish oil, the microporous structure will make it easier to suffer the oxidation and deterioration. In addition, the change of pH can also cause negative effects for calcium alginate gel bead stability. It has been reported that alginate is insoluble in low pH that can form a membrane to inhibit the release of core materials, it will be converted into soluble form and release the encapsulated materials under a higher pH (Natrajan et al., 2015). The intestinal pH can also lead to the degradation of the cross-linking structure (Krasaekoopt et al., 2004, Kim et al., 2008). The chemical environment contains high concentration of monovalent ions, or the presence of Ca^{2+} chelating agents can also lead to the disintegration of calcium alginate gel beads (Zanjani et al., 2014). It will increase the difficulty for core materials that are entrapped into calcium alginate gel beads to have a controllable release in intestinal metabolism.

1.4.3 Chitosan coating technology for calcium alginate gel bead

To improve the beads property and change the microporous structure of calcium

alginate gel beads, the coating technologies have been employed as a commonly used strategy (Simó et al., 2017). Chitosan has been used for coating calcium alginate gel beads and has improved the bead property effectively (Chávarri et al., 2010). Chitosan is a linear chain polysaccharide (Aranaz et al., 2009), which contains N-acetylglucosamine and glucosamine linked by β-1,4-link (Kim et al., 2008). It is an important derivate of chitin and is one of the most abundant natural polymers (Rinaudo, 2006). Chitosan has been employed in medical industry for its antibacterial and anticancer activity (Arjunan et al., 2016). Despite of that, several studies have found that chitosan has the effect on stimulating the growth of plants and increasing their yield, it also can induce positive effects on the immune system of plants (Boonlertnirun et al., 2017).

Although most commercial chitin and chitosan are usually extracted from the shellfish source such as shrimps (Khor and Lim, 2003), related research has shown that chitosan can also be extracted from cell wall of *Chlorella*, one species of green seaweed (Alishahi and Aïder, 2012). It suggests that the requirement of vegetarian can be achieved by using seaweeds source chitosan to replace animals source chitosan.

Figure 1.5 Chemical structure of chitosan (Kumar, 2000)

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As a kind of biopolymer, chitosan has extraordinary properties including non-toxic, biodegradable, biocompatible (Luo and Wang, 2013, Lertsutthiwong and Rojsitthisak, 2011), and it has shown great mucoadhesive and adsorption activities (Azevedo et al., 2014). When the alginate is converted into soluble form at high pH, chitosan is still insoluble that can support the bead structure (Natrajan et al., 2015).

Some studies have already pointed out that hydrogel system formed by alginate and chitosan obtained higher stability than the gel produced with single polymer of alginate (Segale et al., 2016). Actually, the hydrogen gel formed by alginate and other biopolymers have already been used in medical industry for wound dressing and burns healing (Straccia et al., 2015). During the process of chitosan coating calcium alginate gel beads, chitosan interacts with alginate and form a polyelectrolyte complex that is a semi-permeable membrane. This membrane can inhibit the release of core material (Krasaekoopt et al., 2004, Peniche et al., 2004), and improve the mechanical strength (Simó et al., 2017).

It has been reported that thickness of this membrane is influenced by the molecular weight, concentration of chitosan, and the pH condition (Peniche et al., 2004). Moreover, the encapsulation efficiency of chitosan coating calcium alginate gel beads is also influenced by the molecular weight of chitosan, so the lowered molecular weight tends to provide low efficiency encapsulates (Wang et al., 2011).

1.5 Depolymerization of alginate

Most commercially available alginates have large molecular weight that are around 32,000 to 400,000 g/mol (Lee and Mooney, 2012). It means that the viscosity of alginates is generally quite high even in a low concentration. It makes the maximum concentration for alginate used for microencapsulation with calcium chloride is only about 2-4% (Belščak-Cvitanović et al., 2015). The alginate with large molecular will cost much longer time to metabolize and the alginate chain cannot be degraded in human body, this helps to hold the structure and release bioactives slowly into the system (Li et al., 2010; Kristiansen et al., 2011).

The depolymerization process has been investigated as a novel strategy to modify the alginate molecular weight and viscosity and can convert alginate from polysaccharide into oligosaccharide and monosaccharides (Aida et al., 2010). After depolymerization, the alginate still can form gel with calcium cations (Kristiansen et al., 2011). In fact, the oligosaccharide alginate depolymerized from polysaccharide alginate by enzyme induced depolymerization or acid hydrolysis has attracted lots of interest for its application in the drug delivery (Szekalska et al., 2016).

Moreover, sodium alginate has been depolymerized utilising the mechanism of oxidative degradation, and it has shown that the low molecular alginate after modified has much better solubility which usually means more easily to digest (Mao et al., 2012). The previous studies have shown that the increase of alginate concentration in a certain range will increase the emulsion solution stability and fish oil encapsulation efficiency (Chan, 2011). The depolymerized alginate with higher solubility will achieve higher concentration than unmodified alginate, this may improve the alginate gel beads encapsulation efficiency and stability for fish oil. Moreover, it has been reported that the depolymerized alginates have a stronger anti-oxidation activity than the unmodified alginate (Kelishomi et al., 2016).

1.6 Research gaps

More than 200 kinds of alginate have been produced from different sources and have shown the difference properties including the content for G and M residues, length of blocks, and molecular weight (Pawar and Edgar, 2012, Lee and Mooney, 2012). The different types of alginate may have totally different gel properties in fish oil encapsulation. In addition, the depolymerization process has shown the ability to

decrease the molecular weight and viscosity of alginate to a controllable degree. However, the influence of depolymerization process on alginate base gel properties in fish oil microencapsulation has not been investigated yet.

1.7 Aims and hypothesis

1.7.1 Aims of this project

To determine the influence of different type of alginates on the encapsulation of fish oil

1.7.2 Objective

- To establish the method to identify the alginate gel beads properties.
- To identify the alginates gel beads properties of chemical synthesis alginate and seaweed source alginate in fish oil encapsulation.
- To identify the influence of depolymerization process of alginate gel properties in fish oil encapsulation.

1.7.3 Hypothesis

The chemical synthesis alginate and seaweed source alginate will show obviously different encapsulation capacities for fish oil. After depolymerization, the alginate with lower molecular weight and viscosity will form more stable gel bead for fish oil encapsulation.

1.8 Significance of the research in biotechnology

To optimise the alginate base gel beads composition for fish oil encapsulation, it is important to understand the influence of the types of alginates on beads properties. Moreover, the depolymerization has shown the ability to decrease the molecular weight

and viscosity of alginate into a controllable degree. If the depolymerization process has positive effects on alginate gel properties, it will become a novel strategy to modified and improve the commercial alginate to satisfy multiple requirements of alginates in food industry.

CHAPTER 2: Materials and Methods

2.1 Materials

Chemical synthesised alginate (Product number: W201502) and the brown seaweed sourced alginate (Product number: A2033) were purchased form Sigma-Aldrich Pty Ltd. Depolymerized alginate was modified form seaweed sourced alginate performed by Mr. Dhrub Ghimire (Maters student), and the molecular was decreased from 800 to 100 (g/mol). The fish oil (Product number: F8020) and all the reagents were all from Sigma-Aldrich Pty Ltd, including chitosan (Product number: 448877), hexane (Product number: 270504), acetic acid(Product number: A6283), and calcium chloride (Product number: C1016). The sodium alginates properties which had been identified in previous experiments are shown in Table. 2.1.

Table 2.1 The properties of three types of alginates used in this research

Alginates	Viscosity (mPa/s)	Solubility pH (1%) (w/v)	G/M Ratio*	Molecular weight (kDa)
Alginate 1: Chemical synthesized alginate	4-12	5-8	1.05	250-500
Alginate 2: Seaweed sourced alginate	300-500	5-8	0.32	750-1100
Alginate 3: Seaweed sourced alginate after depolymerization	4-12	3-5	0.39	100-200

* The data was from previous results of Mr. Peng Su and Dr. Reinu Elsa Abraham, molecular weight and G/M ratio was determined by HPLC adopted from Abraham et al. (Abraham et al., 2019). The solubility and viscosity was determined using 1% alginate solution.

2.2 Bead preparation

2.2.1 Calcium alginate gel bead

The calcium alginate gel beads for fish oil microencapsulation was prepared as described by Chan (Chan, 2011) with some modified cations. In the previous method, the amount of fish oil and emulsion was counted by volume, however, they are both easily to adhere to the container which makes the loss need to be considered during transfer process. In this project, the beads preparation process is tracked by weigh to decrease the influence from transfer for quantitative control.

The 150mM $CaCl_2$ gelling solution was prepared by dissolving the calcium chloride powder in distilled water. The emulsion solution was prepared with fish oil mixed with alginate solution following the desired concentration (i.e., 1% (w/v) alginate emulsion solution with 10% (v/v) fish oil loading emulsion solution was prepared with 1g sodium alginate, 90 mL distilled water and 10 mL fish oil. For 20% (v/v) and 30% (v/v) oil loading emulsion solution, the distilled water was decreased to 80 mL and 70 mL to maintain the total emulsion solution was 100 mL). Sodium alginate had been pre-dissolved with distilled water in a 200 mL beaker, and the beaker had been weighted before as W_1. Desired volume of fish oil had been added into alginate solution on balance, the weight of fish oil is recorded as W_2. Then beaker contained fish oil and sodium alginate solution was weighted, as W_3.

The alginate solution and fish oil was mixed using ultrasonic mixer about 1 min to get the emulsion solution, under room temperature. 10 mL emulsion solution from 100 mL was transferred into 50mL syringe, with 23 x G needle. The total weight of emulsion solution and syringe with needle, was recorded as W_4. Emulsion solution was dropped

into 100 mL $CaCl_2$ solution manually through needle, while the $CaCl_2$ solution was stirred by hot plate stirrer at 150 rpm and room temperature. The syringe and needle were weighed again, after the dropping process as W_5. The beads were incubated in $CaCl_2$ solution for 1 h until beads were completely formed and hardened. The accurate amount of fish oil dropped into this system W_o was calculated as below.

$$W_o = (W_4-W_5) \times W_2 / (W_3-W_1)$$

Then, the wet beads were extracted from gelling solution using mesh, it was stored at -80 ºC freezer for 6 h for further freeze drying. The freeze-drying process was performed in a freeze vacuum dryer for another 24 h.

2.2.2 Chitosan coating calcium alginate gel bead

The chitosan coating technology is referred to Kim's method (Kim, 2008). Chitosan was dissolved in the gelling solution ($CaCl_2$), meaning the coating process occurred at the same time with the cross-linking of calcium alginate. Chitosan powder was dissolved in 0.5% (w/v) acetic acid, after that, desired amount of $CaCl_2$ was added into the chitosan solution to produce the 150 mM gelling solution. The emulsion solution was prepared followed the same procedures described previously. To maintain the total concentration of gelling material stayed the same as before, the concentration of alginate was decreased for chitosan coating calcium alginate gel beads (i.e., for 1% (w/v) chitosan coating calcium alginate beads, it is prepared with 0.5% (w/v) alginate dropping into gelling solution containing 0.5% (w/v) chitosan. The concentration of alginate and chitosan were 1:1. 10 mL emulsion solution was dropped into 100 mL gelling solution with chitosan to form the chitosan coating calcium alginate gel. The gelling solution was stirred by hot plate stirrer, at 150 rpm and room temperature.

After beads formed and incubated for 1 h in gelling solution that contained 0.5% (w/v) chitosan, they were extracted from gelling solution and washed with 150 mM $CaCl_2$ solution to remove the unbinding chitosan from beads surface. The wet beads were extracted and freeze dried following the procedure of calcium alginate gel beads preparation. Moreover, the accurate amount of fish oil dropping into this system were calculated as described before.

2.3 Encapsulation efficiency

The procedure to determine the encapsulation efficiency of fish oil loading beads is modified from the method of Bannikova et al (Bannikova et al., 2018) and Chatterjee and Judeh (Chatterjee and Judeh, 2015). The beads used for encapsulation efficiency determination in this project are wet beads extracted form gelling solution. The encapsulation efficiency was determined based on the unencapsulated oil in gelling solution and wet beads surface before freeze drying process.

All wet beads produced from 10 mL emulsion solution were extracted from gelling solution by sieve. Beads were transferred into a glass vial, shaking with 20 mL hexane to extract the fish oil on the beads surface, for only 1 min to avoid the beads were degraded and releasing fish oil inside. Another 20 mL hexane was shaking with the gelling solution in a separatory funnel, the fish oil was extracted into the hexane layer. The concentration of fish oil in hexane was determined by the UV-spectroscopy at 269 nm (Chatterjee and Judeh, 2015). The cuvette used in this experiment was quartz cuvette, and experiment was performed in fume hood. The absorbance of unencapsulated oil can be transferred into the amount of fish oil according to the standard curve of fish oil in hexane. The formula modified from Bannikova et al

(Bannikova et al., 2018) and Chatterjee Chatterjee and Judeh (Chatterjee and Judeh, 2015) was shown below.

$$The\ EE\ (\%) = [(Wo - W_U) / Wo] \times 100\%$$

W_U is the total amount of unencapsulated oil. W_O is the accurate amount of fish oil dropping into this system from method 2.2.1.

2.4 Stability

To determine the beads stability after freeze drying, dried beads were stored in Petri dish packed by parafilm to isolate the beads away from air. The weight of Petri dishes was measure before use. These Petri dishes contained beads were incubated for 7 days in desiccator at room temperature. The oil leaking was measured by determining the surface oil and the oil on Petri dish surface. Beads were transferred into a glass vial, shaking with 20 mL hexane for 1 min. The amount of oil leaking on beads surface was determined by UV-spectroscopy, the same as the encapsulation efficiency test. By the standard curve, the surface oil was calculated as W_S, the oil on surface of container was weighted as W_C by determination the change of the Petri dish.

$$W_L = W_S + W_C$$

$$Oil\ leaking\ (\%) = [W_L / W_O] \times 100\%$$

W_L was the total amount of oil leaking in 7 days. The formula is modified form the methods of Bannikova et al (Bannikova et al., 2018) and Chatterjee and Judeh (Chatterjee and Judeh, 2015).

2.5 Determination of bead size and shape

The determination of size and shape of beads was modified from the method of Piornos et al (Piornos et al., 2017). The dried bead was transferred into Petri dishes and captured

by a digital camera, a ruler was captured with beads as standard. The diameter of beads was determined by the image analyze software, ImageJ (Abràmoff et al., 2004). 50 beads were randomly selected for the measurement. The dimeter data was processed with GraphPad, a versatile statistics software (Anderson et al., 2016) to check the size distribution of this experiment.

2.6 Scanning electron microscopy

The influence of fish oil on alginate gel beads structure was imaged with scanning electron microscopy (SEM). The procedure is referred to the method of Abraham et al (Abraham et al., 2019). The dried bead was fixed on the aluminum stub, and it was sputter coated with 10 nm platinum at 25 mA. The imaging was captured at the 5 kV accelerating voltage by the secondary electron detector.

2.7 Statistical analysis

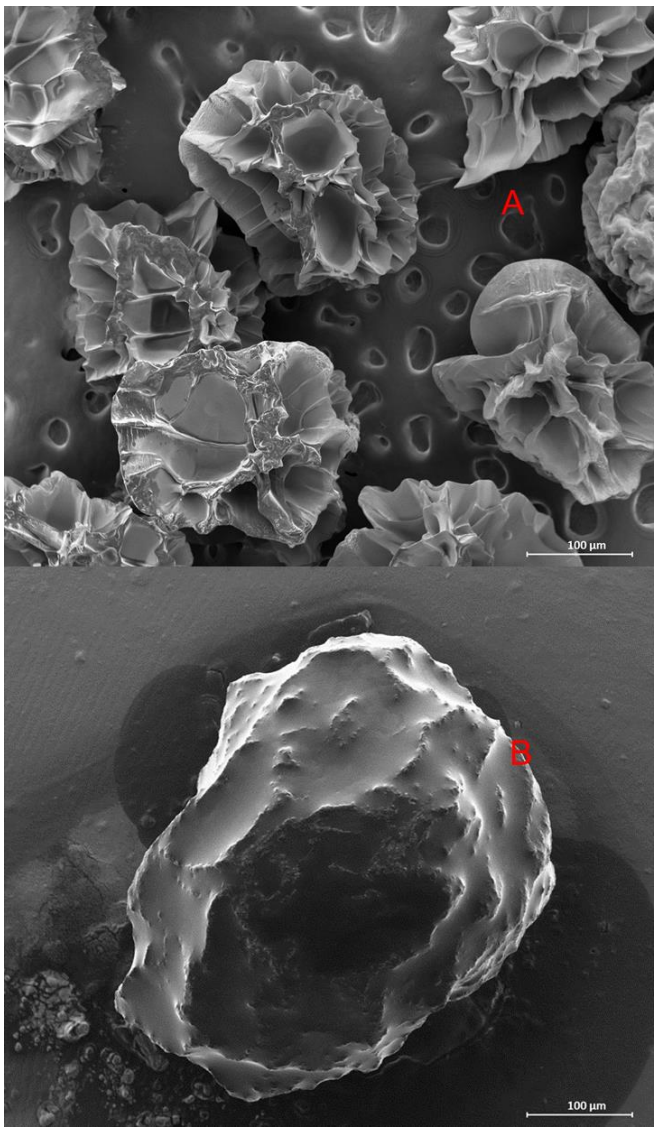
All experiments and tests were performed at least two replicates and values were shown with mean and standard error. Standard curve of fish oil in hexane had shown linear relationship ($R^2 = 0.9998$). 50 beads diameters were measured to determine the size and shape of beads per sample.

CHAPTER 3. Results

3.1 The effects of the fish oil loading on beads structure

To identify the influence of fish oil on calcium alginate gel beads structure, the dried calcium alginate gel beads with 10% fish oil loading and control group beads without fish oil loading were observed by SEM.

Figure 3.1 SEM observation of calcium alginate gel beads.



(A) Calcium alginate gel beads without fish oil; (B) Calcium alginate beads with 10% (v/v) fish oil loading. Beads were produced with 1% (w/v) seaweed sourced alginate.

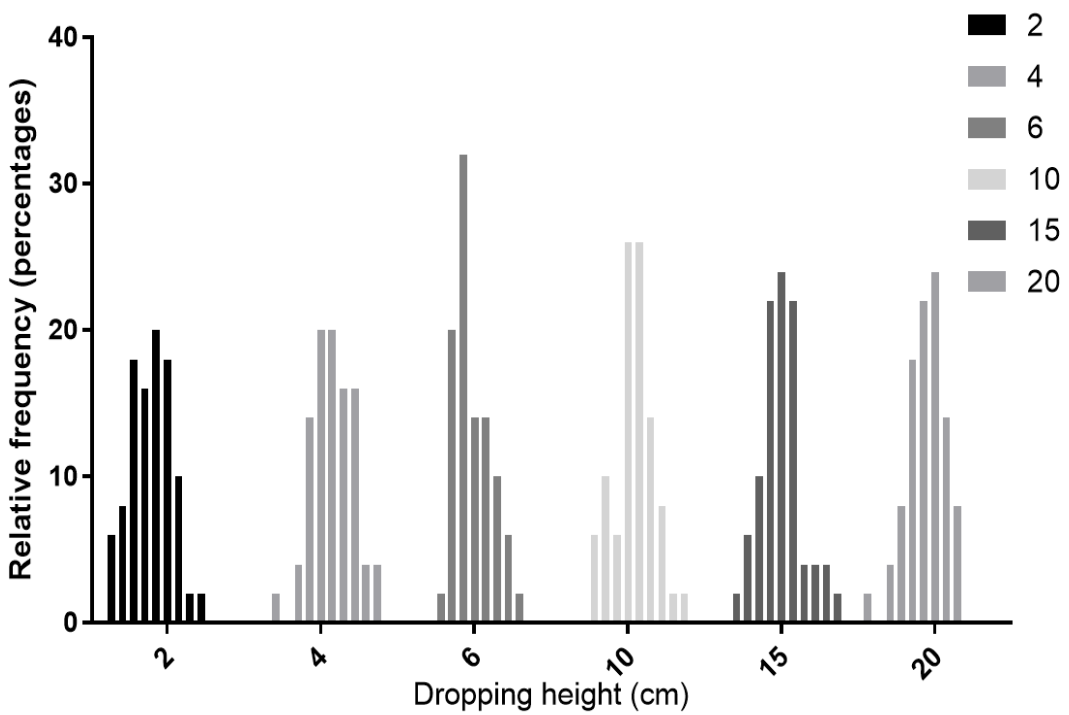
After freeze the calcium alginate bead still maintain the shape. According to the SEM

image, the fish oil loading beads tended to have more plump structure while the calcium alginate gel beads without oil loading showed numerous pores on beads surface after freeze drying (Fig 3.1). These results indicated that the fish oil might be involved in the composition of beads, supporting the beads structure. It also showed that the fish oil was still entrapped in the calcium alginate gel beads after freeze drying.

3.2 Effects of dropping height on beads formation

This study was conducted to understand the influence of dropping height form 2 cm to 20cm on beads size and shape formation.

Figure 3.2 Effects of dropping height on beads size distribution.



The emulsion solution was prepared with 0.5% (w/v) seaweed sourced alginate with 10% (v/v) fish oil loading yield, dropping from 2, 4, 6, 10, 15, and 20 cm height form the gelling solution surface respectively. The bin is from 1.7 mm to 2.9 mm, 0.1 mm per bin.

According to Fig 3.2, the diameter size distribution of different height levels showed a narrow ranged around 2.3 mm. It means that the error from different height of dropping process would not cause obvious influence on the beads size and shape.

3.3 Effects of different types of alginate on fish oil encapsulation properties

3.3.1 Encapsulation efficiency

To determine the gel properties of alginate and optimize the fish oil encapsulation, the 0.5% (w/v) and 1% (w/v) concentration of seaweed sourced alginate and chemical synthesized alginate were used to prepare fish oil loading gel bead. The fish oil loading yield was 10% (v/v), 20% (v/v), and 30% (v/v) respectively by decreasing the concentration of fish oil in emulsion solution.

Table 3.1 Encapsulation efficiency of fish oil encapsulated by seaweed sourced alginate and chemical synthesized alginate gel beads

Alginate	Alginate concentration (%) (w/v)	Fish oil loading (%) (v/v)	EE (%)
Chemical synthesized alginate	0.5	10	89.26 ± 10.50
		20	95.23 ± 3.04
		30	96.84 ± 1.51
Seaweed sourced alginate	0.5	10	98.29 ± 0.03
		20	97.99 ± 0.52
		30	98.16 ± 0.36
Chemical synthesized alginate	1	10	97.81 ± 0.16
		20	98.30 ± 0.10

		30	98.31 ± 0.25
		10	98.21 ± 0.44
Seaweed sourced	1	20	98.04 ± 0.26
		30	97.99 ± 0.11

* The value shown is mean ± standard deviation (n=2).

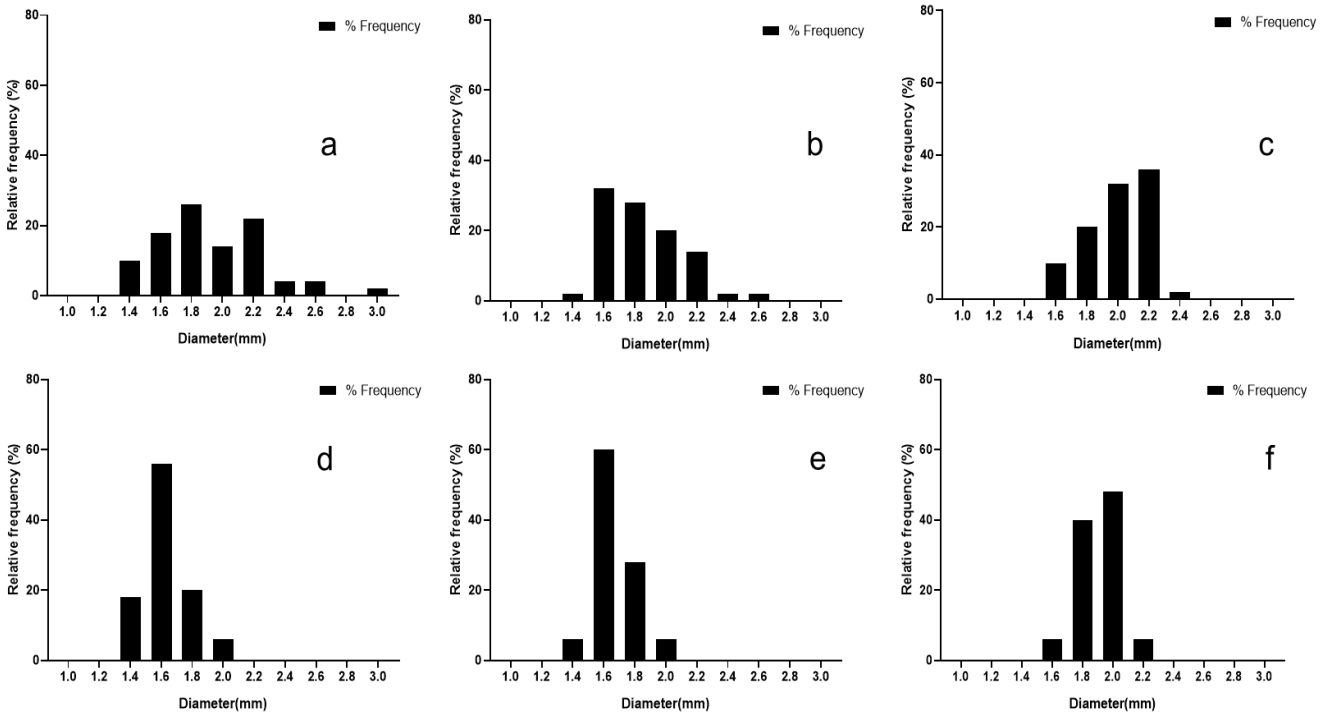
As shown in Table 3.1, the encapsulation efficiency of almost all beads was around 97% ~ 98%. The chemical synthesised alginate had a little lower efficiency at 0.5% (w/v) concentration, especially for 10% (v/v) fish oil loading beads, the encapsulation efficiency was decreased to 89.26% while the standard deviation was 10.5%. It indicated that this group of data will need to repeat again in the further experiment to get the accurate results.

While the fish oil loading yield was increasing, the efficiency of 0.5% (w/v) chemical synthesized alginate gel beads increased to 95%. When the concentration of alginates was increased to 1% (w/v), both seaweed sourced alginate and chemical synthesised alginates had encapsulation efficiency around 98%.

3.3.2 Size distribution

To determine the influence of alginates types and concentration, as well as the fish oil loading yield on beads size and shape, 50 beads were randomly selected for the diameter measurement per sample.

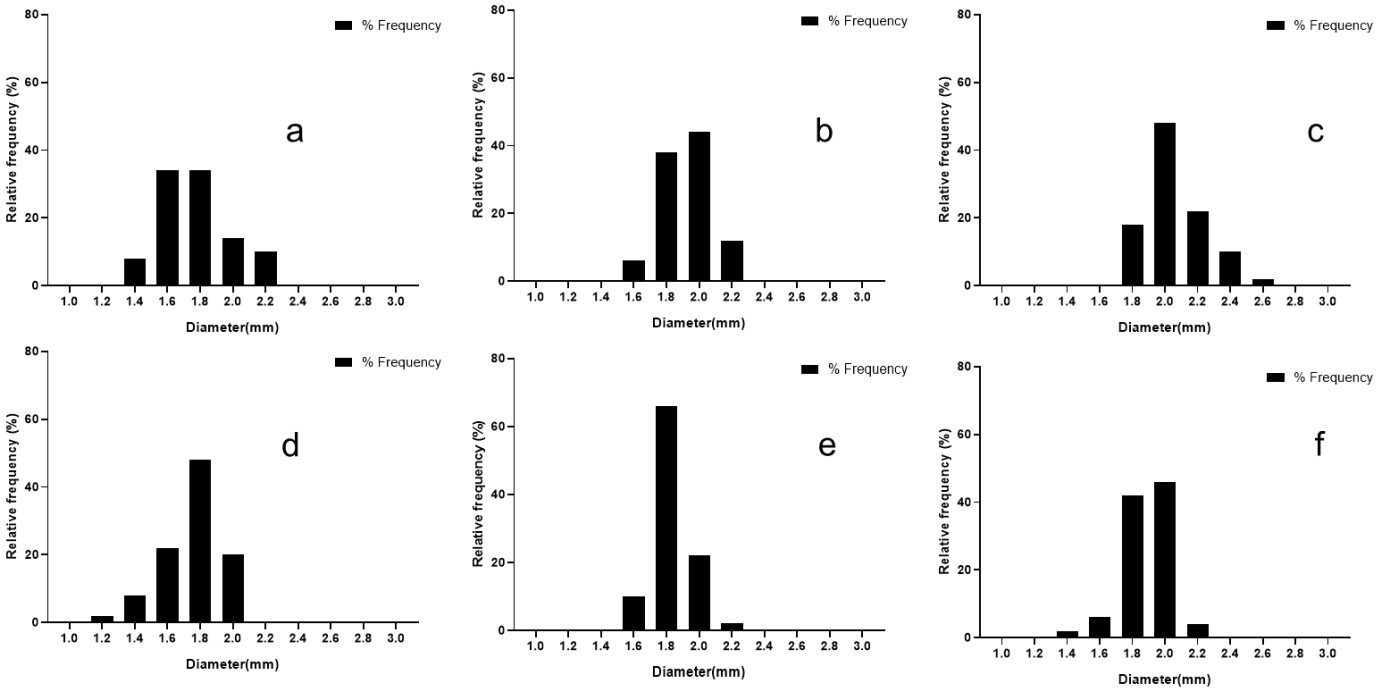
Figure 3.3 Size distribution of 0.5% seaweed sourced alginate and chemical synthesized alginate gel beads.



Beads were prepared with 0.5% (w/v) seaweed sourced alginate and chemical synthesized alginate. a, b, c were prepared with chemical synthesized alginate. d, e, f were prepared with seaweed sourced alginate. Fish oil loading was 10% (v/v), 20% (v/v), and 30% (v/v) respectively, from left to right.

According to the results (Figure 3.3), the beads produced by 0.5% (w/v) seaweed sourced alginate showed narrow diameter range around 1.4 mm to 2.2 mm while the diameter range of 0.5% (w/v) chemical synthesized alginate beads were around 1.4 mm to 3.0 mm. It means that the seaweed sourced alginate tends to have a more regular shape when the concentration of alginate is 0.5% (w/v) with fish oil loading from 10% (v/v) to 30% (v/v). The fish oil loading yield was increased from 10% (v/v) to 30% (v/v), but the influence on beads size and shape of two type of alginate were not obvious.

Figure 3.4 Size distribution of 1% seaweed sourced alginate and chemical synthesized alginate gel beads.



Beads were prepared with 1% (w/v) seaweed sourced alginate and chemical synthesized alginate. a, b, c were prepared with chemical synthesized alginate. d, e, f were prepared with seaweed sourced alginate. Fish oil loading was 10% (v/v), 20% (v/v), and 30% (v/v) respectively, from left to right.

For the 1% (w/v) seaweed sourced alginate gel beads, the diameter were around 1.4 mm to 2.2 mm. The diameter of 1% (w/v) chemical synthesized alginate were around 1.4 mm to 2.6 mm with a narrow range than the 0.5 % (w/v) alginate concentration. By comparing the results of Figure 3.3 with Figure 3.4, it showed that beads produced with seaweed sourced alginate were more regular in both 0.5% (w/v) and 1% (w/v) alginate concentration. The beads prepared with chemical synthesized alginate were not so regular when the concentration was 0.5% (w/v). The fish oil loading yield also did not cause obvious influence for on alginate gel beads size and shape.

3.3.3 Stability

According to the results of encapsulation efficiency and size distribution of two types of unmodified alginate (3.3.1 and 3.3.2), 1% (w/v) concentration of alginate and 10% (v/v) fish oil loading beads were used for stability test to investigate the stability of beads by determine the oil leaking ratio of dried beads.

Table 3.2 Stability in term of oil leakage of seaweed sourced alginates and chemical synthesized alginate gel beads

Alginate	Alginate concentration (%) (w/v)	Fish oil loading (%) (v/v)	Oil leaking ratio (%)
Alginate 1: Chemical synthesized alginate	1	10	13.71 ± 1.59
Alginate 2: Seaweed sourced alginate	1	10	58.33 ± 0.97

As shown in Table 3.2, the calcium alginate gel beads prepared with alginate from chemical synthesized had the lower oil leaking ratio as 13.71% while the oil leakage of brown seaweed source alginate was more than 50%, indicating that the calcium alginate gel bead formed with chemical synthesized alginate had much better stability.

3.4 The effects of depolymerized alginate on fish oil encapsulation properties

3.4.1 Encapsulation efficiency

The depolymerized alginate from seaweed sourced alginate was used for beads preparation to compare with the unmodified seaweed sourced alginate to identify the influence of depolymerization process on alginate gel bead properties in fish oil encapsulation.

Table 3.3 Encapsulation efficiency of seaweed sourced alginate and depolymerized alginate gel beads

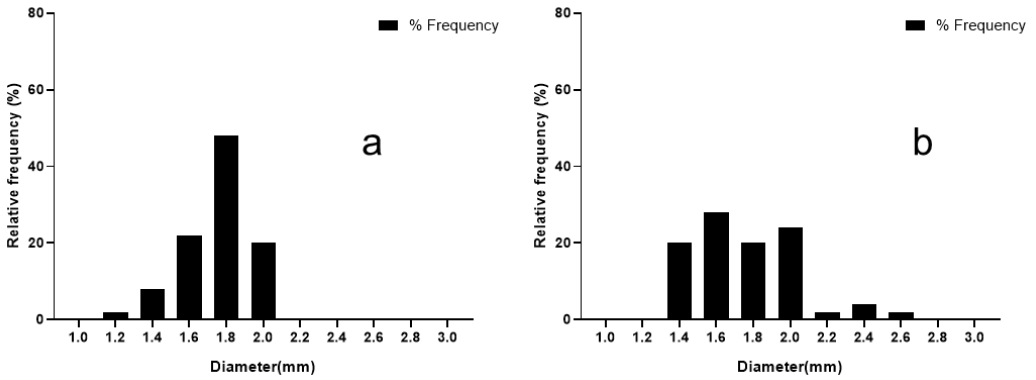
Alginate	Alginate concentration (%) (w/v)	Fish oil loading (%) (v/v)	EE (%)
Alginate 2: Seaweed sourced alginate	1	10	98.71 ± 0.27
Alginate 3: Depolymerized alginate	1	10	97.06 ± 0.25

As the results shown in Table 3.3, the encapsulation efficiency of depolymerized alginate was 97.06% while the efficiency of seaweed sourced alginate was 98.71%, It suggested that almost 100% fish oil was entrapped into wet beads during the beads preparation process for both depolymerized alginate and unmodified seaweed sourced alginate.

3.4.2 Size distribution

The diameter of beads prepared with modified alginate was measured to compared with the unmodified alginate (seaweed source) gel beads for determining the influence of depolymerization of alginate on beads size and shape.

Figure 3.5 Size distribution of seaweed sourced alginate and depolymerized alginate gel beads.



Beads were produced with 1% (w/v) alginate concentration with 10% (v/v) fish oil loading. a, seaweed sourced alginate gel beads. b, depolymerized alginate gel beads.

The diameter distribution range of seaweed sourced alginate gel beads was around 1.2 mm to 2.0 mm, and that was around 1.4 mm to 2.6 mm (Figure 3.5) for depolymerized alginate gel beads. The unmodified seaweed sourced alginate had narrower diameter range.

3.4.3 Stability

The oil leakage of dried beads after one week incubation was measures to determine the influence of depolymerized alginate on gel bead stability in fish oil encapsulation.

Table 3.4 Stability in terms of oil leakage of seaweed sourced alginates and depolymerized alginate gel beads

Alginate	Alginate concentration (%) (w/v)	Fish oil loading (%) (v/v)	Oil leaking ratio (%)
Alginate 2: Seaweed sourced alginate	1	10	58.33% ± 0.97
Alginate 3: Depolymerized alginate	1	10	28.21% ± 0.96

*The stability of alginate 2 was used as control group, referred to result 3.3.3.

The depolymerized alginate had less oil leakage than the seaweed sourced alginate. About 30% (v/v) oil leakage was reduced after depolymerization process (Table 3.4). It indicated that the calcium alginate gel beads prepared with depolymerized alginate had higher stability than seaweed sourced alginate.

3.5 The effects of chitosan coating depolymerized and seaweed sourced alginate on fish oil encapsulation properties

To optimize the beads composition and determine the influence of depolymerization of alginate on chitosan coating calcium alginate gel beads, chitosan coating technology was used for beads preparation in fish oil encapsulation with depolymerized alginate and seaweed sourced alginate.

3.5.1 Encapsulation efficiency

To determine of the effects of chitosan coating on the encapsulation efficiency of depolymerized alginate and seaweed sourced alginate gel bead in fish oil encapsulation, chitosan is used as 1:1 ratio with alginate that is randomly selected. Beads were prepared with 0.5 % (w/v) concentration of alginate and 10% (v/v) fish oil loading. The

gelling solution was 150 mM $CaCl_2$ solution with 0.5% (w/v) chitosan. The unencapsulated oil was determined with the same procedure as calcium alginate gel beads.

Table 3.5 Encapsulation efficiency of chitosan coating seaweed sourced alginate and depolymerized alginate gel beads (at 1:1 ratio)

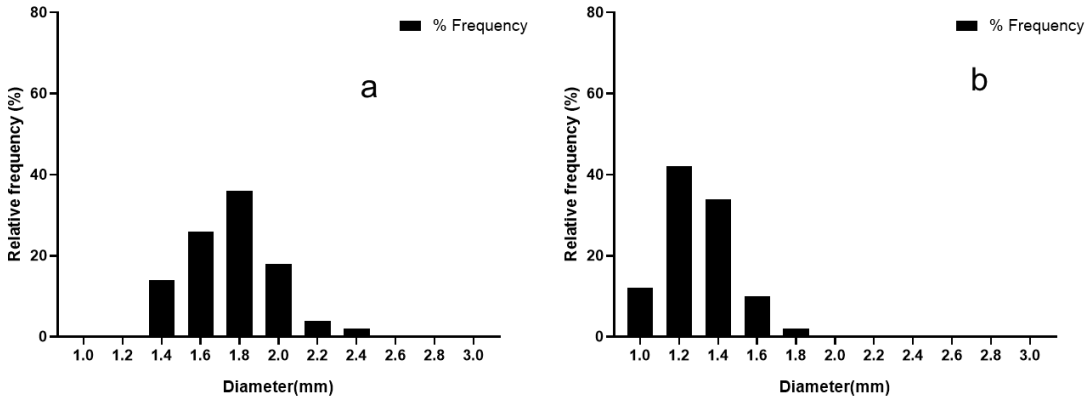
Alginate	Alginate concentration (%) (w/v)	Chitosan concentration (%) (w/v)	Fish oil loading (%) (v/v)	EE (%)
Alginate 2: Seaweed sourced alginate	0.5	0.5	10	98.98 ± 0.25
Alginate 3: Depolymerized alginate	0.5	0.5	10	98.29 ± 0.54

The encapsulation efficiency of unmodified alginate (seaweed source) reached up to 98.98% while the efficiency of depolymerized alginate was 98.29%. It indicated that the chitosan coating process had no obvious negative effects on fish oil encapsulation efficiency for both seaweed sourced alginate and depolymerized alginate (from seaweed sourced alginate).

3.5.2 Size distribution

To determine of the effects of chitosan coating on the bead size distribution of depolymerized alginate and seaweed sourced alginate gel bead in fish oil encapsulation, the diameter of 50 dried beads per sample produced with depolymerized alginate and seaweed sourced alginate were measure followed the same procedure with calcium alginate gel beads.

Figure 3.6 Size distribution of chitosan coating seaweed sourced alginate and depolymerized alginate gel beads (at 1:1 ratio)



Beads were prepared with 0.5% (w/v) alginates with 10% fish oil loading. Gelling solution was 150mM $CaCl_2$ solution with 0.5% (w/v) chitosan: (a) seaweed sourced alginate, (b) depolymerized alginate.

The diameter of beads prepared with unmodified seaweed sourced alginate was around 1.4 mm to 2.4 mm while the depolymerized alginate had the beads diameter range around 1.0 to 2.2 mm. For the depolymerized alginate, the chitosan coated beads had shown lower diameter than the beads produced without chitosan coating, comparing with the results 3.4.2.

3.5.3 Stability

To identify influence of chitosan coating on depolymerized alginate and seaweed sourced alginate gel bead stability in fish oil encapsulation, the oil leakage of dried chitosan coating calcium alginate gel beads was measured after one week incubation. The measurement was performed with the same procedure with calcium alginate gel beads.

Table 3.6 Stability in terms of oil leakage of chitosan coating seaweed sourced alginate and depolymerized alginate gel beads (at 1:1 ratio)

Alginate	Alginate concentration (%) (w/v)	Chitosan concentration (%) (w/v)	Fish oil loading yield (%) (v/v)	Oil leaking ratio (%)
Alginate 2: Seaweed sourced alginate	0.5	0.5	10	23.05 ± 6.48
Alginate 3: Depolymerized alginate	0.5	0.5	10	Unstable

For seaweed sourced alginate, the oil leakage for the chitosan coating beads was decreased to 23.05% than the calcium alginate gel beads without chitosan coating. The oil leakage of beads produced with depolymerized alginate was too high that cannot be calculated by current standard curve (Table 3.6), which still needs further experiment.

CHAPTER 4: Discussion

4.1 The gel bead properties of seaweed sourced alginate and chemical sourced alginate in fish oil encapsulation

For 0.5% and 1% alginate concentration beads, almost all calcium alginate beads had the encapsulation efficiency ranging from 97% to 98% with 10% (v/v), 20% (v/v) and 30% (v/v) fish oil loading yield. The chemical synthesised alginate had lower encapsulation efficiency when the concentration of alginate was 0.5% (w/v) with 10% (v/v) fish oil loading yield, but it was still about 90%. When the concentration of alginate was 1%, both chemical synthesised and seaweed sourced alginate showed encapsulation efficiency over 97%. It means that almost 100% fish oil has been entrapped into calcium alginate gel beads, for both seaweed source and chemical synthesis alginate. Hence, the 1% (w/v) concentration of alginate with 10% (v/v) fish oil loading beads were determined for further stability test. However, the oil leaking of beads was quite serious after one week. The seaweed sourced alginate even showed about 60% oil leaking ratio while the chemical synthesised alginate only showed about 10% oil leaking ratio.

The freeze drying process of beads could be one possible reason for the oil leaking. It has reported that the freeze drying process would form a microporous structure and decrease the shelf-life of beads (Kaushik et al., 2015). Some other studies also used hot air to dry the beads instead of freeze drying process such as spray drying. However, it has been reported that the hot air would cause the oxidation of fish oil during the spray drying process (Kaushik et al., 2015). Therefore, it is important to find a possible strategy to decrease the negative effects from drying process to improve the microcapsules stability and shelf-life in further research. Calcium alginate gel beads prepared with seaweed sourced alginate had shown more a spherical and regular shape,

however, the oil leaking was almost 60% after one week. Comparing with that, the oil leaking of chemical synthesis source sodium alginate was only 13.71%. The higher G/M ratio of chemical synthesis source alginate could be one possible reason because only the G residues are involved in the cross-linking of alginate with calcium cations, and influence the encapsulation capacity (Ching et al., 2017). The high G residues content of chemical synthesis source sodium alginate makes it has more strongly cross-linking with calcium cation (Rehm, 2009).

4.2 The improvement of fish oil encapsulation stability by depolymerized alginate

To determine the influence of depolymerized alginate gel bead property in fish oil encapsulation, the depolymerized alginate and unmodified seaweed sourced alginate were used for calcium alginate gel beads and chitosan coating calcium alginate beads preparation. The G/M ratio of seaweed sourced was 0.32 while that of modified alginate was 0.39, indicating that the depolymerization had no obvious influence on the G/M ratio of alginate. After modification, the molecular weight of seaweed sourced alginate was decreased from 750-1100 kDa to 100-200 kDa, and viscosity decreased to 4-12 m Pa/s from 300-500 Pa/s. According to the encapsulation efficiency of both calcium alginate gel beads and chitosan coating calcium alginate beads, the depolymerized alginate had no negative effects for beads forming process. Moreover, the stability of alginate gel bead showed a remarkable improvement with the oil leakage decreased from 58% to 28%. It exhibits that the depolymerized alginates could form much more stable beads in the fish oil microencapsulation process.

The chitosan coating strategy had improved the stability of seaweed sourced alginate gel beads, reducing about 30% oil leakage, but it caused some negative effects on

depolymerized alginate beads resulting the oil leakage was too high to determine. It supposed to be the concentration of fish oil in hexane was over the linear relationship range of standard curve. However, this result indicated that the oil leakage of chitosan coating depolymerized alginate gel bead was extremely high. One possible reason of the high oil leakage is the 1:1 ratio of chitosan coating, which decreased the concentration of alginate. It has shown that chemical synthesised alginate with lower viscosity and molecular weight (750-1100 kDa) than seaweed sourced alginate (100-200 kDa) had the lowest encapsulation efficiency at 0.5% concentration, as 89.26%. When the concentration increased to 1%, the bead encapsulation was increasing up to 97%. For the depolymerize alginate with low viscosity and molecular weight, the low concentration may cause the low encapsulation efficiency and stability for fish oil.

4.3 Conclusion

The results indicated that the chemical synthesised alginate with higher G residues (G/M ratio as 1.05) formed gel bead has only 13.71% oil leakage while the seaweed sourced alginate (G/M ratio as 0.32) beads showed 58.33% oil leakage. The depolymerized alginate from seaweed sourced alginate formed gel bead with less oil leakage (28.21%). For the commercial alginates with lower G residues content and large molecular weight, the depolymerization will be a potential strategy to improve the alginate gel properties in encapsulation. However, the impact of alginate properties such as molecular weight, viscosity, and G/M ratio on the alginate bead encapsulation stability and efficiency has not been fully understood for the limitation of available alginates.

4.4 Further work

In the further research, more types of alginate should be investigated to understand the impacts of alginate properties on gel beads properties in encapsulation. In addition, the influence of depolymerized alginate on gel bead bioavailability should also be investigated by determine the release of fish oil in simulated gastro-intestinal test in the further application in food industry, as well as the fish oil oxidation.

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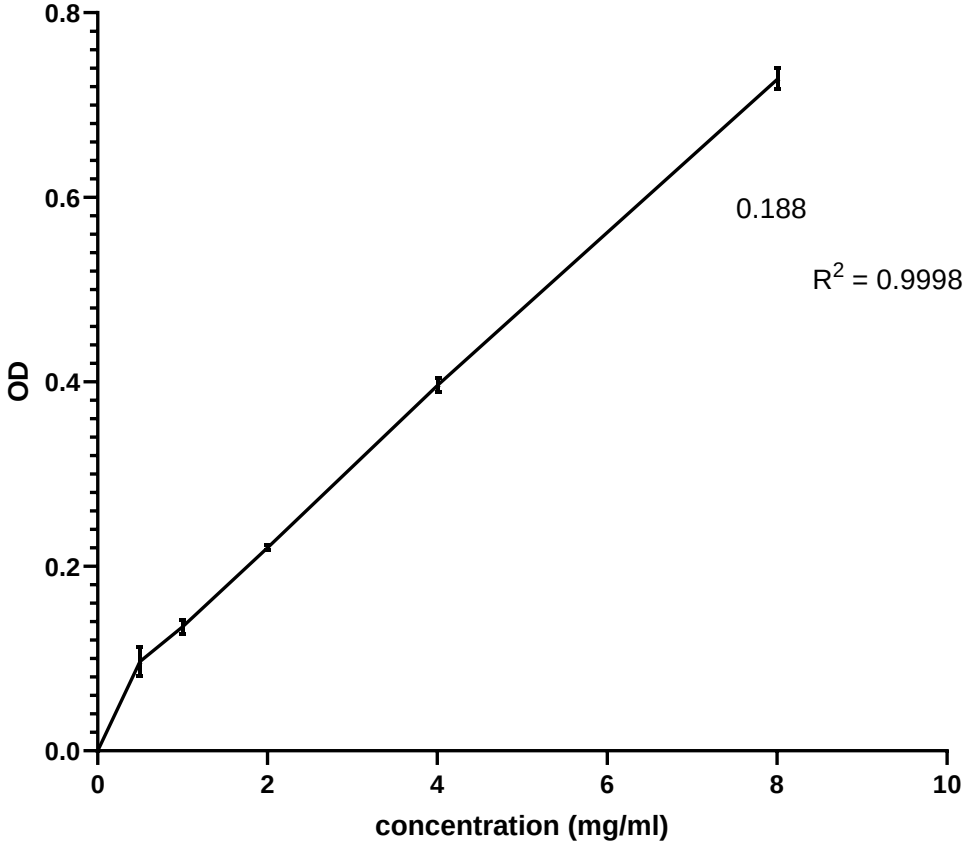
Appendices

Appendices 1 The standard curve of fish oil in hexane



The standard curve is built on fish oil in hexane, determined by the UV-spectroscopy at 269 nm. It only showed the linear relationship from 0.5 mg/mL to 8.01 mg/mL, so this range was used to build the standard curve for the determination of encapsulation efficiency and stability. The baseline of this standard was based on the absorbance of hexane. The value shown is mean ± standard deviation (n=3).