การพัฒนาวิธีการเอนแคปซูเลชันของวัสดุชีวภาพระดับนาโนโดยใช้ไครโอเจลของไคโตซาน

นางสาวณฐพร โสวสด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์นาโนและเทคโนโลยี (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
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DEVELOPMENT OF ENCAPSULATION TECHNIQUE FOR NANO-BIOMATERIALS USING CHITOSAN-BASED CRYOGEL

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Nanoscience and Technology
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Thesis Title	DEVELOPMENT OF ENCAPSULATION TECHNIQUE FOR NANO-BIOMATERIALS USING CHITOSAN-BASED CRYOGEL
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ณฐพร โสวสด : การพัฒนาวิธีการเอนแคปซูเลชันของวัสดุชีวภาพระดับนาโนโดยใช้ไครโอเจลของไคโตซาน. (DEVELOPMENT OF ENCAPSULATION TECHNIQUE FOR NANO-BIOMATERIALS USING CHITOSAN-BASED CRYOGEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.ธวัชชัย ชรินพาณิชกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. กิตติคุณ ดร.วิวัฒน์ ตัณฑะพานิชกุล, Assoc. Prof. Kyuya Nakagawa, D.Eng., 176 หน้า.

งานวิจัยนี้เลือกสารเคอร์คูมินเป็นโมเดลของวัสดุชีวภาพ กระบวนการเตรียมและบรรจุที่เลือกใช้คือไครโอทรอปิกเจเลชัน (cryotropic gelation) โดยมีวัตถุประสงค์เพื่อการพัฒนาและศึกษาการเอนแคปสารเคอร์คูมินซึ่งละลายในวัฏภาคน้ำมันด้วยระบบโฮโดรเจล การบรรจุสารเคอร์คูมินเริ่มจากการเตรียม O/W (oil in water) นาโนอิมัลชันโดยใช้สารคอลลอยด์ของไคโตซาน จากนั้นนาโนอิมัลชันถูกเปลี่ยนกลายเป็นเจลโดยใช้กระบวนการไครโอทรอปิกเจเลชัน การบรรจุอนุภาคน้ำมันในไครโอเจลนี้จัดเตรียมโดยใช้ระบบ 3 องค์ประกอบของไคโตซาน, **K**-คาราจีแนน และ คาบอกซีเมททิลเซลลูโลส (NaCMC) แล้วทำการศึกษาผลของปัจจัยของกระบวนการเอนแคปซูเลชันได้แก่เงื่อนไขของกระบวนการแช่แข็ง (กระบวนการแช่แข็งในทิศทางเดียว) ทั้งกรณีที่ไม่บ่มตัวอย่างและบ่มตัวอย่างหลังแช่แข็ง ตลอดจนผลกระทบของความเข้มข้นของไคโตซาน (1.5, 2.0 and 3.0%), อัตราส่วนของความเข้มข้นระหว่างคาราจีแนน กับคาบอกซีเมททิลเซลลูโลส (1:9, 4:6 and 6:4) และน้ำหนักโมเลกุลของไคโตซาน (ต่ำ กลาง และ สูง) โดยเน้นผลของอัตราทำให้เย็นระหว่างกระบวนการแช่แข็ง (-0.5, -1, and -2 ˚C/min) ต่อโครงสร้างสัณฐานของไครโอเจล และลักษณะการปลดปล่อยสารเคอร์คูมินจากแคปซูลได้ถูกศึกษา อนึ่งผลของ pH ของฟอสเฟตบัฟเฟอร์ต่อการบวมตัวของตัวอย่างก็ถูกศึกษาเช่นกัน ผลการศึกษาปรากฏว่าผลได้ของการผลิตนาโนแคปซูลอยู่ในช่วง 83.9 ถึง 99.6% %w/w เมื่อใช้ไคโตซานที่มีน้ำหนักโมเลกุลสูงโดยขึ้นกับอัตราทำให้เย็นระหว่างกระบวนการแช่แข็ง ข้อมูลนี้ชี้ให้เห็นว่าไคเนติกส์ของการเกิดเจลมีความสัมพันธ์แนบแน่นกับระดับของการเอนแคป กราฟของการปลดปล่อยของสารเคอร์คูมินซึ่งมีทั้งอัตราที่เป็นแบบรวดเร็วและแบบลำดับหนึ่ง (burst release and first order) เกิดขึ้นจากการเปลี่ยนแปลงอัตราการทำให้เย็นระหว่างกระบวนการแช่แข็ง เคอร์คูมินสามารถมีการปลดปล่อยอย่างต่อเนื่องนานกว่า 4 วันและปริมาณการปลดปล่อยสารเคอร์คูมินมีค่าสุดท้ายมากสุดในช่วง 41.1 ถึง 59.9%. การวิจัยนี้ประสบความสำเร็จในการควบคุมการปลดปล่อยสารเคอร์คูมินภายใต้เงื่อนไขการทำให้แข็งที่รวดเร็วที่ -2 ˚C/min โดยไม่ขึ้นกับค่าสัดส่วนของวัฏภาคน้ำมัน จากการศึกษาเปรียบเทียบพบว่าตัวอย่างที่ผ่านการบ่มมีอัตราการปลดปล่อยสารเคอร์คูมินช้ากว่าตัวอย่างที่ไม่ผ่านการบ่มเพราะการบ่มทำให้โครงสร้างของตัวอย่างเกิดการเชื่อมโยงโครงข่ายมากขึ้น

ภาควิชา สหสาขาวิทยาศาสตร์นาโนและเทคโนโลยี ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาศาสตร์นาโนและเทคโนโลยี ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2555.....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5187777920 : MAJOR NANOSCIENCE AND TECHNOLOGY
KEYWORDS: ENCAPSULATION / CRYOGEL / CONTROLLED RELEASE / CURCUMIN / CHITOSAN / CARRAGEENAN

NATAPORN SOWASOD: DEVEOPMENT OF ENCAPSULATION OF NANO - EMULSION BIOMATERIAL USING CHITOSAN BASED CRYOTROPIC GELATION. ADVISOR: ASSOC. PROF. TAWATCHAI CHARINPANITKUL, D.Eng., CO-ADVISOR: EMERITUS PROF. WIWUT TANTHAPANICHAKOON, Ph.D., ASSOC. PROF. KYUYA NAKAGAWA, D.Eng., 176 pp.

Curcumin was chosen as the present model biomaterial. A technique was developed and investigated to encapsulate the oil-soluble curcumin into a hydrogel system. The curcumin loaded oil-in-water nano-emulsion was prepared using a chitosan colloidal suspension, and the emulsion was converted into hydrogel by using cryotropic gelation. The cryogel based oil encapsulation was carried out with a ternary system of colloidal chitosan, κ-carrageenan, carboxy methylcellulose sodium salt (NaCMC) suspensions and the effects of the freezing condition (unidirectional freezing rate with and without post-freezing incubation) as well as initial concentration ratio of carrageenan to NaCMC on the properties of the nanocapsules were experimentally studied. The effects of chitosan concentration (1.5, 2.0 and 3.0%), Κ-carrageenan:NaCMC ratio of the polymer suspension (1:9, 4:6 and 6:4) and MW of chitosan (low, medium and high) on sol-gel formation were investigated. The effects of cooling rate during unidirectional freezing (-0.5, -1, and -2 ◦C/min) on the morphology of the freeze-dried cryogel specimens and the release behavior of curcumin from the specimens were determined. So were the effects of pH of the phosphate-buffered media on the swelling of the specimens. The key findings are as follows. The encapsulation yields were found to vary from ca 83.9 to 99.6% when a high-MW chitosan was used, and they were influenced by the cooling protocols during freezing, indicating that the gel formation kinetics was related to the degree of encapsulation. The release curves showed that both a burst release and a first order release were achieved simply by changing the freezing conditions. Controlled release of the encapsulated curcumin in an aqueous system could be maintained for 4 days, and the releasable amount of curcumin was found to range from 41.1 to 59.9%. The encapsulation yield as well as the release pattern and releasable amount of curcumin were influenced by the cooling protocol used during freezing. Irrespective of the introduced oil phase composition, controlled release of curcumin was achievable when the cooling rate was sufficiently high at -2 ^{o}C/min. When cold incubation was carried out, the release rate became slower than the corresponding case of no incubation because crosslinking was enhanced during the incubation period.

Department: Nanoscience and Tecnology Student's Signature.....

Field of Study: Nanoscience and Tecnology Advisor's Signature.....

Academic Year:.....2012.....Co-advisor's Signature.....

CHAPTER I

INTRODUCTION

1.1 Background

Nano/micro-encapsulation is an important process for food and pharmaceutical industries by which core materials such as oils are packaged within wall material. The advantages of this technique were protection of core materials from heat, light, and oxygen. In the last decades, nanoencapsulation technology is widely interested in the food and pharmaceutical industry for various applications such as efficiency of protection and controlled release in active food ingredients (Lakkis, J.M., 2007) such as vitamin, polyphenol etc. Spray drying is the most widely accepted encapsulation technique in industry, which is the transformation of a feed from a fluid state (solution, dispersion, emulsion) to dried particulate form by spraying the feed into a hot drying medium (Atmane, M. et al., 2006). Other problem with spray drying for this technology produces no uniform microcapsules and not good for heat-sensitive material (Desai, K.G.H. et al., 2005; Gouin, S., 2004; Bylaitë, E., 2001). This traditional process still cannot be adapted to encapsulate an extremely sensitive substances such as proteins. Several researchers are interested to prepare hydrogels for encapsulation to apply drug delivery system because hydrogels are three-dimensional networks of hydrophilic polymers that are insoluble and swell in water, it has a large amount of space for stabilizing biologically active substances. The size of particles from encapsulation process into hydrogel may be classified as: macro

(>5000 mm): micro (1.0–5000 mm); and nano (<1.0 mm). Capsules below 1.0 mm in size are frequently referred to as nanocapsules, which are often made by very specialized nanoencapsulation methods. In this study, we select nano-size a food active ingredient for hydrogel encapsulation by controlled release in the body. The nano - scale holes were formed in hydrogel structures by polymeric chains. The selected proper polymers can control release of a system and optimizing the gelation degree. We have to select polymer that biocompatibility, biodegradability, nontoxicity and biological properties (such as chitosan, carrageenan, PVP, PLGA, alginate etc.). The main disadvantage of these methods is to change the pH and temperature influence form the gel and using a cross-linking agent to form gel. However, hydrogel preparation methods usually requires to use critically low (or high) pH conditions, or to use high temperature conditions for initiating cross-linking reactions. This seriously limits use of the hydrogels by such conditions for encapsulation bioactives. These problems can be overcome by using cryotropic gel formation because this process uses low temperature for encapsulation materials. Lozinsky *et al*. suggest that cryotropic gelations are interesting hydrogel preparation method in food and pharmaceutical applications such as drug delivery, skin tissue engineering and controlled release of food because cryotropic gelation is sol-gel transition induced by the concentration increase of the substrates due to the dehydration by freezing (Giannouli, P. et al., 2003; Orrego, C. E. et al., 2009; Vrana, N. E. et al., 2008; Ho, MH., et al., 2004).

In this study, formulation a ternary of chitosan-carrageenan/ carboxymethyl cellulose (CMC) based cryogel system for encapsulating oil phase of a food active ingredient (such as curcumin, polyphenol and flavonoid) in modified suspension are

investigated by freeze drying. The nano-emulsion (oil in water) is prepared by high speed homogenizer and the suspension is prepared by ultrasonic homogenizer. A suspension of oil phase and water phase were converted into cryogels after freezing. After freeze drying, microstruture of frozen cryogel is analyzed by scanning electron microscopy (SEM). The polymeric structures of cryogels are investigated for clear the role of freezing. Release behavior of a food active ingredient is investigated in the pH of phosphate-buffer at selected pH conditions. The influences of freezing condition on the release characteristics will also be investigated.

1.2 Objectives of study

1.2.1. To develop nano-emulsion for encapsulating oil phase into cryogel matrices.

1.2.2. To investigate controlled release characteristics of active food ingredients from cryogels system.

1.3 Scope of research

1.3.1 Preparation of hydrogels using chitosan based cryotropic gelation (chitosan, carboxymethyl cellulose and κ-carrageenan ternary system) for encapsulating active food ingredients dissolved in oil.

1.3.2 Determine the optimal ratio of oil phase that can be encapsulated in the prepared cryogel;

- The mass ratio of active food ingredients: chitosan (solution); 5:95, 10:90 and 20:80

1.3.3 Determine influence of NaCl on cryogel formation by varying as follow; 0%, 1%, 2% and 5% of NaCl concentration.

1.3.4 Determine the effect of the molecular weight of chitosan.

- Three types of chitosan: low (Mw=220,000Da), medium (Mw=680,000Da) and high (Mw=1,300,000Da) molecular weight.

1.3.5 Determine the optimal ratio of κ-carrageenan: carboxymethyl cellulose (CMC); 1:9, 2:8, 3:7, 4:6 and 6:4

1.3.6 Preparation cryogel at ratio of κ-carrageenan: carboxymethyl cellulose (NaCMC); 1:9 (set 1), 4:6 (set 2), and 6:4 (set 3)

- Without incubation at cooling rate: -0.5°C/min, -1°C/min and -2°C/min, in which the cooling plate temperature was set at -20°C and maintained for 24 hours at a chamber pressure of around 10-20 Pa.

- Cold incubation at cooling rate: -0.5◦C/min, -1◦C/min and -2◦C/min, set -10°C and maintained 10 hr and then set at -20°C and maintained for 24 hours at a chamber pressure of around 10-20 Pa.

1.3.7 Investigation of freezing conditions and the effect of ratio of Carrageenan to NaCMC 4: 6 and 6 : 4 on swelling, yield of gel fraction and the release behavior of curcumin at without incubation and cold incubation.

1.3.8 Characterize of the obtained cryogel.

- Observation of prepared nanoparticles of freeze-dried samples, freeze - thawed sample and oil phase in nano-emulsion sample are investigated by SEM, TEM and Optical microscope respectively.

- Yield of encapsulation is analyzed by High-Performance Liquid Chromatography (HPLC).

- Average size and Zeta potential

- Yield of gel fraction

- Release curve of active food ingredients is analyzed by High-Performance Liquid Chromatography (HPLC).

1.4 Expected benefits

An understanding of how to prepare a ternary of chitosan-carrageenan/ carboxymethyl cellulose (CMC) based cryogel system for encapsulating oil phase of a food active ingredient by using ice-crystal templates and three different freezing methods will be obtained.

CHAPTER II

FUNDAMENTAL KNOWLEDGE AND LITERATURE REVIEW

2.1 Encapsulation technology

Nano/microencapsulation is an important process for food and pharmaceutical industries, in which core materials such as oils or flavours are packaged within wall material. Typical advantage of this technique is protection of core materials from heat, light, and oxygen. The techniques of encapsulation can be classified according to process characteristics (spraying processes, coating processes, and suspension processes) and capsules formation mechanism (physical, chemical, and physicochemical)

An important step in encapsulating a food ingredient is the selection of a suitable wall material, basically a film-forming biopolymer, from a wide variety of natural or synthetic polymers, depending on the core material and characteristics desired in the final microcapsules. In general, controlled release is another major expectation of encapsulating a food ingredient.

With encapsulation, the release of active ingredient can be controlled by retaining a core structure in the encapsulating matrix. Many different systems of this encapsulation have been established and proposed for food application, such as spray drying, spray chilling or spray cooling, extrusion coating, fluidized-bed coating, air suspension coating, multi-orifice centrifugal extrusion, coacervation/ phase

separations, liposome entrapment, inclusion complexion, co-crystallization, interfacial polymerization and rotational suspension separation (Desai, K.G.H. et al., 2005; Yuliani, S., et al., 2004; Liu, X.-D., et al.) .

2.1.1 Type of encapsulation

2.1.1.1 Reservoir type

2.1.1.2 Matrix type

2.1.1.3 Combination

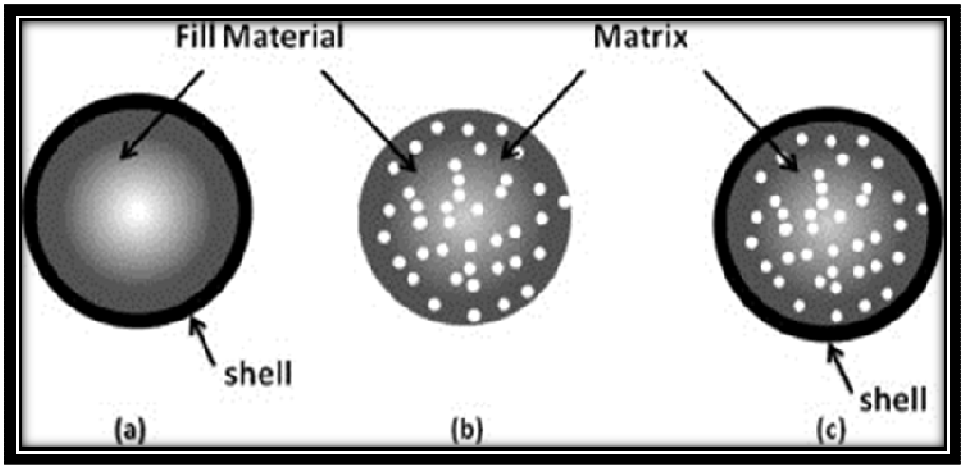

Figure 2.1 Schematic representation of encapsulation systems

2.2 Hydrogel based encapsultion

Hydrogels have been of great interest to biomaterial scientists for many years. Hydrogels are three-dimensional, hydrophilic, polymeric networks with chemical or physical cross-links capable of imbibing large amounts of water or biological fluids (Coviello, T., et al., 2007). Their affinity to absorb water is attributed to the presence of hydrophilic groups such as –OH, –CONH–, –CONH2–, and –SO3H in polymers

that form hydrogel structures. Hydrogels may be chemically stable or they may degrade and eventually disintegrate and dissolve. They are called ‘reversible’, or ‘physical’ gels when the networks are held together by molecular entanglements, and/or secondary forces including ionic, H-bonding or hydrophobic forces. When a polyelectrolyte is combined with a multi-valence ion of the opposite charge, it may form a physical hydrogel known as an ‘ionotropic’ hydrogel (Hoffman, A. S., 2002).

Hydrogels based on both natural and synthetic polymers have continued to be of interest for encapsulation. Hydrogels are of special interest in controlled release applications because of their soft tissue biocompatibility, the ease with which the active ingredients are dispersed in the matrix and the high degree of control achieved by selecting and modifying the physical and chemical properties of the polymer network (Hamidi, M. et al., 2008). Hydrogels consist of polymer chains crosslinked to each other to create a tangled mesh structure, providing a matrix for the entrapment of drugs.

2.3 Cryogel based encapsulation

Polymeric cryogels

Among the polymeric materials utilized in bioseparation, gel matrices are the most widely used, for instance, as carriers in chromatography, media for electrophoresis, isotachophoresis and isoelectric focusing, as media for immuno-diffusion assays, etc. From the view point of modern polymer chemistry, gels (or more exactly lyogels in the jargon of colloid chemistry) present structured polymer-

immobilized solvent systems in which macromolecules form a three-dimensional network fixed by relatively stable, temporally non-fluctuating bonds. The morphology of the network (monophase or heterophase) is determined by the chemical nature of the bonds and the method of gel production. Solvent (water in the case of hydrogels) bound by the polymer network plays a crucial role in maintaining the stability of the gel, preventing it from collapsing into a compact polymer mass, and allowing the diffusion of solutes into and out of the gel (Kudela, 1987; Tanaka, 1987). The majority of gel systems are capable of undergoing pronounced reversible deformations without leading to flow (Ross-Murphy & McEvoy, 1986). There are two main ways of producing gels (Figure 2.2): The first is via limited swelling of a non-crosslinked polymer (a block, a film, a powder or fibres) or via swelling (maximal at equilibrium) of a xerogel (a polymer network produced by chemical synthesis without solvent or by drying a lyogel).

The second is via formation in a liquid system. This is the most common method employed. In this case, the initial system consists of either a solution of monomers in which gelation takes place as a result of branched polymerization, or a solution of polymer in which gel formation is the result of chemical cross-linking, self-gelation upon the change of thermodynamic quality of the solvent, or phase transition of sol into a gel.

Gels can be divided into covalently cross-linked, ionotropic gels where the macromolecules are bound by electrostatic interactions, and physical gels, where the macromolecules are bound by hydrophobic interactions and hydrogen bonds. Aging of sols usually produces heterophase gels with complicated morphology.

When gel formation takes place in a liquid solvent, the latter is immobilized by the network of the forming gel. In general, the structured polymeric system loses its ability to flow, but the diffusional mobility of solvent molecules within the bulk network decreases only slightly allowing the dissolved substances to diffuse in the gel.

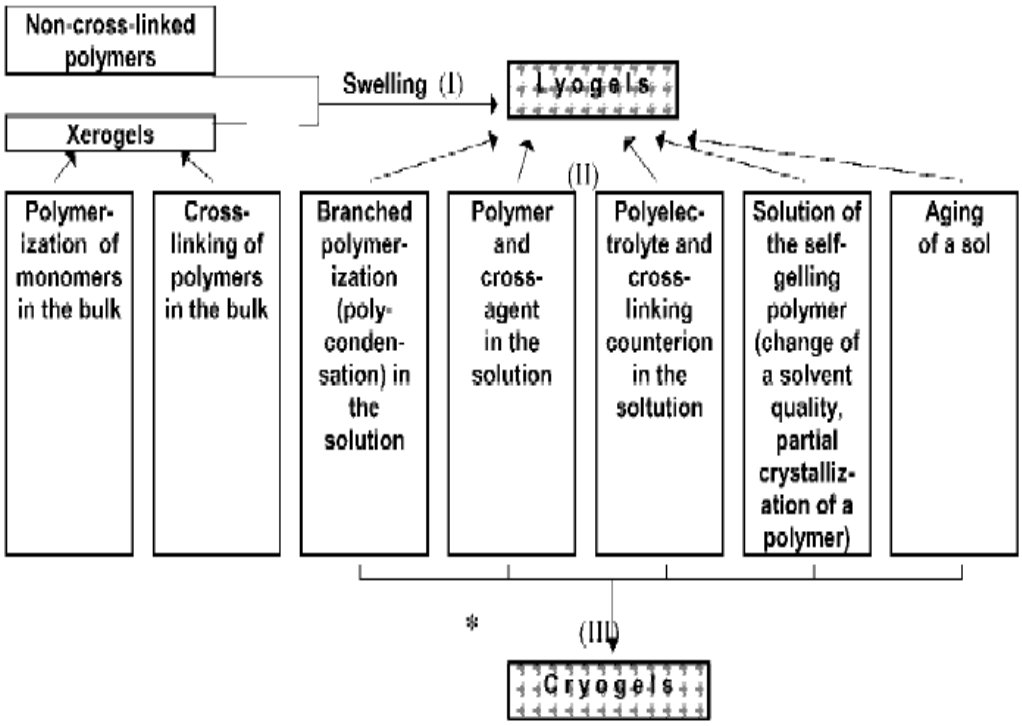

Figure 2.2 Classification of gelling systems (re-drawn from Lozinsky, 1994a)

What will happen upon freezing the initial solution or the sol of a gel-forming precursor. In other words, what will happen if the solvent crystallizes upon decreasing temperature. Gel-like systems may also be formed in this case as well and all types of gel formation combined under II in Figure 2.2 can be realized through appropriate regimes of freezing, storage in the frozen state and thawing. Cryotropic gelation (cryogelation or cryostructuration are often used synonyms) is a specific type of gel formation which takes place as a result of cryogenic treatment of systems potentially capable of forming gels. The products of such cryogelation obtained under the conditions of frozen samples have been named as cryogels (from the Greek κριοσ (kryos) meaning frost or ice) (III in Figure 2.2). Cryotropic gel formation can take place (i) during freezing, e.g., in the case of gelatinized starch pastes or aqueous solution of locust bean gum (Lozinsky et al., 2000e) and results in thermoreversible physical cryogels; (ii) during storage of the samples in the frozen state mainly as chemically cross-linked cryogels (Lozinsky et al., 1998a; Rogozhin et al., 1982);

Cryotropic gel formation can take place

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(ii) during storage of the samples in the frozen state mainly as chemically cross-linked cryogels (Lozinsky et al., 1998a; Rogozhin et al., 1982);

(iii) when thawing frozen samples, which is typical for cryotropic formation of gels from aqueous poly(vinyl alcohol) (PVA) solutions (Lozinsky & Damshkaln, 2000; Mori et al.,1997).

The essential feature of cryogelation is crystallization of the solvent (Figure 2.3), which distinguishes cryogelation from the cooling-induced gelation, where gelation takes place upon decreasing the temperature e.g., the gelation of gelatin, or agar-agar solutions, which proceeds without any phase transition of the solvent. The latter gels can obviously be termed psychrotropic gels, in analogy to the widely used terminology psychrophilic microorganisms or psychrometry. What follows is a description of the production of cryogels, the properties which distinguish them from other gels, and potential applications of cryogel-type materials, especially in bioseparation.

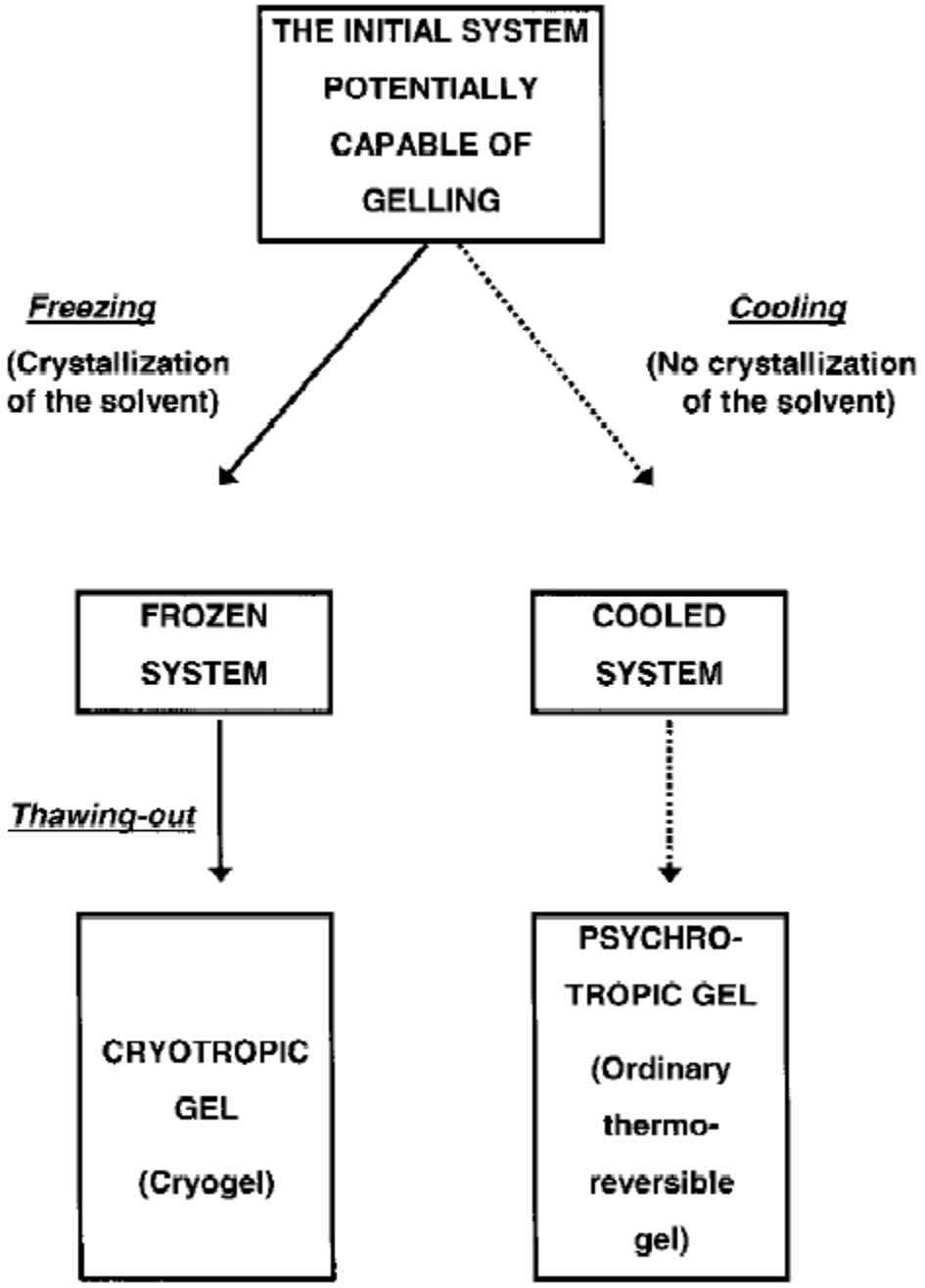


Figure 2.3 Distinctions between chilling-induced and freezing-induced gelation (re-drawn from Lozinsky, 1994a)

Cryotropic gelation is a sol-gel transition induced by the concentration increase of the substrates due to the dehydration by freezing (ice formation). This cryotropic gelation would be useful for entrapping dispersed oil microspheres into a gel network because the network formation could simply be controlled by a freezing operation. Processes of cryotropic gelation of polymeric systems involve the non-deep freezing, storage in the frozen state, and thawing of the solutions or colloidal dispersions containing monomeric or polymeric precursors potentially capable of producing gels. Polymeric materials formed under these conditions were termed as cryogels (from the Greek word “κριοσ” (cryos) - frost, ice), and they possess some specific features as compared to conventional gels formed at temperatures higher than the crystallization point of the solvent. The general scheme of cryotropic gelation is shown in Figure 2.4 (Lozinsky, V.I., 2008).

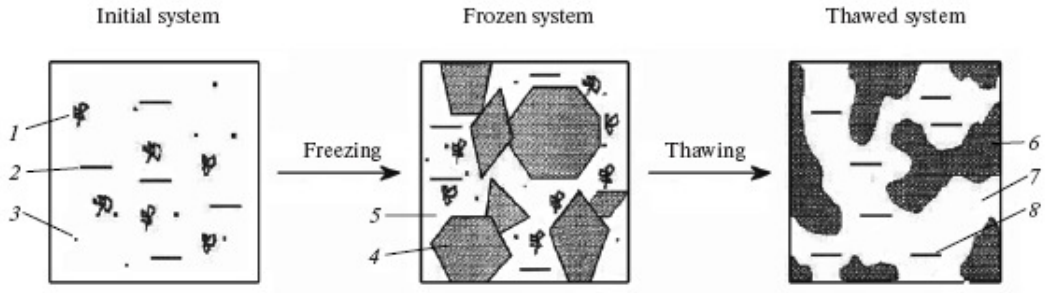


Figure 2.4 Principal scheme for the formation of the polymeric cryogels: (Lozinsky, V.I., et al., 2002) 1. high-molecular-weight precursor, 2. solvent, 3. low-molecular-weight precursors or soluble substances, 4. polycrystals of the frozen solvent, 5. unfrozen liquid microphase, 6. polymeric framework of the cryogel (gel walls of macropores),7. macropores; 8. defrosted solvent.

In general, an initial system (A in Figure 2.4) could include any of the gel-forming systems. It is critical that the gelation rate is not too high, otherwise sufficient gelation will take place already in the liquid sample before it freezes. The final product in this case has a combined morphology, consisting partially of that of a traditional gel (probably destroyed in some way by frozen solvent) and that of a cryogel formed from a part of the polymer, which remained non-gelled till the sample is frozen.

The frozen system is heterogeneous (B in Figure 2.4) and consists of solid phase (crystals of frozen solvent) and unfrozen liquid microphase. The volume of the microphase depends on the nature of the solvent, the initial concentration of dissolved substances, the thermal history of the sample during freezing, the presence of soluble and insoluble admixtures etc. Unfrozen microphase deliberately is presented in Figure 2.4 on a scale not reflecting the real ratio between frozen and non-frozen parts.

After thawing of the frozen sample, the gel formed has a macroporous structure (C in Figure 2.4). Crystals of the frozen solvent play the role of pore-forming agent, or porogen. Melting of these crystals leaves cavities in the cryogel, which became filled with liquid solvent. Surface tension at the interface of the gel and the liquid causes the shape of the initially sharply angled cavities to become rounded. Together with macropores in between polymeric walls, the latter have micropores of their own between macromolecules forming these walls. Heterophase and heteroporous (a combination of macro- and micropores) morphology of cryogels endows them with a unique combination of physical properties of polymeric cryogels.

2.4 Freeze-drying process

Freeze drying, also termed “lyophilization”, is a drying process whereby water or another solvent is removed from the frozen product by sublimation (Swarbrick et al., 2002; Velardi et al., 2008). Sublimation occurs when a frozen solvent goes directly to the gaseous phase without passing through the liquid phase. The removal of solvent by sublimation creates an open network of “pores”, which allows pathways for escape of solvent vapor from the product. Here, only the solution system of water and solutes is focused in this study.

The lyophilization technique is performed through several successive steps as follows.

2.4.1 Freezing stage

In general, the first step of freeze-drying process is the formation of the ice from the water contained in product solution, which this process is also termed as a freezing stage (Jennings, 1999; Swarbrick et al., 2002). Nakagawa (2007) elucidated the temperature profile during unidirectional freezing process which is represented in Fig. 2.5. Firstly, the starting solution is cooled down at constant freezing rate until some supercooled level which is the starting point of ice nucleation. Next, the temperature rapidly increases due to the heat evolution caused by the sudden ice crystallization, and then the temperature slightly decreases due to the latent heat generates by ice-crystal growth up to the end of ice-crystal growth period corresponding to the total ice freezing. Finally, the temperature continues to decrease until complete solidification of cryoconcentrated phase.

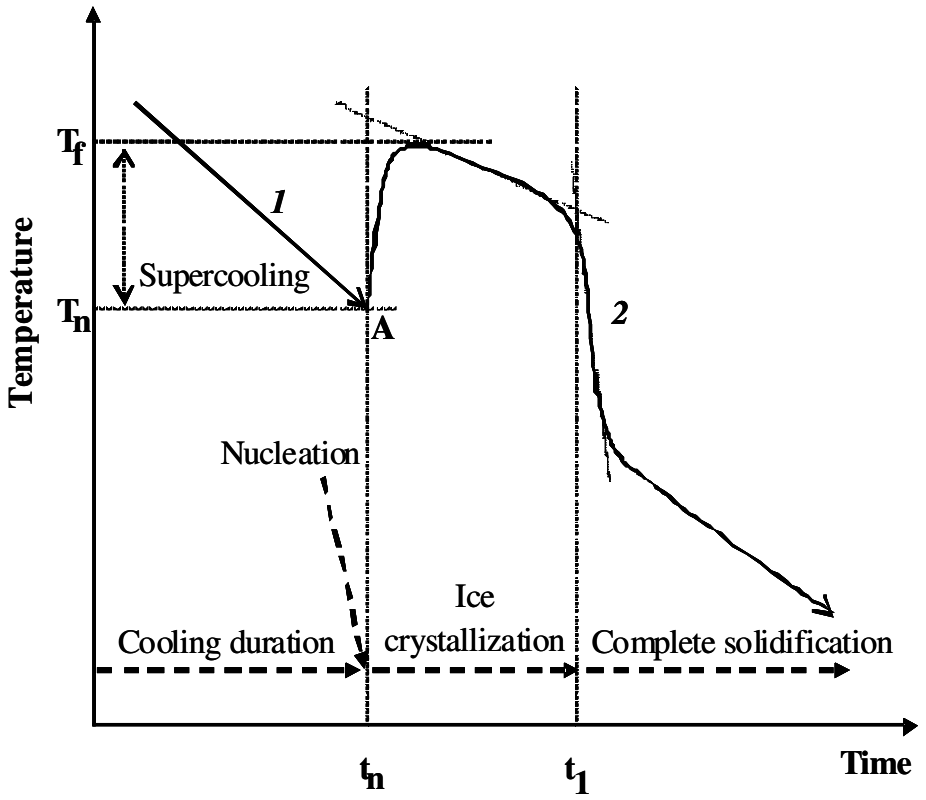


Figure 2.5 Schematic of temperature profile during freezing process (Nakagawa, 2007)

In general, there are two common freezing parameters in the definition of the freezing process, i.e. the degree of supercooling and the rate of ice crystallization (Jennings, 1999; Swarbrick et al., 2002).

2.5 Literature review of cryogel preparation by freeze-drying process

Lozinsky et al. (1986) investigated the influence of the temperature of preparation of cross-linked polyacrylamide cryogels on the dynamics of their formation and on the properties of the materials formed. It was shown that, for the given system, dependences of gelation rate and gel-fraction yield upon temperature within the range – 10 ◦C to -30 ◦C were of extremal character. They have found that the temperature of cryostructurization markedly influences the macrostructure of the porous polymeric gel.

Guesv et al. (1993) prepared the gel by free radical copolymerization of acrylamide and N, N'-methylene-bis-acrylamide in a frozen aqueous medium. They studied some features of the formation of polyacrylamide cryogels by means of ^{1}H- and ^{2}H-NMR. Using the spectral changes during polymerization, the cryotropic gelation may be conventionally subdivided into three stages during which the narrow NMR signals due to the low molecular weight components disappear, while the broad signals attributed to the formation of the polymeric compound develop. It was demonstrated that the dynamics of polymerization depended on the freezing procedure, i.e. on the thermal prehistory of a sample.

Lozinsky et al. (1996) prepared poly (viny1 alcohol) cryogels from aqueous solutions of the polymer by freezing and thawing, and the cryogels were employed as matrices for cell immobilization. In their experiment, the swelling behavior of these macroporous gel carriers in pure water and in solutions of certain compounds (salts, amino acids, and glucose) was studied to elucidate the osmotic properties of the cryogels during long-term exposure to aqueous media. It was shown that after the

initial sol fraction was washed out, the residual gel matrix possessed high stability even at extreme pH conditions (acid or alkali concentration up to 1.0 mol.l^{-1}) or in the presence of strong chaotropic salts such as sodium rhodanide. Although the macroporous supermolecular structure of the carriers under consideration underwent certain changes as a result of aging processes during prolonged washing of the gel, the highly porous morphology of the material was retained.

Konstantinova and Lozinsky (1997) prepared the ovalbumin by using cryogelation process and studied the process features. In their experiment, ovalbumin was a cryoresistant protein, and simple freezing-thawing of ovalbumin solutions did not form cryogels, which were formed only as a result of the cryoinduced denaturation of the protein molecules under specific conditions. The results obtained indicate that there is a direct correlation between the phase state of frozen samples and the kinetic features of cryogelation, properties and structure of ovalbumin cryogels.

Lozinsky and Plieva (1999) prepared the poly (vinyl alcohol) cryogels by the freeze-thawing of concentrated aqueous solutions of the polymer as promising gel carriers for cell immobilization. In their experiment, these carriers possess definite advantages when compared to other hydrogels commonly used for the same purposes. They found that the common benefits were as follows: (i) PVA cryogels had very high micro- and macro-porosities which provide favored conditions for the nonhindered mass transfer of substrates and metabolites; (ii) the rheological characteristics of the nonbrittle matrix are excellent and allow the use of these carriers in most types of reactors; (iii) thermostability of PVA cryogels exceeds that of other commonly used thermoreversible gel carriers; (iv) the cryogels are highly resistant to biological degradation as well as having a low sensitivity to culture media

compositions; (v) PVA itself is a biologically compatible, nontoxic, and readily available low-cost polymer.

Nakagawa (2007) developed a mathematical model that simulates temperature profiles during freezing process of standard pharmaceutical formulations (mannitol and BSA based) in two dimensional axsymmetric space, and the ice crystal mean sizes were semi-empirically estimated from the resulting temperature profiles. In this study, water vapor mass transfer permeability values during sublimation step were also estimated from ice phase morphological parameters. All these numerical data were compared with experimental data, and quite good agreement was observed that confirmed the adequacy of the present model calculations. It was confirmed that, for a given formulation, the mass transfer parameters during freeze-drying were strongly dependent on morphological textural parameters, and consequently, on the nucleation temperatures that fix the ice phase morphology. The influence of freezing rate was also predicted from the simulations, proving that an increase of cooling rates led to slower primary drying rates.

Bejirapha et al. (2011) determined the effect of freezing process in the absence or presence of excipients on the stability of capsicum loaded nanocapsules (NC) during freeze-thawing and freeze-drying procedures. In their experiment, capsicum loaded nanocapsules were prepared by the modified-emulsion–diffusion method combined with the microfluidization method. The prepared samples were frozen at freezing temperatures of -40, -20, and -15 ◦C to study the effects of cooling temperature on the properties of the capsicum-oleoresin-loaded nanoparticles. It was found that high freezing temperature had an effect on the maintenance of nanocapsule size after freeze-thawing and freeze-drying. The morphologies of NC made with

gelatin or κ–carrageenan were conserved after freeze-drying showing spherical forms according to electron microscope observations.

Nakagawa et al. (2011) produced a sample of dry food by using freeze drying process. They prepared a nanocapsule (NC)–gelatin suspension which was freeze-dried. The obtained dried product was rehydrated, and the dispersibility of the nanocapsules was investigated. It was found that the prepared freeze-dried food had different dispersion characteristics at different positions in the dried bulk sample, and this heterogeneity was dependent on the cooling program used during the processing. They suggested that the gel network formation of NC–gelatin would be advantageous for producing excellent NC dispersion characteristics after drying. A slow cooling condition is favourable for promoting sol–gel transition in the solution. However, the large scale of ices formed during slow freezing would damage the gel networks, leading to poor dispersibility. They discussed the key processing factors for controlling the dispersion characteristics of nanocapsules stabilized in a freeze-dried food sample.

Tanthapanichakoon et al. (2007) prepared curcumin nanoencapsulated in chitosan cross-linked with tripolyphosphate (TPP) via the technique of evaporation of o/w/o emulsion. The resulting nanocapsules were characterized and the release characteristics of curcumin from the nanocapsules were investigated. It was found that the nanocapsules in the emulsions of interest were somewhat spherical with average particle size of 250 nm, as measured with the Dynamic Light Scattering (DLS) method. After freeze-drying, the observed nanocapsules were found by TEM analysis to be about 150 nm in size. The yield of encapsulation ranged from 17.98 - 51.91

%w/w. The nanocapsules were found to display extended release profiles lasting up to 24 hours.

Lersutthiwong *et al*. (2009) prepared chitosan-alginate biopolymeric for encapsulation of turmeric oil using a three-step procedure of o/w emulsification, gelation and solvent removal in order to investigate the effects of the molecular weight of chitosan, the chitosan/alginate mass ratio, and the order of addition of chitosan and calcium chloride. The results show that chitosan with a low molecular weight was required to produce small nanocapsules. This report suggested that the use of a LM-chitosan/alginate ratio of 0.1:1 with addition of the chitosan after calcium chloride gives nanocapsules with good physical stability.

Risbud *et al*. (2000) developed a pH-sensitive chitosan / polyvinyl pyrrolidone (PVP) based controlled drug release system for antibiotic delivery. They synthesised the hydrogels by crosslinking chitosan and PVP blend with glutaraldehyde to form a semi-interpenetrating polymer network (semi-IPN). In this study, semi-IPNs, air-dried and freeze-dried, were compared for their surface morphology, wettability, swelling properties and pH-dependent swelling. In this report, the pore diameter of obtained freeze-dried samples was about 39.20 ± 2.66 μm. Freeze-dried membranes released around 73% of the amoxicillin (33% by air-dried) in 3 h at pH 1.0 and thus, had superior drug-release properties to air-dried hydrogels.

Sakayama *et al*., (1993) prepared the polyelectrolyte complex gel by mixing chitosan and κ-carrageenan solutions in the presence of NaCl in order to investigate the swelling equilibrium at various pH. To prepare from natural polysaccharides in this study, initial formation of κ- carrageenan, which has sulfonate group, was used as polycation anion and chitosan, which has amino groups, was used as polycation

cation. The results show that, in the presence of 5.7 % NaCl, homogeneous gel was obtained. They also suggested that the swelling of the complex gel was revealed to be sensitive to a rather narrow range of pH. In a NaOH solution of pH 10.5, the maximum swelling occurred. At pH below 9 and at pH 13, no swelling was observed.

Mitsumata *et al*., (2003) synthesized the polyelectrolyte complex hydrogel consisting of chitosan and κ- carrageenan and carboxymethyl cellulose sodium salt (CMC) in order to investigate swelling properties of the gel under various pH conditions. In this study, the polyelectrolyte complex gel is formed by the electrostatic interaction between polyelectrolytes. As this report, gelation and swelling properties of the complex gel strongly depend on κ-carrageenan / carboxymethylcellulose sodium salt (NaCMC) and salt concentration. The results show that the gels showed a maximum degree of swelling around pH 11-12.

Giannouli *et al*. (2003) developed the xanthan gum cryogel by cryogelation technique. In this study, xanthan solution (0.2-2.0 wt %) was prepared in deionised water at 80 ˚C. Cryogels were prepared by feezzing rapidly by transfer to a freezer at -20 ˚C, held at -20 ˚C for 24 h, and allowed to thaw in the refrigerator at 5 ˚C. They have found that much strong networks are obtained when solutions of xanthan were frozen and thawed.

Podorozhko *et al*. (2010) prepared and studied the composite heterophase poly (vinyl alcohol) (PVA) cryogels containing entrapped small droplets of Vaseline oil. They also studied the oil droplets (5-45 wt %) of o/w emulsions. In this study, the oil-filled cryogels were formed via freeze–thaw treatment of freshly prepared oil-in-water emulsions containing varied volume fraction of lipophilic phase, and the influence of the amount of this phase, as well as the effects of freezing conditions on the physico-

mechanical (shear moduli) and thermal (gel fusion temperature and fusion enthalpy) characteristics of resulting composites have been explored. It was shown that, over certain range of PVA concentrations in aqueous phase and a range of volume fraction of the hydrophobic phase, its microdroplets performed as ‘‘active’’ fillers causing an increase in both the gel strength and the heat endurance of composites. The light microscopy data on the morphological features of such filled PVA cryogels revealed the effect of diminution in size of oil droplets entrapped in the gel matrix as compared with the initial emulsions. They attributed this to the disintegrating action of crushing and shear stresses arising upon the system freezing and growth of ice crystals. The oil-filled PVA cryogels were found to be capable of gradually releasing the lipophilic constituents (the Rose hips oil, in this case) in response to the cyclic mechanical compression. The morphological features show oil droplets entrapped in PVA cryogel matrix.

CHAPTER III

EXPERIMENTAL

3.1 Materials

3.1.1 Model active food ingredient

Curcumin (Purity: 79.78 %, Thai-China Industry, Bangkok, Thailand)

3.1.2 Wall materials

1) Chitosan (Tokyo Kasei Kogyo, Japan). Chitosan granules consisting of low MW chitosan (LM-chitosan, number-averaged molecular weight (Mw) = 220,000 Da), medium MW chitosan (MM-chitosan, Mw =68,0000Da) and high MW chitosan (HM-chitosan, Mw =1,300,000Da) were supplied from Tokyo Chemical Industry Co., Ltd., Japan. The degrees of deacetylation of the corresponding 3 types of chitosan were held at 78%, 87% and 82%, respectively, as measured by the author using the reported 1H-NMR technique (Hirai, Odani & Nakajima,1991).

2) κ-carrageenan (Sigma, Germany)

3) Carboxymethyl cellulose sodium salt (Fluka, Switzerland)

3.1.3 Analytical chemicals

4) Acetic acid (Purity: 99.9 %, Wako Pure Chemical Industries Co.,Ltd, Japan)

5) Acetronitrile (Purity: 99.8 %, Kishida Chemical, Japan)

6) Triolein (Kanto Chemical, Japan)

7) Tween 80 (Sigma-Aldrich, Germany)

8) Distilled water

9) Sodium chloride (Purity: 99.5 %, Katayama Chemical, Japan)

10) Sodium dihydrogen phosphate, 2-hydrate (Kishida Chemical, Japan)

11) Di-sodium hydrogen phosphate, 2-hydrate (Kishida Chemical, Japan)

All of the other chemicals used in this work, Tween 80, acetic acid, ethanol were analytical grade. All chemicals were used as received.

3.1.4 Laboratory Instruments

1) Freeze dryer

2) Spray dryer (Model B-290 BUCHI Co., Ltd., Switzerland)

3) High speed homogenizer (Heung Bo Tech., Korea)

4) High pressure Homogenizer (Panda plus 2000, USA)

5) Ultra Sonic Homogenizer (Model UH-300, SMT company, Japan)

6) Water bath shaker (T-22S, Thomas Kagaku, Japan)

3.1.5 Analytical Instruments

1) Scanning Electron Microscopy (Model S-2400, Hitachi, Japan)

2) Transmission Electron Microscopy (Model Jeol, JEM-2100, Japan)

3) Optical Microscope (Model VH-Z500R, Keyence, Japan)

4) High-Performance Liquid Chromatography (Model STD-10AV VP, Shimadzu, Japan)

3.2 Experimental procedures

3.2.1 Experimental Procedure

The overall approach in this research is shown in **Figure 3.1** In brief the methodology goes as follows: First, O/W emulsion (a) was mixed with polymer suspension (b). Then 5 % (w/v) of sodium chloride was added to the prepared O/W emulsion before mixing with the polymer suspension (b) consisting of κ-carrageenan, NaCMC and 5% of sodium chloride. The mixture was stirred at room temperature for 180 second using an ultrasonic homogenizer to obtain a colloidal suspension (c). The suspension was unidirectionally was frozen to obtain frozen cryogels (d). After that gel formation may be improved by using cold incubation of the cryogels (e). The obtained frozen cryogels were subsequently freeze-dried to obtain dried cryogels. In this research, I will investigate the yield of encapsulation, yield of gel fraction, swelling characteristics and release characteristics of the active ingredient (curcumin) embedted in the dried cryogels.

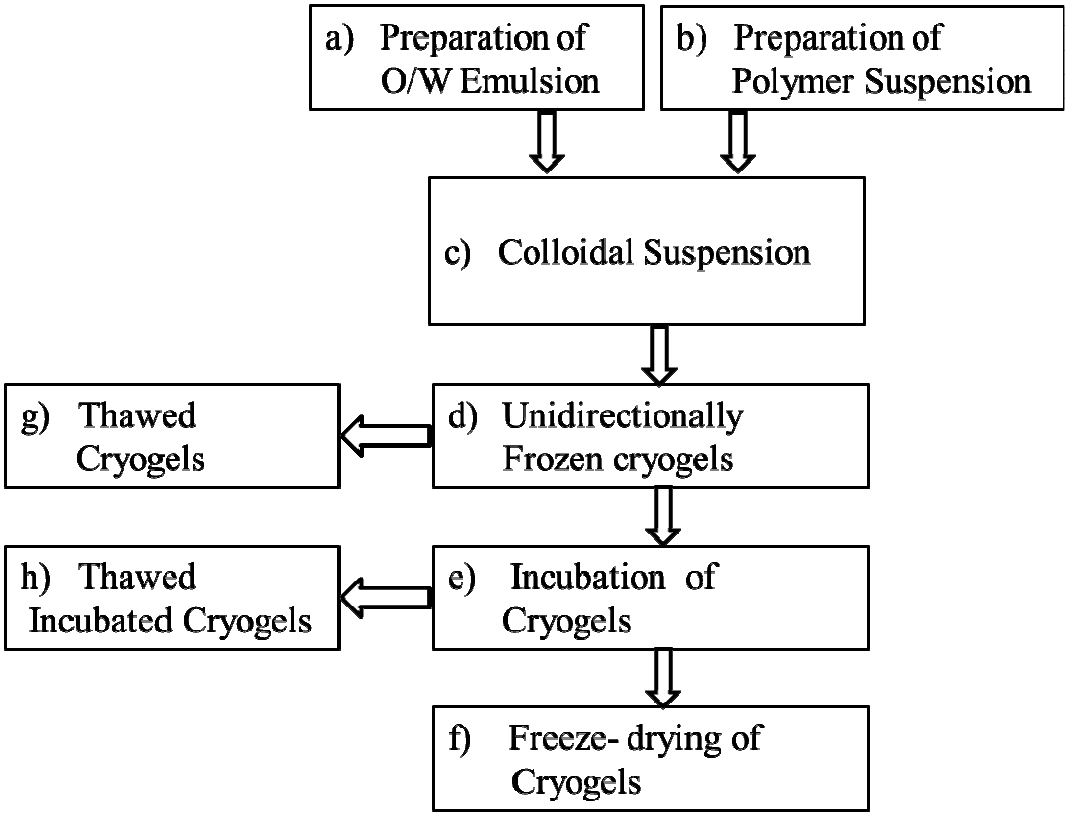


Figure 3.1 Experimental Procedure

3.2.2 Procedure for Unidirectional Freezing and Freeze-drying

The procedure is schematized in **Figure 3.2**. The colloidal samples were stabilized at 20°C for 30 min on the heat exchange plate and frozen unidirectionally. The sample temperature was reduced to -40°C at programmed cooling rates, namely, -0.5, -1.0 or -2.0°C/min. Freeze-drying of the frozen samples (with incubation and without cold incubation) was subsequently carried out on the same heat exchanger where the cooling plate temperature was set to -20°C and maintained for 24 hours in

order to completely remove the ice at a chamber pressure of around 10-20 Pa. The experimental prodedure is explained in more detail in section 3.2.4.

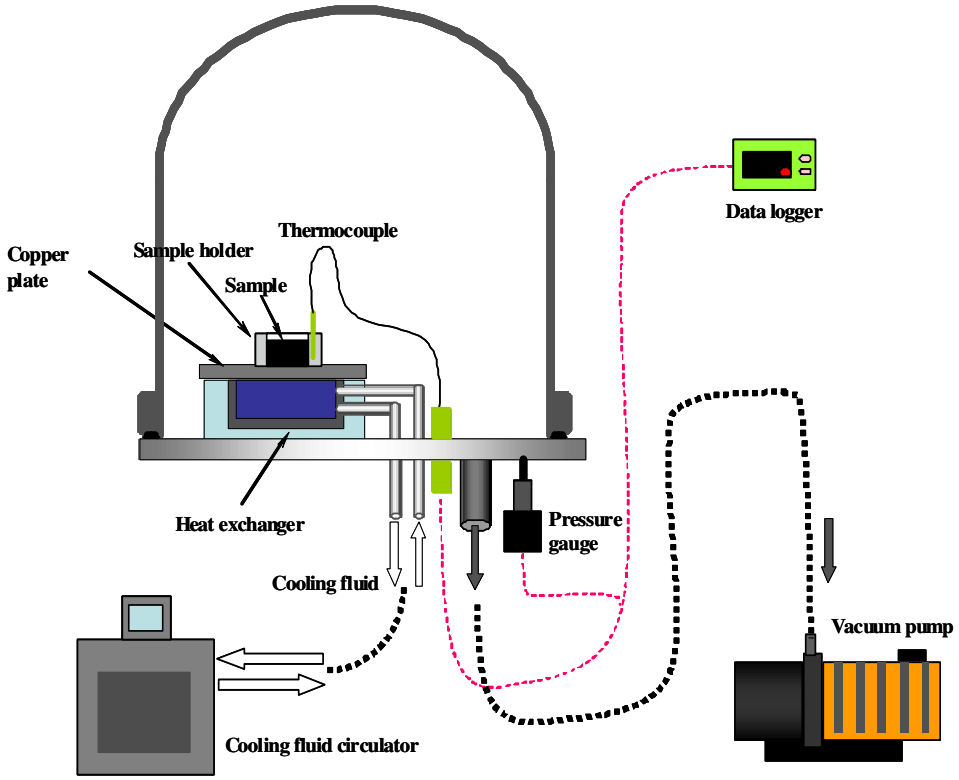


Figure 3.2 Schematic diagram of the freeze-dryer (Nakagawa, 2007)

3.2.3 Screening experiments of gel preparation

Variation of parameters in the formulation

Preparation of O/W emulsion (step a)

1) Find suitable concentration of chitosan solution 1.5%, 2% 3% v/v in 2% acetic acid

2) Find suitable MW of the three types of chitosan molecular weight at low (MW=220,000Da), medium (MW=680,000Da) and high (MW=1,300,000Da

Preparation of Polymer suspension (step b)

1) Find suitable K-carrageenan:CMC ratio of hydrogel at 1:9, 2:8, 3:7 and 4:6 ratio

2) Find suitable concentration of NaCl in the formulation at 0%, 1%, 2% and 5% of NaCl. When get concentration of 5% NaCl, do experiment by added NaCl in Chitosan solution and carrageenan:CMC solution before and after mixing.

3.2.4 Preparation of unidirectionally frozen and freeze-dried cryogels

3.2.4.1 The Case of Without Incubation

3.2.4.1.1 Preparation of emulsion and polymer suspension

The methodology for preparing cryogel is shown schematically in **Figure 3.3** In brief, the methodology goes as follows: First, 0.3 wt% of curcumin (oil phase) dissolved in triolein (a) was mixed with a 3% chitosan solution in a mixture of acetic acid (2% v/v) containing 5 wt% of Tween80 as an emulsifier (b) using an

Ultra-Turrax T25 homogenizer at 12,000 rpm for 5 min. The ratio of the oil phase to the aqueous phase is set at 5 %, 10% and 20% (v/v). Then 5 % (w/v) of sodium chloride was added to the prepared O/W emulsion (c) before mixing with a polymer suspension (d) consisting of κ-carrageenan, NaCMC and 5% of sodium chloride. The mixture was stirred at room temperature for 180 second using an ultrasonic homogenizer to obtain a colloidal suspension.

3.2.4.1.2 Procedure for Unidirectional Freezing and Freeze-drying

The obtained colloidal suspension was subsequently frozen unidiectionally with a plate heat exchanger as shown in **Figure 3.2**. A PTFE sample holder (equipped cylindrical sample space, diameter D=10mm, Height H=10mm) was used for supporting the solution on the cooling plate. The sample sets were stabilized at 20°C for 30 min on the plate, and the temperature was, then, cooled down to -40°C at programmed cooling rates, namely; -0.5, -1.0 and -2.0°C/min. Freeze-drying was subsequently carried out on the same heat exchanger where the cooling plate temperature was set to -20°C and maintained for 24 hours at a chamber pressure of around 10-20 Pa. Some of the frozen samples were thawed just after the freezing to confirm the cryogel formation.

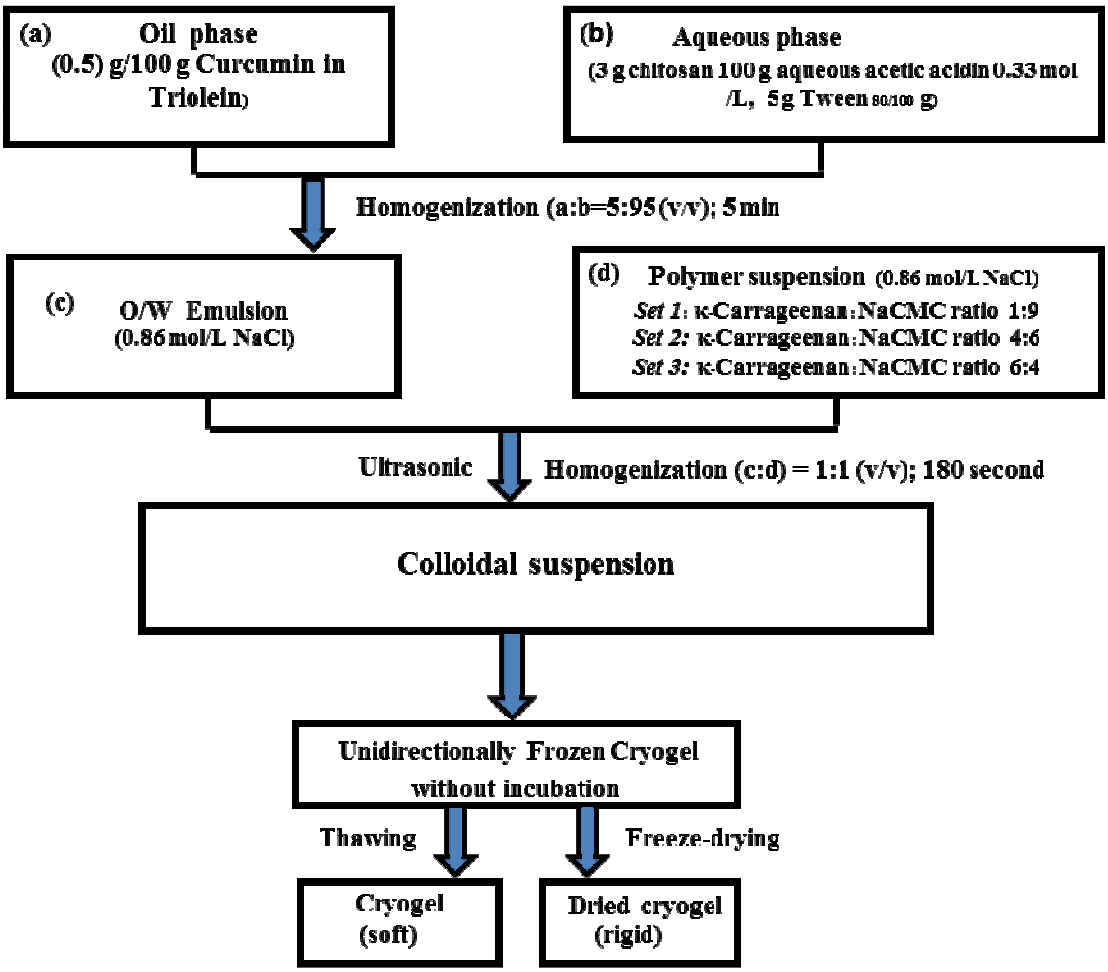


Figure 3.3 Preparation procedure of cryogel containing curcumin without incubation

3.2.4.2 The case of Cold Incubation

3.2.4.2.1 Preparation of emulsion and polymer suspension

The cryogel nanocapsule preparation process is schematized in **Figure 3.4**. First, a triolein solution containing 0.3 g of curcumin/100g (oil phase) was mixed with 3 g of chitosan/100g aqueous suspension containing 0.33 mol/L of acetic acid and 5 g of Tween80/100g. The ratio of the oil phase to the aqueous phase was set at 0.05 ml/ml or 5 vol% and this mixture was emulsified with a high-speed homogenizer (Heung Bo Tech. Co. Ltd., Seoul, Korea) at 12000 rpm for 5 min. Sodium chloride at a concentration of 0.86 mol/L was added to the o/w emulsion, then the emulsion was mixed with another polymer suspension consisting of κ-carrageenan, NaCMC and sodium chloride at 0.86 mol/L. As indicated in **Figure 3.4**, two sets of the polymer suspension with different weight ratios of carrageenan to CMC were prepared, i.e. carrageenan:CMC = 4:6 (set 2) and 6:4 (set 3). The resulting mixture was again homogenized at 12000 rpm for 5 min to obtain a homogeneous colloidal suspension containing an O/W emulsion with 5 vol% oil phase.

3.2.4.2.2 Procedure for Unidirectional Freezing and Freeze-drying

The obtained nanocolloidal suspension was frozen unidirectionally with a plate heat exchanger, whose temperature was controlled with an external cooling device. A PTFE sample holder (cylindrical space, diameter D = 10 mm, height H = 10 mm) was used to keep the solution on the cooling plate, where each

sample was first stabilized at 20°C for 30 min. Then the temperature was reduced down to -40°C at the programmed cooling rate, either -0.5 or -2.0°C/min and then maintained at -10°C for 10 hours. Freeze-drying was subsequently carried out on the same heat exchanger at a chamber pressure of around 10-20 Pa, in which the cooling plate temperature was set and maintained at -20°C for another 24 hours.

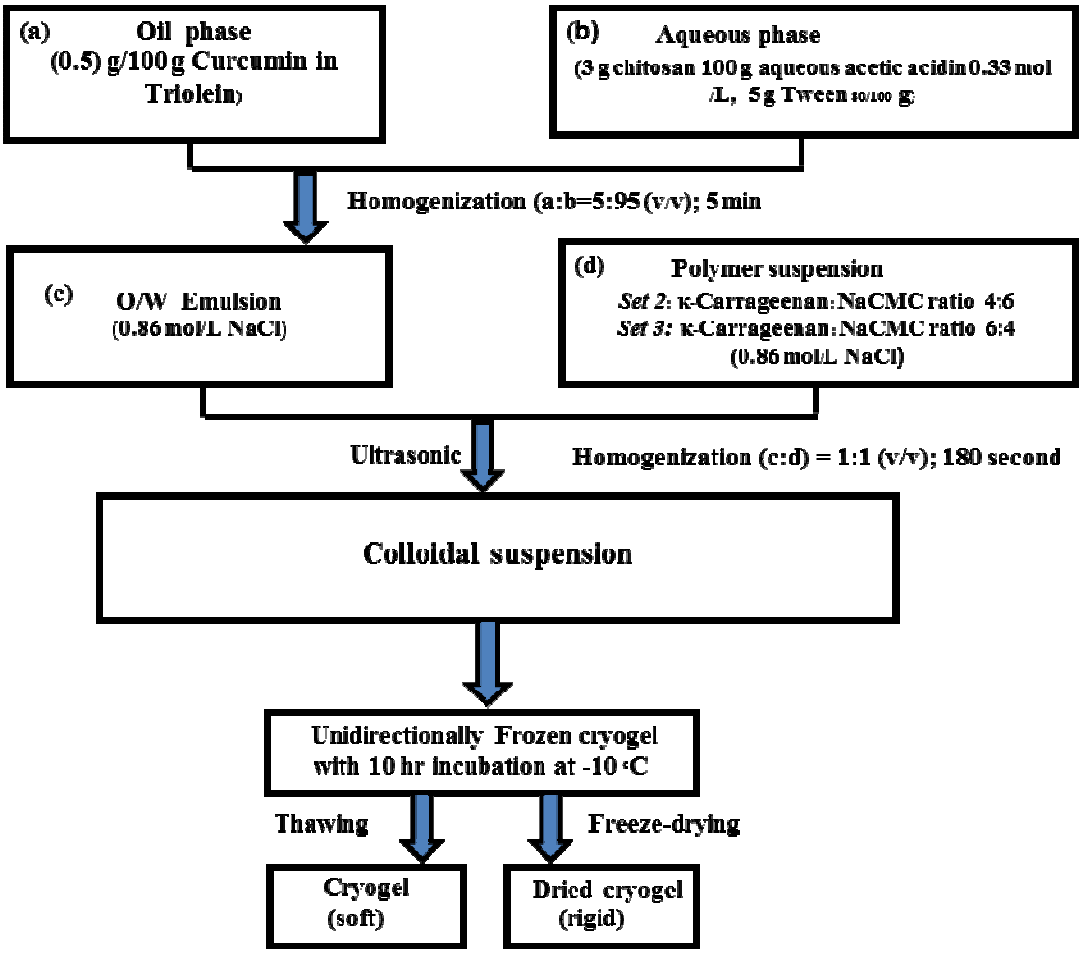


Figure 3.4 Preparation procedure of cryogel containing curcumin cold incubation

3.3 Characterization of cryogel

3.3.1 Curcumin encapsulation yield measurement

The encapsulation yields were estimated by measuring the amount of curcumins extracted from freeze-dried specimen. The mass of extracted curcumins (extracted from a freeze-dried sample soaked in 2-ml ethanol solution for 24 hours) was devided by the total mass of curcumin loaded in a sample to obtain the yield value. Curcumin concentration was analyzed by High Performance Liquid Chromatography (HPLC) with UV-spectrometer. A peak detected at wave length 254 nm was used for the analysis. The yield of encapsulation (Y) was calculated as follows:

$$Y = (L_c/T_c) \times 100 \quad (\%w/w) \qquad (1)$$

where Lc is the extracted mass of curcumin from the specimen
and Tc is the total mass of curcumin introduced in the preparation

3.3.2 In-vitro release study

The release curves of curcumin were obtained in phosphate buffer solutions at pH 7.4 (1.3 mM phosphate). A freeze-dried cryogel specimen was placed in a test tube containing 5 mL of the buffer solution. Then the test tube was tightly capped and installed in a shaking water bath whose temperature was maintained at 37ºC. One mL of the solution was withdrawn periodically, and the same make-up volume of fresh buffer solution was added to the test tube. The curcumin concentration in each

withdrawn sample was analysed with the HPLC and the obtained value was used to calculate and obtain a data point for the accumulated curcumin released on the curve. To facilitate the pattern identification, the released amount of curcumin at any time t was normalized by the total mass of curcumin introduced at the start of each preparation and shown as percentage. The measurements were made in triplicates.

Curcumin concentration was analyzed by High Performance Liquid Chromatography (HPLC) with UV-spectrometer as detector (STD-10AV VP, Shimadzu). The extracted sample was injected into a C18 column (VP-ODS 150 mm×4.6 mm, Shimadzu Co.Ltd, 5-μm particle size). The mobile phase consisted of a mixture of acetonitrile and 10% acetic acid in water (50:50 %v/v). (Tanthapanichakoon, W. et al., 2007).The injection volume was 20 μl with isocratic elution at a flow rate of 0.8 ml/min. The retention time of curcumin was 8.7 min. The peak detected at wavelength 254 nm was used for the curcumin analysis.

3.3.3 Swelling properties of the freeze-dried speciments

Swelling behaviour of the freeze-dried specimens was observed by placing the prepared freeze-dried specimen of interest in a petri dish filled with the selected phosphate buffer solution. Images of the swelling specimen were taken continually with a digital camera for 24 hours in order to monitor and analyse the changing size. The swelling ratio of the cryogel was determined with the following equation:

$$\text{Swelling } (\%) = (W_s - W_d)/ W_d \times 100 \quad (2)$$

where W_d is the original diameter of the cylindrical freeze-dried specimen and W_s is the observed swollen diameter at time t.

3.3.4 Yield of gel fraction

To find out the extent of cryotropic gelation and the effect of incubation after unidirectional freezing, colloidal suspensions without the oil phase in **Figure 3.3 and 3.4** were prepared and frozen at a cooling rate of interest with and without 10-hour incubation at -10°C. Then 0.5 g of these specimens were thawed and added with 2 mL of DI water. The resulting suspension was centrifuged to obtain a supernatant solution and the polymer concentration in it was analyzed with UV-Vis spectroscopy at 300-nm wavelength. The yield of gel fraction was calculated as follows:

$$\text{Yield of gel fraction } (\%) = ((C_0-C_s)/C_0) \times 100 \quad (3)$$

where C_0 is the similarly obtained polymer concentration in the colloidal suspension before undergoing cryotropic gelation and C_s is the supernatant polymer concentration obtained after thawing.

3.3.5 Microstructure observation

3.3.5.1 Scanning Electron Microscopy

The microstructural morphology of freeze-dried specimens was investigated with Scanning Electron Microscope (SEM) (Model S-2400, Hitachi, Japan). A specimen of interest was vertically cut in half in the order to observe its internal microstructure on the cross section. The sliced piece was mounted on

aluminum foil and the surface coated with platinum under vacuum. Photomicrographs were taken at different levels of magnification.

3.3.5.2 Transmission Electron Microscopy

The freeze-thawed samples of the encapsulated oil phase in the cryogel matrix were investigated by Transmission Electron Microscopy (TEM) (Model Jeol, JEM-2100, Japan).

3.3.5.3 Optical Microscope

O/W emulsion samples of the oil phase encapsulated in chitosan were observed by an optical microscope (Model; VH-Z500R, Keyence, Japan).

3.4 Characterization of emulsion of cryogel

3.4.1 Particle size analysis

To find out the effect of post-freezing incubation, frozen cryogels with and without incubation were thawed out to yield colloidal suspensions and the size distribution of nanocapsules were determined respectively by dynamic light scattering and zeta potential probe (Zetasizer, NanoZS, Malvern Instruments Ltd., Worcestershire, UK). The measurements were carried out at 25 $^{\circ}$C in triplicates

3.4.2 Zeta potential measurement

To find out the effect of post-freezing incubation, frozen cryogels with and without incubation were thawed out to yield colloidal suspensions and the zeta potential of nanocapsules were determined respectively by dynamic light scattering and zeta potential probe (Zetasizer, NanoZS, Malvern Instruments Ltd., Worcestershire, UK). The measurements were carried out at 25 ˚C in triplicates

3.5 Procedure for Spray Drying the colloidal Suspension

As shown in **Figure 3.5**, I wanted to apply high pressure homogenization before spray drying the colloidal suspension. However, the suspension was too viscous and we had to dilute the concentration of O/W emulsion and polymer suspension by 10 times. Microcapsules were obtained by spray drying the fine colloidal suspension (with 5% w/v of dextrin as a stabilizer) in a Mini Spray dryer Model B-290 (BUCHI Co., Ltd., Switzerland) equipped with a 0.7 mm diameter spray nozzle. The spray dryer consisted of a cylindrical chamber with 60 cm I.D. and 110 cm height, followed by a cyclone separator of 50 cm height. The operational conditions were: air inlet temperature of 130 °C, air outlet temperature of 65 ± 10 °C, feed rate of 1 mL/min, and air flow rate of 50 kg/h.

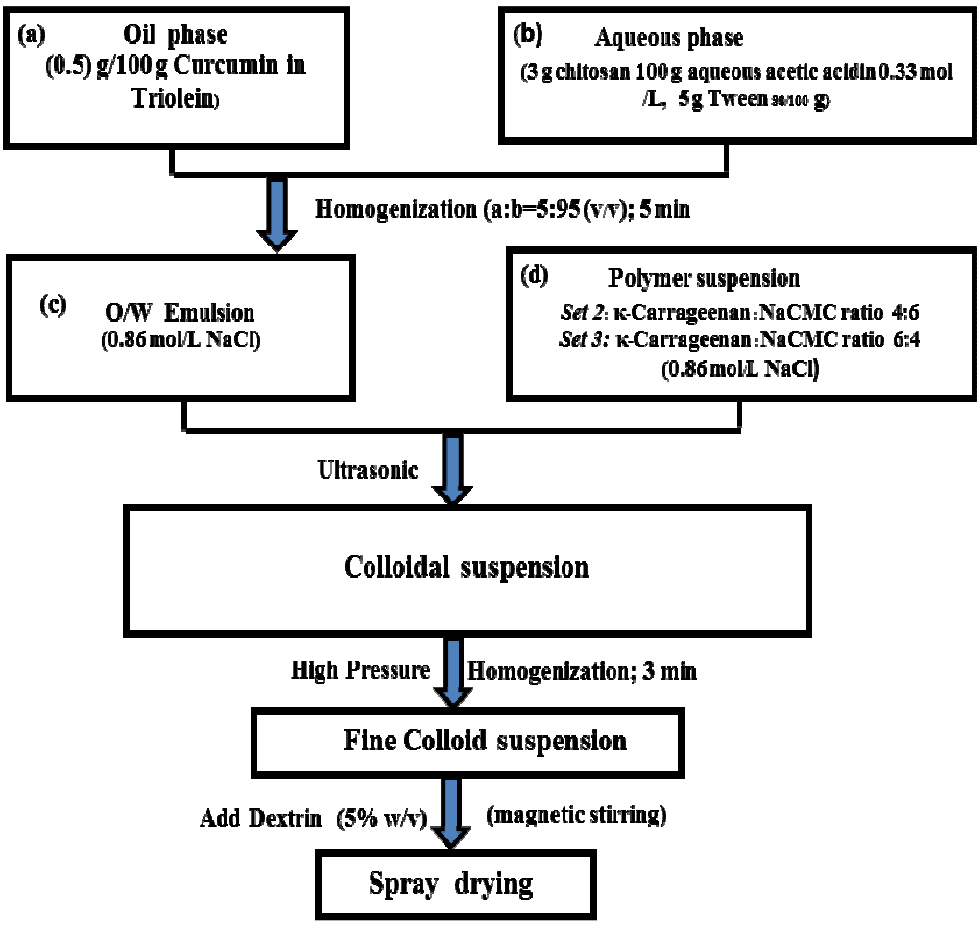


Figure 3.5 Preparation procedure of cryogel containing curcumin by using Spray dryer.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preparation of chitosan/κ-carrageenan/NaCMC cryogels

4.1.1 Screening experiments

Screening experiments were carried out to find promising formulations for making homogeneous gel suspensions suitable for forming cryogels. First, the suitable concentration of NaCl in the O/W emulsion and polymer suspension, and the correct order of NaCl addition shown in **Figure 3.3** were found by varying the NaCl concentration at 0, 1, 2 and 5% and by adding NaCl either before or after mixing the emulsion with the polymer suspension, respectively.

Table 4.1 summarizes the first batch of screening experiments and the observed appearance of the resultant polymer suspensions. Sol-gels denote low-viscosity milk-like gel-sol mixtures that were not promising for making and failed to form cryogels, whereas homogenous gel suspensions (HS) were found to be rather suitable. Obviously, NaCl had to be added before mixing the O/W emulsion with the polymer suspension.

Table 4.1 Screening experiments in the development of the preparation procedure shown in **Figure 3.3** (% Oil phase: 5%)

Formulation ID.	Chitosan in Solution	MW of Chitosan	Ratio of Carrageenan: CMC (set1) 1:9, (set2) 4:6	Obtained Suspension
1-A**	1.5%	Low	Set1	Sol-Gel
2-A	1.5%	Medium	Set1	Sol-Gel
3-A	1.5%	High	Set1	Sol-Gel
4-A	2%	Low	Set1	Sol-Gel
5-A	2%	Medium	Set1	Sol-Gel
6-A	2%	High	Set1	Sol-Gel
7-A	3%	Low	Set1	Sol-Gel
8-A	3%	Medium	Set1	Sol-Gel
9-A	3%	High	Set1	HS*
10-A	1.5%	Low	Set2	Sol-Gel
11-A	1.5%	Medium	Set2	Sol-Gel
12-A	1.5%	High	Set2	Sol-Gel
13-A	2%	Low	Set2	Sol-Gel
14-A	2%	Medium	Set2	Sol-Gel
15-A	2%	High	Set2	HS
16-A	3%	Low	Set2	HS
17-A	3%	Medium	Set2	HS
18-A	3%	High	Set2	HS

* HS: Homogeneous suspension
** A: % Oil phase = 5%

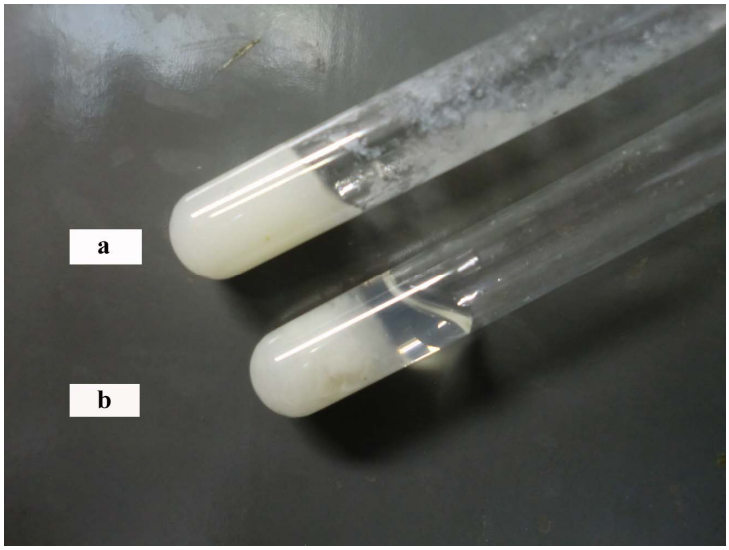


Figure 4.1 Effect of NaCl concentration on homogeneous gel formation:

(a) 5% NaCl (b) 0, 1 and 2% NaCl concentration

Figure 4.1(a) showed that the obtained gel was homogeneous at 5% NaCl whereas **Figure 4.1(b)** showed a typical sample of phase separation observed when NaCl concentration was 0, 1 and 2%.

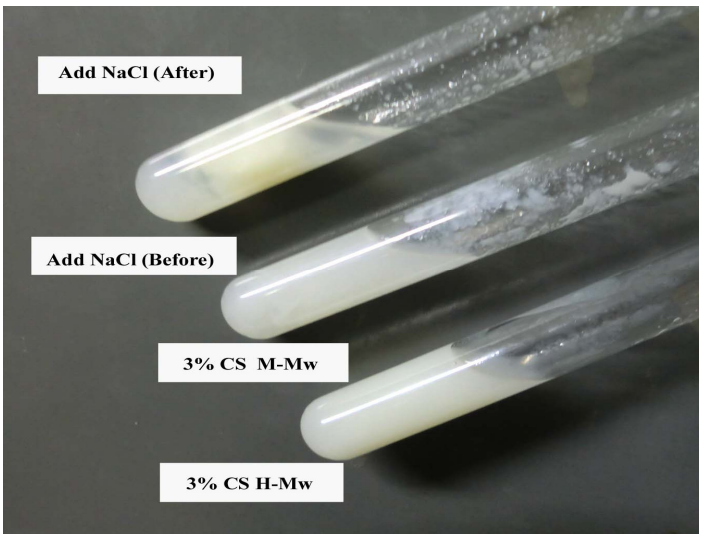


Figure 4.2 Visual comparison between the addition of 5% NaCl before (see bottom two photos) and after (see top photo) mixing the O/W emulsion and the polymer suspension

The correct order of NaCl addition was obvious in **Figure 4.2**, which compares between the addition of 5% NaCl before and after mixing the O/W emulsion and the polymer suspension. Prior addition of NaCl to the O/W emulsion and polymer suspension is necessary to obtain homogeneous suspensions. In addition, since 5% NaCl was found to be most suitable for homogeneous gel formation, the author decided to use 5% NaCl in all subsequent formulations.

In short, the resulting emulsions exhibit homogenous gelation (forming reasonably good gels) when the chitosan concentration in the aqueous phase (b) in **Figure 3.3** was 3% and NaCl was added before mixing the O/W emulsion and the polymer suspension, whilst the ratio of κ-carrageenan to carboxymethylcellulose sodium salt ratio was mainly 4:6 (set 2). The associated gel formation phenomena may be explained as follows. Hydrogel formation was modulated by ionic interactions,

which occurred spontaneously via electrostatic attraction between the positive charges of chitosan and the negative charges of carrageenan. **Figure 4.3(a)** shows that, when NaCl was added *after* the chitosan-containing O/W emulsion and the carrageenan-containing suspension had been mixed, segregated fibrous matters were obtained because chitosan and carrageenan immediately formed dense gels by strong attraction between the positive charges of the amino groups of chitosan and the negative charges of the sulfonate groups of κ-carrageenan. As a consequence, the subsequent addition of NaCl could not retard the gel formation rate and there was not sufficient lead time to encapsulate the oil droplets in the polymer matrices. On the contrary, **Figure 4.3(b)** confirms that desirable colloidal suspensions or soft gels were obtained when NaCl was added *afterwards*. Therefore, the latter procedure was adopted throughout the remaining experiments.

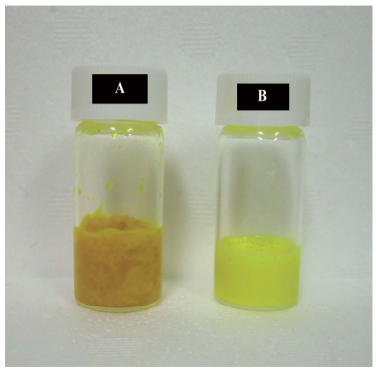


Figure 4.3 Effect of the NaCl addition sequence:

(A) nonhomogeneous suspension (B) homogeneous suspension

As pointed out in Section 2.2, the role of NaCMC was to fine-tune the nature and rate of gelation, thereby making the system better suited for oil-phase encapsulation. The final colloidal suspension was converted into cryogel (frozen

hydrogel) by the use of cryotropic gelation via unidirectional freezing. Unless specified otherwise, the employed cooling rate during freezing was -0.5, -1, or -2 ^{o}C/min. Hydrogel was obtained after the cryogel was thawed out, whereas further freeze-drying yielded dried cryogel.

4.1.2 Preparation of frozen and freeze-dried cryogels

The samples preparation procedure was schematized in **Figure 3.3** As mentioned above, the role of the added sodium chloride was to retard spontaneous gel formation between cationic and anionic polyelectrolytes (chitosan and carrageenan, respectively) caused by electrostatic attraction (Sakiyama et al., 1993) (Note that their aim of NaCl addition was to control swelling equilibrium of the gels by changing the charge balances). Because the author aimed to control sol-gel transition caused by dehydration during unidirectional freezing, selected amount of NaCl was added to the original polyelectrolyte suspensions in order to avoid rapid and strong hydrogel formation at ambient temperature (Lozinsky et al., 1996). Here κ-Carrageenan is the counterpart polyelectrolyte to chitosan. An interesting idea given by Mitsumata et al. (2003) was that the gelling nature of chitosan and κ-carrageenan was tunable by replacing κ-carrageenan with a weaker anionic polyelectrolyte, namely NaCMC. Their main objective in the report was to control the swelling characteristics of the hydrogels. However, the idea would be useful and applicable for modifying cryotropic gel formation, as reported in a later section.

Naturally, it is desirable to increase the proportion of the encapsulated oil phase in the nanocapsules. **Table 4.2** summarizes the combined effect of increasing the oil-phase composition and chitosan MW on the gelation condition. Obviously, as the composition of the oil phase increased to 20%, a higher ratio of carrageenan to CMC at 4:6 was more suitable for gel formation. An excess of CMC apparently contributed to excessive retardation of gel formation. At the same time, a shortage of carrageenan contributed to insufficient crosslinking of the polymer matrix, thereby resulting in structural weakness of the cryogels.

Table 4.2 Effect of increasing the oil-phase composition and chitosan MW on the gelation condition

Formulation	% Oil phase	Obtained suspension before freezing	Gel condition after freeze-thawing
7-A*	5%	Sol	Sol
8-A	5%	Sol, highly viscous	Gel
9-A	5%	Sol, highly viscous	Gel
7-B**	10%	Sol	Sol
8-B	10%	Sol, highly viscous	Gel
9-B	10%	Sol, highly viscous	Gel
7-C***	20%	Sol	Sol
8-C	20%	Sol, viscous	Sol - Gel
9-C	20%	Sol, viscous	Sol - Gel
16-A	5%	Sol, viscous	Sol - Gel
17-A	5%	Sol, highly viscous	Gel
18-A	5%	Sol, highly viscous	Gel
16-B	10%	Sol, viscous	Sol - Gel
17-B	10%	Sol, highly viscous	Gel
18-B	10%	Sol, highly viscous	Gel
16-C	20%	Sol, viscous	Sol - Gel
17-C	20%	Sol, highly viscous	Gel
18-C	20%	Sol, highly viscous	Gel

* A: % Oil phase = 5%
** B: % Oil phase = 10%
*** C: % Oil phase = 20%

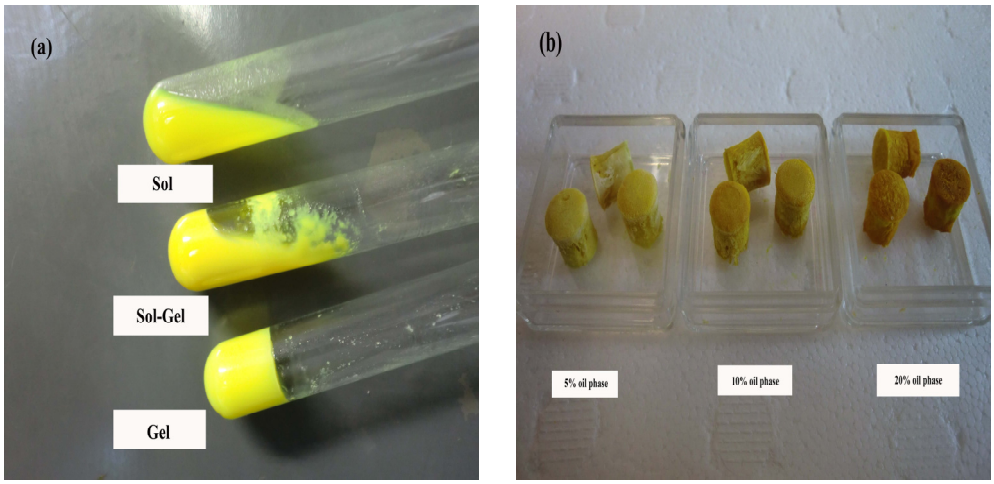


Figure 4.4 Examples of the effect of the oil-phase composition and chitosan MW

(a) Freeze-thawed specimens (b) Freeze-dried specimens

Figure 4.4(a) shows the effect of the gel condition of the suspensions on the thawed specimens. When the suspension conditions were sol, sol-gel and gel, we obtained after freeze-thawing a liquid suspension, wet powder and wet rigid gel, respectively. **Figure 4.4(b)** shows the corresponding effect of oil phase percentage on the freeze-dried specimens. The observed results were fully consistent with the above reasoning. In short, high MW chitosan and high carrageenan-CMC ratio gave the structurally strongest specimens for encapsulating 20% oil phase. Therefore, unless stated otherwise, these conditions were adopted in all subsequent experiments.

4.2 Morphology of Cryogels

4.2.1 Observation of morphology using SEM

Figure 4.5 shows SEM images of the morphology of dried cryogel specimens at 2 different magnifications (150 and 400 times). The frozen specimens were obtained for three oil phase compositions (5, 10 and 20%, respectively) by unidirectionally freezing them at -20 °C for 24 hour, then thawing and natural drying them at 25 °C. At the lower magnification, Fig. 4.5 (a), (c) and (e) show the general shape of the cryogel networks. At the higher magnification, Fig. 4.5 (b), (d) and (f) reveal nano-size oil droplets encapsulated in the cryogel matrix.

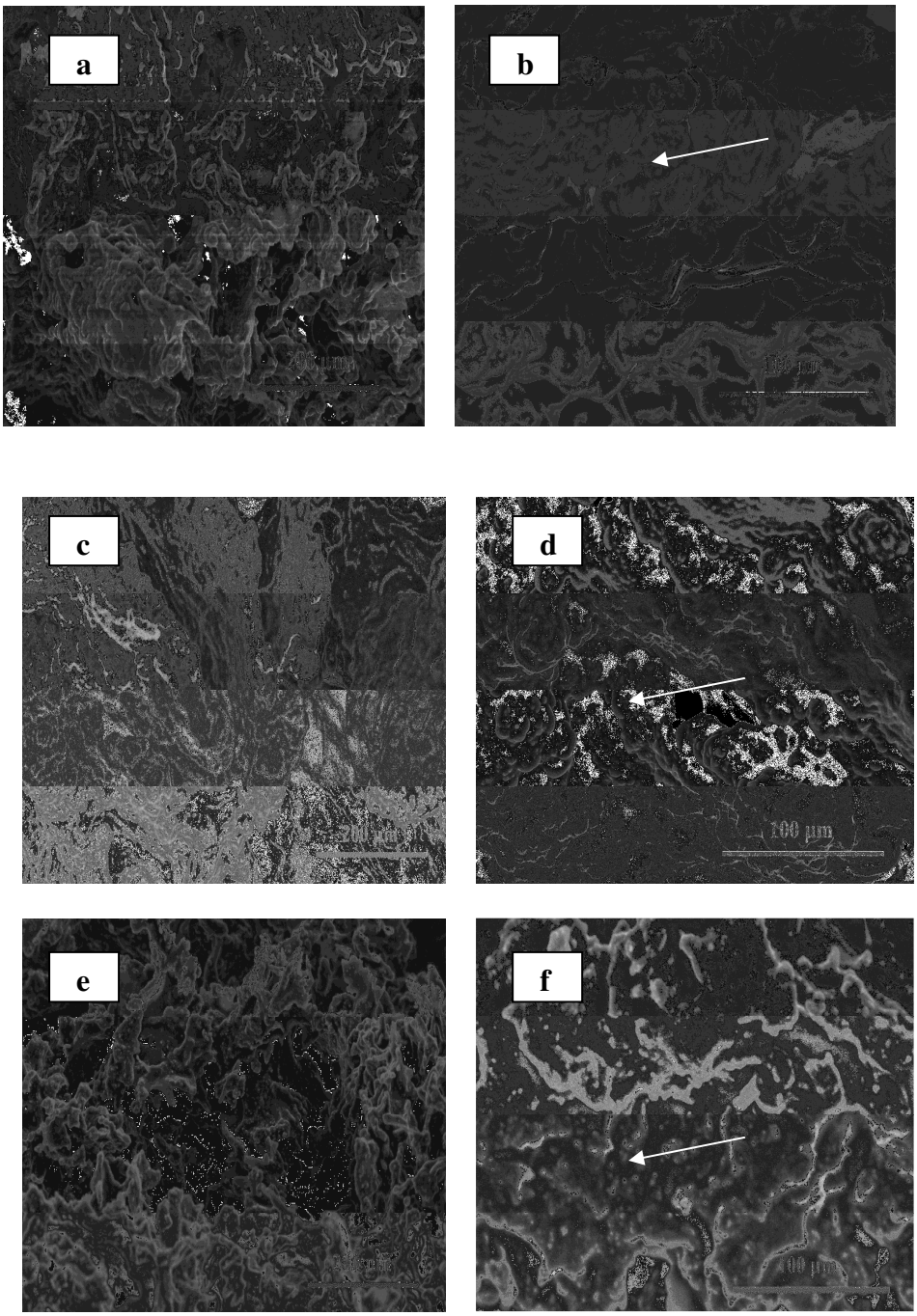


Figure 4.5 SEM micrographs of the cross-sections of cryogels obtained with a cooling rate of -2 °C/min, then thawed and dried at 25 °C: (a-b) 5%, (c-d) 10%, and (e-f) 20% oil phase

4.2.2 Observation using TEM

Figure 4.6 shows an example of the O/W emulsion which was freeze-thawed and observed with TEM. Nano scale particle formation was confirmed in the image, where the core material (oil droplets) was surrounded by polymer shell membrane.

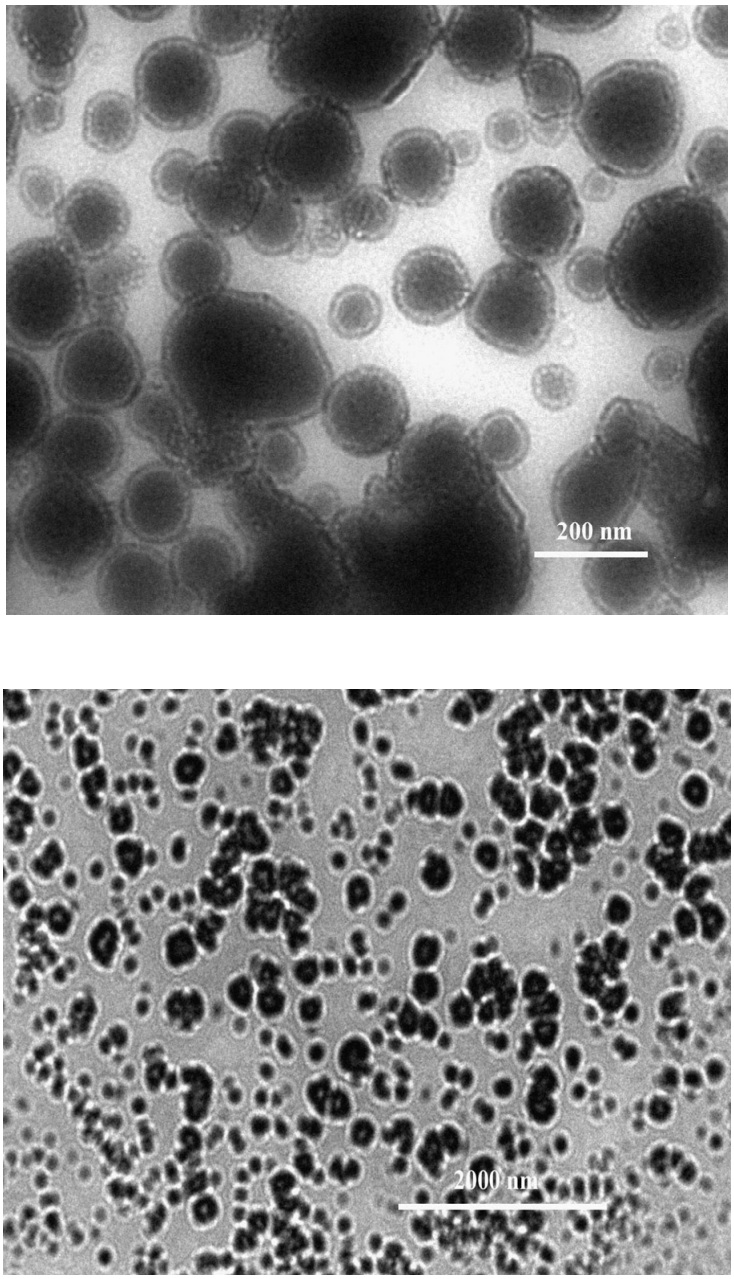


Figure 4.6 TEM micrographs of the cross-sections of the cryogels obtained

4.2.3 Observation using optical microscope

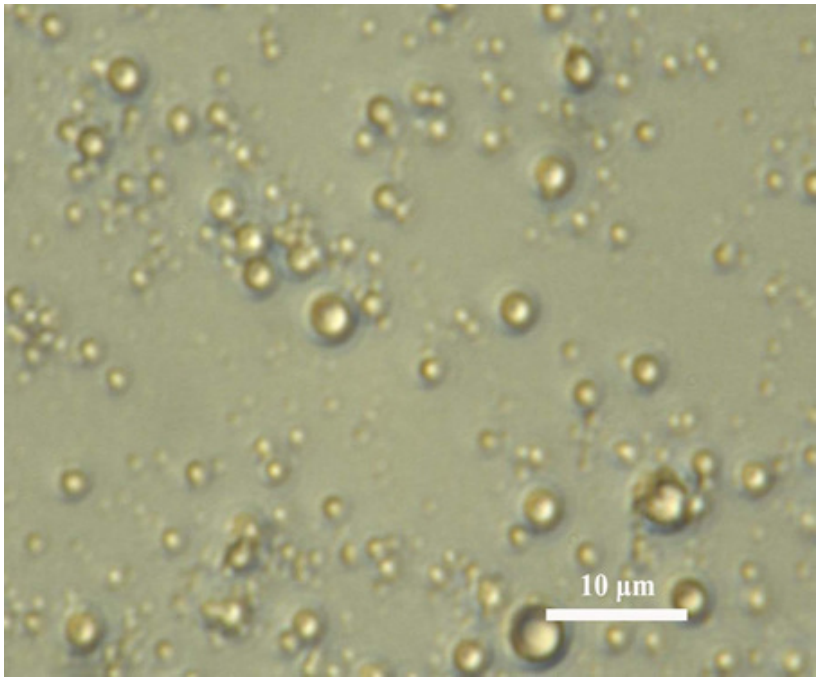


Figure 4.7 Morphology of oil droplets in O/W emulsion

Figure 4.7 shows an example of the observed morphology of oil droplets in the O/W emulsion, whose number-averaged size was 128 nm. The optical image shows essentially spherical oil droplets and it may be considered that there were a large number of nano-sized droplets that cannot be seen with the optical microscope.

4.3 Influence of cryogel preparation conditions on the properties of the nanocapsules in the absence of incubation

4.3.1 Effect of chitosan MW and oil phase composition on the yield of curcumin encapsulation

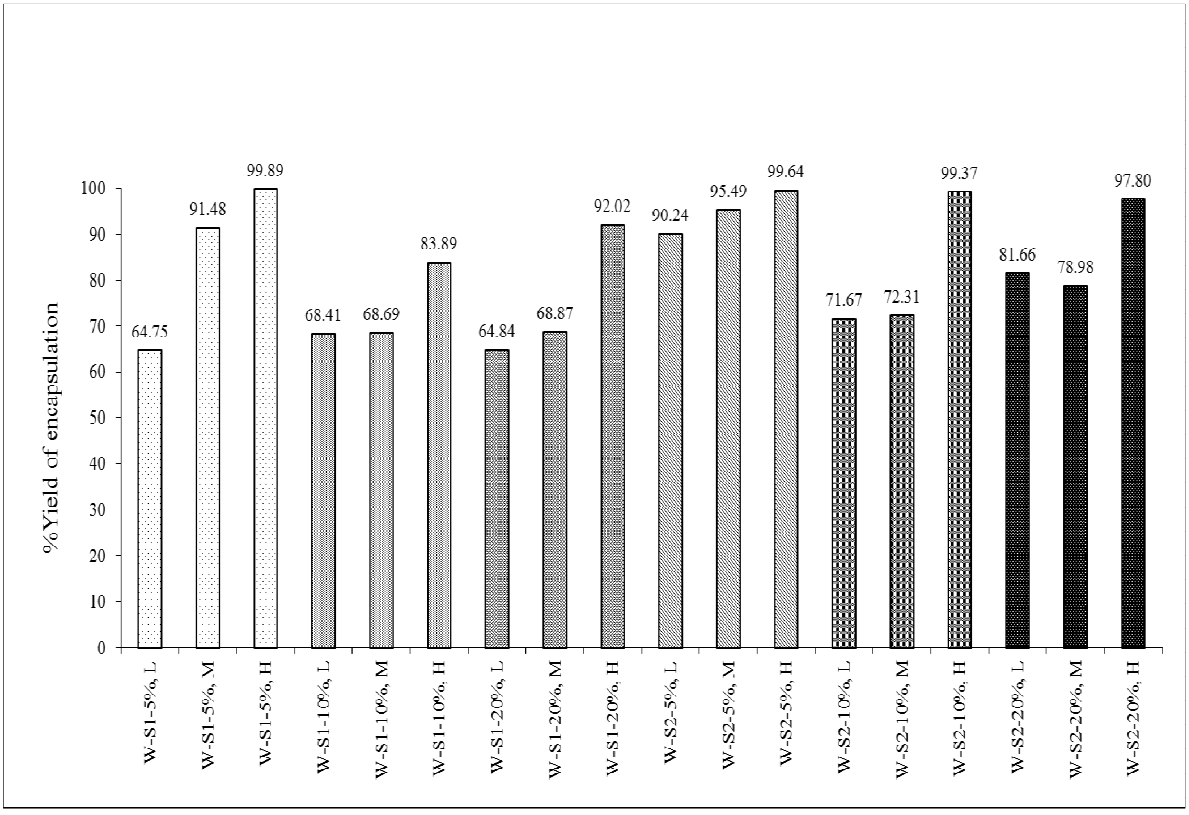


Figure 4.8 Effect of chitosan MW (low, medium, high) and oil phase composition (5, 10, 20%) on the yield of curcumin encapsulation for freeze-dried cryogel specimens containing different ratios of carrageenan to CMC: (set1) 1:9 (set2) 4:6 at the cooling rate of -2 ^{o}C/min.

Figure 4.8 summarizes the combined effect of chitosan MW and oil phase composition on the yield of curcumin encapsulation for freeze-dried cryogel specimens. Obviously, the molecular weight of chitosan had a significant positive effect on the yield. The trend is consistent with that reported by Xu *et al*.'s (Xu & Du, 2003). The encapsulation yield for the two sets of polymer suspensions was found to range from 83.9 to 99.6% when high-MW chitosan was used and the cooling rate during unidirectional freezing was -2 ^{o}C/min.

4.3.2 Effect of the freezing rate and oil phase composition on the yield of curcumin encapsulation

Table 4.3 Encapsulation yield of curcumin.

Specimen Code	% Oil phase	Used polymer suspension Set 1 or 2	Cooling rate during freezing [K/min]	Yield of encapsulation [%]	Total amount of curcumin released at 4 days [%]
W-S1-05-5%	5%	1	-0.5	89.45	38.4
W-S1-1-5%	5%	1	-1.0	90.58	41.3
W-S1-2-5%	5%	1	-2.0	99.89	52.3
W-S2-05-5%	5%	2	-0.5	91.94	46.6
W-S2-1-5%	5%	2	-1.0	95.95	36.9
W-S2-2-5%	5%	2	-2.0	99.64	48.7
W-S1-05-10%	10%	1	-0.5	83.89	41.1
W-S1-1-10%	10%	1	-1.0	94.49	48.7
W-S1-2-10%	10%	1	-2.0	99.93	58.5
W-S2-05-10%	10%	2	-0.5	95.15	43.3
W-S2-1-10%	10%	2	-1.0	98.13	48.3
W-S2-2-10%	10%	2	-2.0	99.37	59.9
W-S1-05-20%	20%	1	-0.5	90.65	30.6
W-S1-1-20%	20%	1	-1.0	92.02	48.2
W-S1-2-20%	20%	1	-2.0	98.31	51.3
W-S2-05-20%	20%	2	-0.5	97.41	48.2
W-S2-1-20%	20%	2	-1.0	98.89	50.7
W-S2-2-20%	20%	2	-2.0	99.78	41.9

The yields of curcumin encapsulation are listed in **Table 4.3**. The combined effect of the cooling rate during unidirectional freezing and the oil phase composition on the yield of curcumin encapsulation for freeze-dried cryogels containing high-MW chitosan was investigated. Evidently, as the cooling rate increased, the yield of encapsulation rose in tandem. As expected, the freezing condition was clearly a factor that strongly related to the mechanism of oil encapsulation. The oil loss could be visibly confirmed in a few experimental runs. In the present system, the freezing of a solution progressed directionally from the bottom to the top of the solution. When the oil phase was not incorporated into the cryogelling matrix, the oil droplets accumulated at the freezing front, and a shallow oil layer consequently covered the

frozen sample. However, the author could not observe any of oil layers when rapid freezing conditions were applied. In fact, the cooling rate was found to have not only a significant effect on the yield but also on curcumin release behavior, which will be presented next.

4.3.3 Effect of freezing rate and ratio of Carrageenan to NaCMC on the yield of gel fraction

Unidirectional contact freezing of the starting solution with a plate heat exchanger was used to prepare cryogels for investigating the effect of the formulation. **Table 4.4** shows the observed yields of gel fraction. Evidently, freezing at a faster cooling rate had a slightly positive effect on the yield of gel fraction, although the observed differences might be considered insignificant when the composition was the same.

Table 4.4 Yield of gel fraction of cryogels.

No.	Specimen code	Use polymer suspension set (1,2 and 3)	Cooling rate during freezing (K/min)	% Yield of gel fraction	SD.
1	W-S1-05	set1	-0.5	93.48	0.17
2	W-S2-05	set2	-0.5	94.13	0.75
3	W-S3-05	set3	-0.5	92.85	0.6
4	W-S1-2	set1	-2	95.58	0.25
5	W-S2-2	set2	-2	94.96	0.11
6	W-S3-2	set3	-2	94.32	0.11

4.3.4 Effect of pH of buffer media and composition of the polymer suspension on the swelling ratio

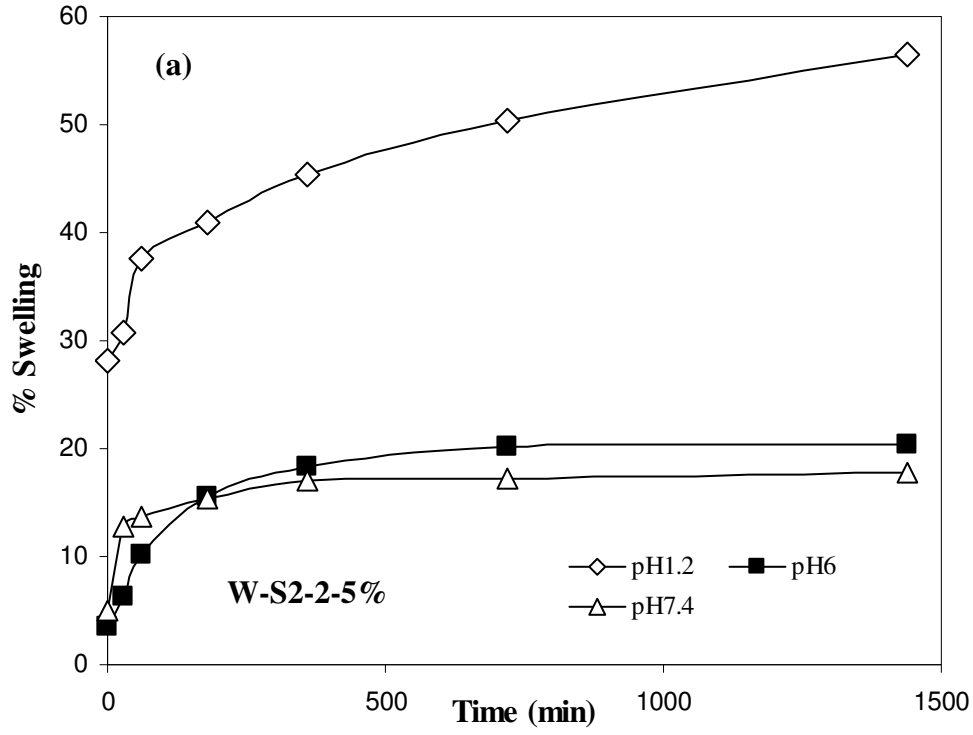
Table 4.5 shows the combined effect of pH (1.2, 3.0, 5.0, 7.4 and 9) of the phosphate buffer media and type of polymer suspension with oil phase composition (5%) on the observed swelling ratio. All of the specimens were obtained using a cooling rate of -0.5 or -2 ◦C/min. Apparently, the one-dimensional swelling ratio increased significantly from around 10-20% to 40-60% as pH decreased from essentially neutral (pH 7.4) to strongly acidic (pH 1.2). The slower the cooling rate, the bigger the swelling ratio became, essentially independent of the introduced oil phase composition. Generally, a bigger swelling ratio means that the structure of the cryogel matrix has fewer crosslinkings and less bonding strength. This provides an important piece of circumstantial evidence that these three samples should possess different microstructures due to the different cooling rate during freezing, though they were prepared from the same formulation. In other words, the resulting gel network structure could depend on its gel formation kinetics during sol-gel transition.

Table 4.5 Swelling ratio of cryogels.

No.	Specimen Code	pH	Use polymer suspension set (1,2 and 3)	Cooling rate during freezing (K/min)	% Swelling	SD.
1	W-S1-05-1	1.2	set 1	-0.5	109.69	0.022
2	W-S1-2-1	1.2	set 1	-2	92.10	0.095
3	W-S1-05-3	3	set 1	-0.5	87.57	0.009
4	W-S1-2-3	3	set 1	-2	67.98	0.021
5	W-S1-05-5	5	set 1	-0.5	45.83	0.053
6	W-S1-2-5	5	set 1	-2	27.23	0.037
7	W-S1-05-7	7	set 1	-0.5	18.85	0.021
8	W-S1-2-7	7	set 1	-2	18.54	0.001
9	W-S1-05-9	9	set 1	-0.5	17.47	0.055
10	W-S1-2-9	9	set 1	-0.5	16.14	0.088
11	W-S2-05-1	1.2	set 2	-0.5	104.28	0.016
12	W-S2-2-1	1.2	set 2	-2	82.21	0.064
13	W-S2-05-3	3	set 2	-0.5	83.25	0.016
14	W-S2-2-3	3	set 2	-2	66.23	0.025
15	W-S2-05-5	5	set 2	-0.5	44.25	0.013
16	W-S2-2-5	5	set 2	-2	23.62	0.052
17	W-S2-05-7	7	set 2	-0.5	18.29	0.015
18	W-S2-2-7	7	set 2	-2	18.41	0.054
19	W-S2-05-9	9	set 2	-0.5	16.58	0.028
20	W-S2-2-9	9	set 2	-2	14.79	0.003
21	W-S3-05-1	1.2	set 3	-0.5	93.04	0.021
22	W-S3-2-1	1.2	set 3	-2	78.52	0.012
23	W-S3-05-3	3	set 3	-0.5	70.62	0.001
24	W-S3-2-3	3	set 3	-2	64.28	0.028
25	W-S3-05-5	5	set 3	-0.5	40.83	0.015
26	W-S3-2-5	5	set 3	-2	23.60	0.031
27	W-S3-05-7	7	set 3	-0.5	18.20	0.011
28	W-S3-2-7	7	set 3	-2	16.96	0.015
29	W-S3-05-9	9	set 3	-0.5	14.54	0.018
30	W-S3-2-9	9	set 3	-2	13.40	0.088

Similarly, **Figure 4.9** shows the combined effect of pH (1.2, 6.0 and 7.4) of the phosphate buffer media and oil phase composition (5, 10, and 20%) on the observed swelling ratio. All of the six tested, set2 polymer containing specimens coded as W-S2-2 were obtained using a cooling rate of -2 ˚C/min. This Figure reveals

that the one-dimensional swelling ratio increased significantly from around 10-20% to 40-60% as pH decreased from essentially neutral (pH 7.4) to strongly acidic (pH 1.2). In the case of **Figure 4.9(a),** the swelling ratio appeared to increase monotonically above 60%, thereby indicating a serious weakening of the crosslinkings in the polymer matrix. Subsequently, the effect of pH on the curcumin release behaviour will further be investigated to determine the range of applicability of the current chitosan-based encapsulation technique.



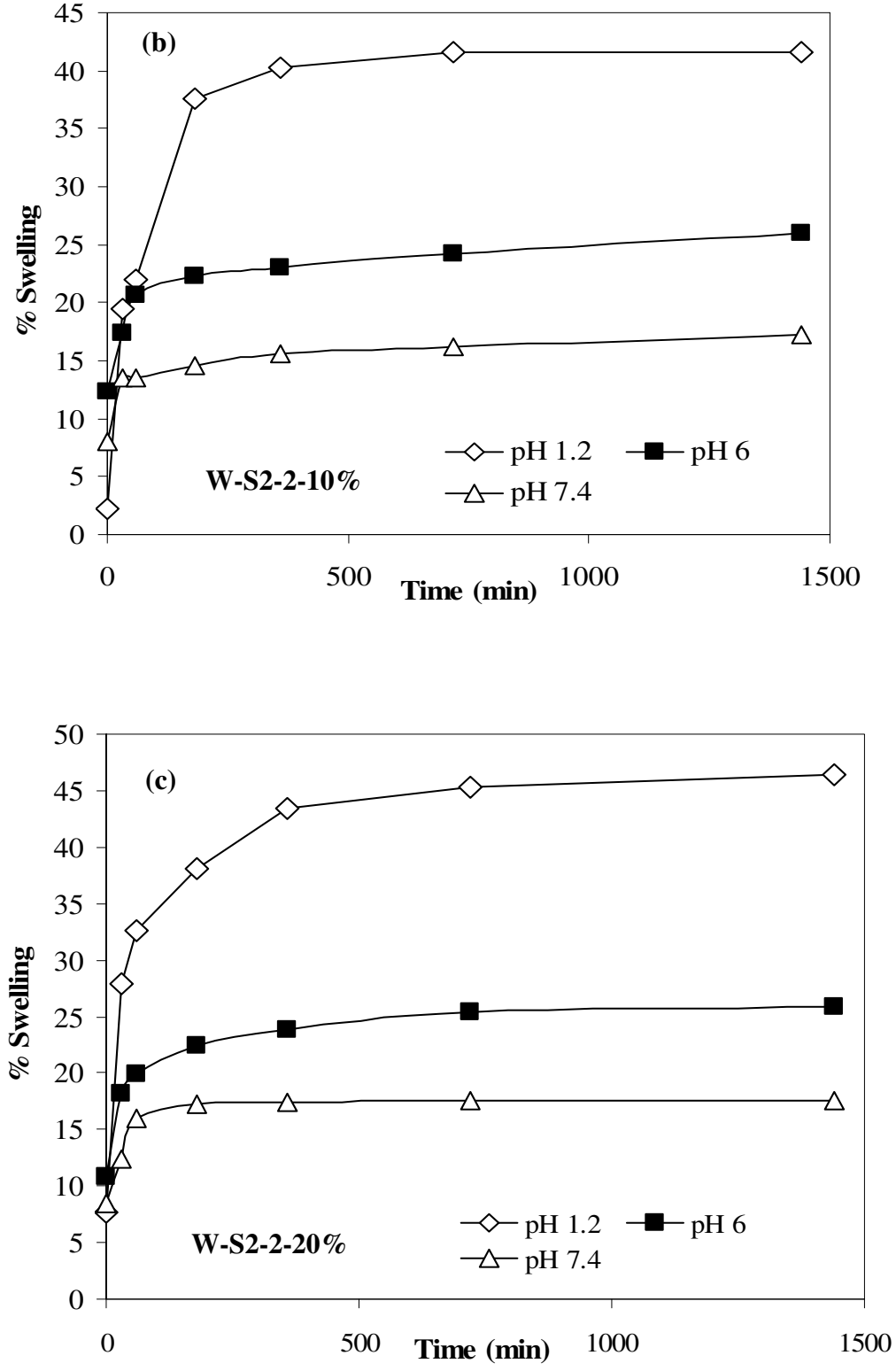


Fig. 4.9 Effect of pH of the phosphate buffer media and oil phase composition on the observed swelling ratio: (a) 5% (b) 10% (c) 20% oil phase

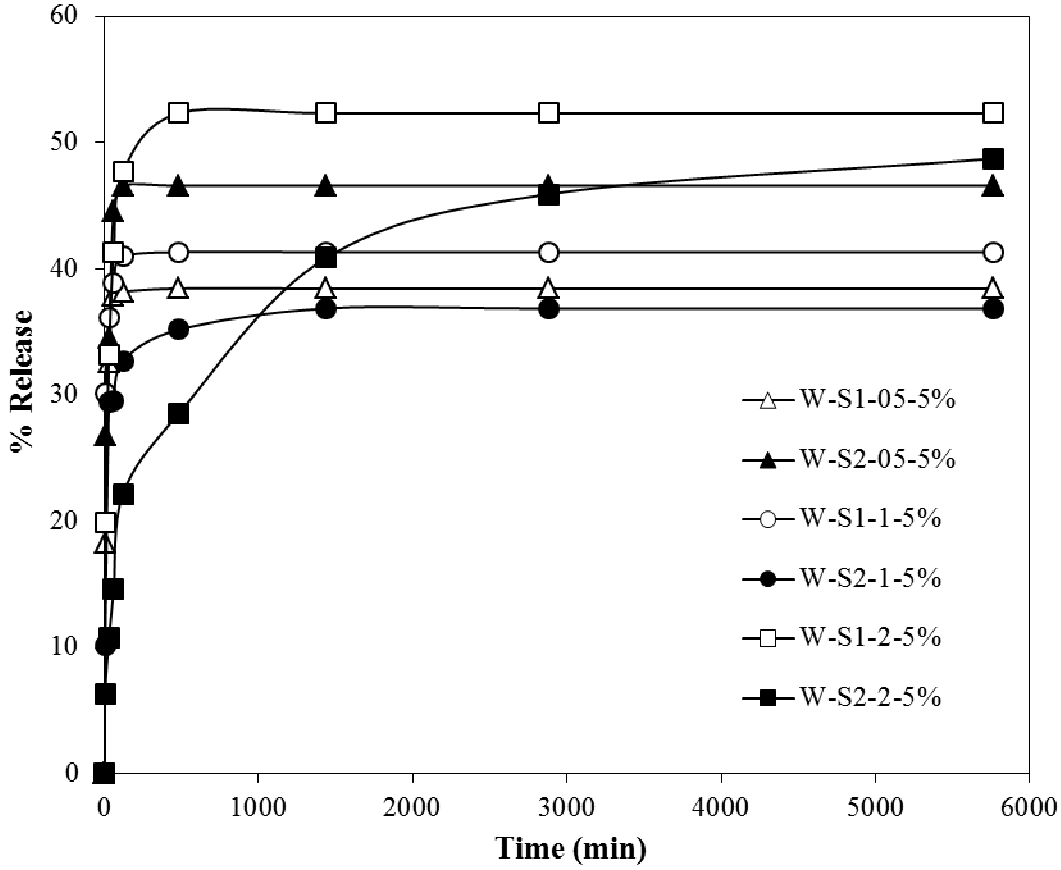
4.3.5 Effect of the freezing rate and oil phase composition on the curcumin release behavior of freeze-dried cryogels

Figure 4.10 shows the effect of the cooling rate (-0.5, -1 and -2 ◦C/min) and oil phase composition (5, 10, and 20%) on the behavior of curcumin release from freeze-dried cryogels in a phosphate buffer solution with pH 7.4. Similarly, **Table 4.3** shows the corresponding effect on the total percentage of curcumin released at 4 days. Note that specimens W-S1-05, W-S1-1 and W-S1-2 were prepared using set 1 polymer suspension in **Figure 3.1**, whereas W-S2-05, W-S2-1 and W-S2-2 employed set 2. **Figure 4.10** reveals two types of release behavior: a burst release (typified by W-S1-2) and a moderate first-order release rate (typified by W-S2-2), essentially irrespective of the introduced oil phase composition.

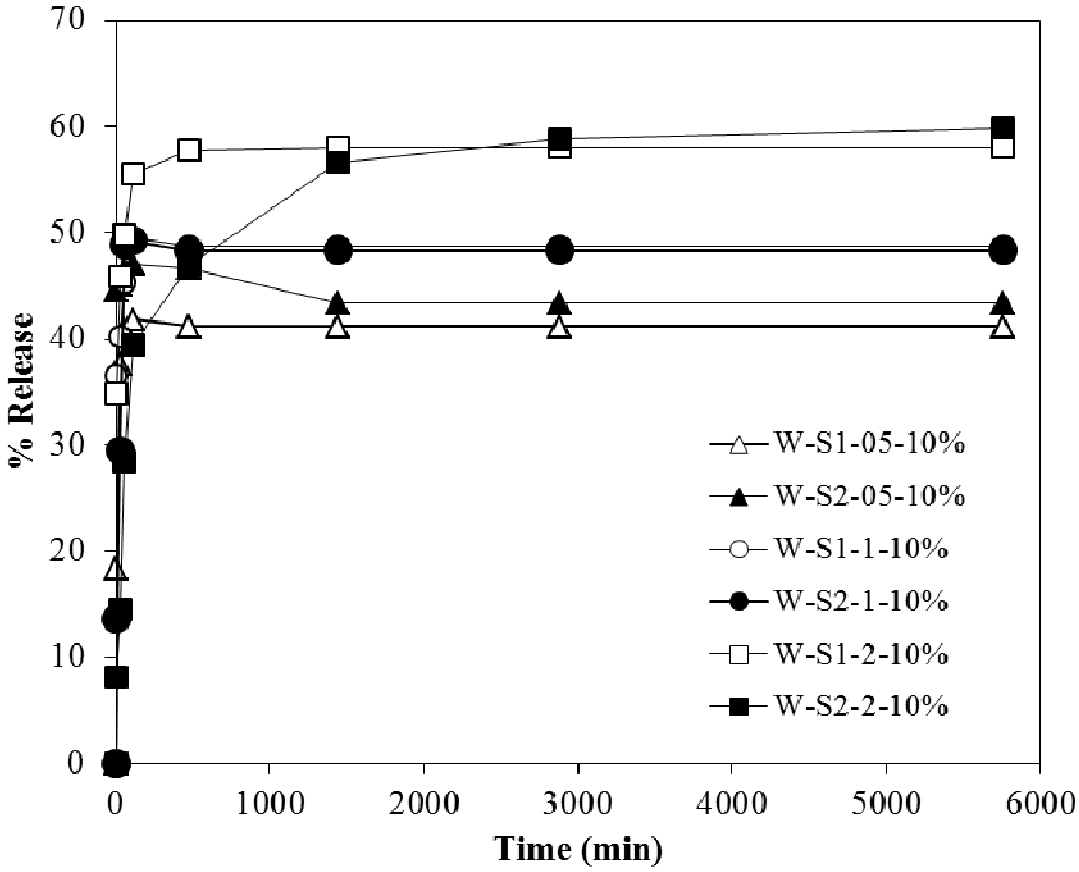
Generally, the slower the rate of release, the more numerous crosslinkings and the higher bonding strength the polymer matrix has. Since specimens W-S1-2 and W-S2-2 were made of different formulations consisting of a carrageenan:CMC ratio of 1:9 and 4:6, respectively, the more balanced ratio was considered to be essential for a stronger microstructure. It is reasonable to consider that a significant structural difference in the gel network should contribute to the difference in mass transfer resistance through the matrix, which consequently led to appreciably different release curve types. During the cryotropic gelation process, some free oil was observed to accumulate towards the freezing front, and a thin oil layer consequently covered the frozen specimen. Normally, these surface free oils could not be removed without thawing before the specimen immediately underwent freeze-drying. Though the specimen surface was cleaned afterwards, some of the surface oil could remain and got counted in the encapsulation yields listed in **Table 4.3**. It is possible that a little

residual surface oil could partly contribute to the observed burst release. It is also conceivable that some oil droplets might not be perfectly encapsulated.

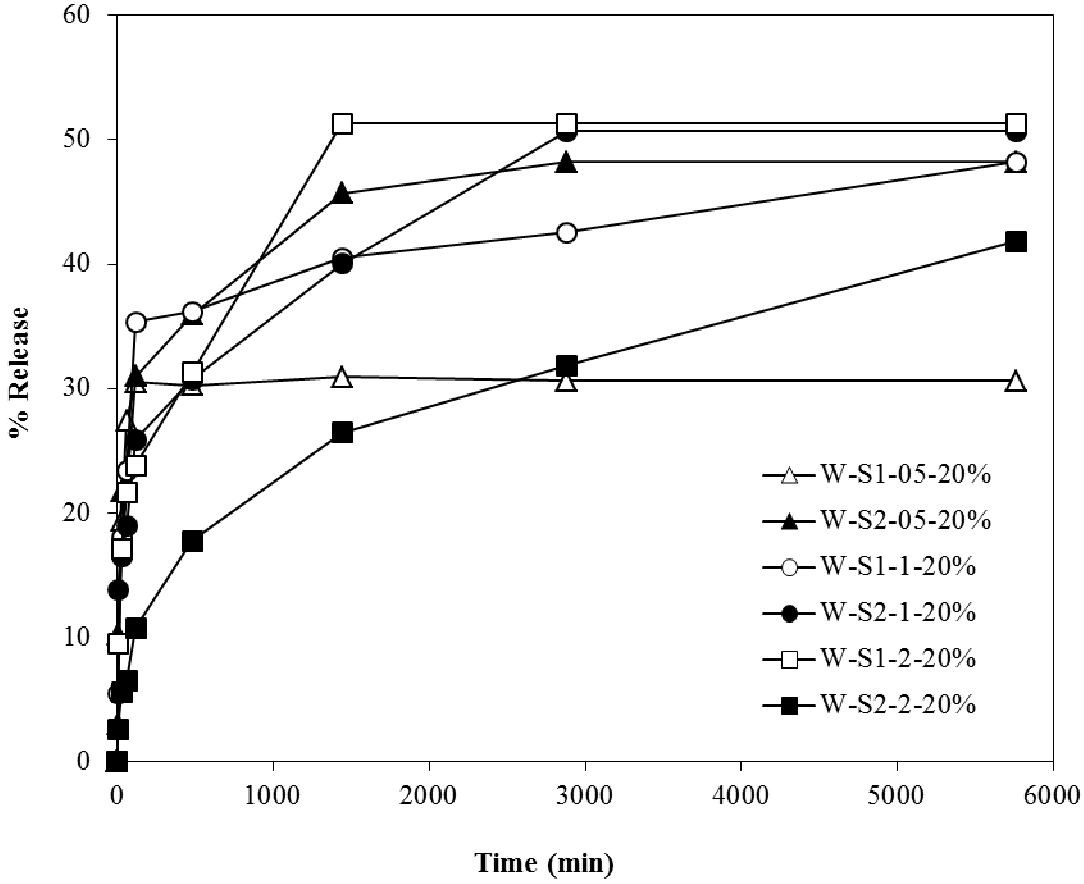
Nevertheless, the facts that the prepared freeze-dried specimens were not an oily stuff and that our SEM images (Figure 4.5 (b, d, f)) clearly show incorporated oil domains in the matrix mean that the burst release might not be solely attributed to the residual oils. At present we still cannot differentiate between well encapsulated and incompletely encapsulated oil droplets in the matrix. Judging from the curves in Figure 4.10, the residual and incompletely encapsulated oils should not exceed from a few up to 10%. The Figure also shows that, irrespective of the introduced oil phase composition, controlled release of curcumin was achievable when the cooling rate was sufficiently high at -2 ^{o}C/min, especially set2 polymer suspension was used. Therefore, it may be postulated that, when the formulation was suitable, the rapid freezing condition would influence the formation of nano-micro structures in the present cryogels that were favourable for controlled release of curcumin.


60
50
40
30
20
10
0
% Release
0
1000
2000
3000
4000
5000
6000
Time (min)
W-S1-05-5%
W-S2-05-5%
W-S1-1-5%
W-S2-1-5%
W-S1-2-5%
W-S2-2-5%

(a) 5% oil phase



(b) 10% oil phase



(c) 20% oil phase

Fig. 4.10 Combined effect of the cooling rate and oil phase composition on the behavior of curcumin release from freeze-dried cryogels in a phosphate buffer solution with pH 7.4: (a) 5% (b) 10% (c) 20% oil phase

4.4 Influence of cryogel preparation condition on the properties of the nanocapsules in the presence of cold incubation

4.4.1 Effect of freezing rate and ratio of Carrageenan to NaCMC on the yields of gel fraction and curcumin encapsulation

Table 4.6 Yield of gel fraction of cryogels

No.	Specimen code	Use polymer suspension set (2and3)	Cooling rate during freezing (K/min)	% Yield of gel fraction	SD
1	W-S2-05	set1	-0.5	94.13	0.75
2	I-S2-05	set1	-0.5	95.20	0.38
3	W-S3-05	set2	-0.5	92.85	0.6
4	I-S3-05	set2	-0.5	94.11	0.04
5	W-S2-2	set1	-2	94.96	0.11
6	I-S2-2	set1	-2	95.92	0.04
7	W-S3-2	set2	-2	94.32	0.11
8	I-S3-2	set2	-2	95.41	0.08

The identification codes and characteristics of the specimens are summarized in Table 4.7. Obviously, post-freezing incubation induced more crosslinks and possibly higher bond strength. **Table 4.6** shows the observed yields of gel fraction. Evidently both post-freezing incubation and a faster freezing rate had a slightly positive effect on the yield of gel fraction, although the observed differences between the two factors might be considered insignificant when the composition stayed the same. Since specimen I-S2-2 displayed the highest gel fraction, it may be considered that, when

the formulation was suitable as in the case of polymer set 2, rapid freezing followed by incubation had the greatest effect on the formation of nano- and micro-structure in the freeze-dried cryogels.

4.4.2 Effect of freezing rate and ratio of Carrageenan to NaCMC on the swelling ratio

In the absence of incubation, it has already been found that the slower the cooling rate, the bigger the swelling ratio of freeze-dried cryogels became, and surprisingly this phenomenon was essentially independent of the introduced oil phase composition. Generally, a bigger swelling ratio means that the microstructure of the cryogel matrix has fewer crosslinks and less bonding strength. Therefore, post-freezing incubation could reasonably be expected to reduce swelling of freeze-dried cryogels. **Table 4.7** shows the observed swelling ratios of cryogels made from polymer sets 1, 2 and 3, respectively, in the presence and absence of post-freezing incubation. Obviously, incubation significantly reduced the swelling ratios in all comparable cases.

Table 4.7-1 Swelling ratio of cryogels (polymer set 1) with and without incubation

No.	Specimen Code	pH	Use polymer suspension set (1,2 and 3)	Cooling rate during freezing (K/min)	% Swelling	SD.
1	W-S1-05-1	1.2	set 1	-0.5	109.69	0.022
2	I-S1-05-1	1.2	set 1	-0.5	97.48	0.008
3	W-S1-2-1	1.2	set 1	-2	92.10	0.095
4	I-S1-2-1	1.2	set 1	-2	80.84	0.037
5	W-S1-05-3	3	set 1	-0.5	87.57	0.009
6	I-S1-05-3	3	set 1	-0.5	84.88	0.078
7	W-S1-2-3	3	set 1	-2	67.98	0.021
8	I-S1-2-3	3	set 1	-2	44.84	0.001
9	W-S1-05-5	5	set 1	-0.5	45.83	0.053
10	I-S1-05-5	5	set 1	-0.5	43.86	0.043
11	W-S1-2-5	5	set 1	-2	27.23	0.037
12	I-S1-2-5	5	set 1	-2	21.32	0.088
13	W-S1-05-7	7	set 1	-0.5	18.85	0.021
14	I-S1-05-7	7	set 1	-0.5	18.31	0.023
15	W-S1-2-7	7	set 1	-2	18.54	0.001
16	I-S1-2-7	7	set 1	-2	15.13	0.064
17	W-S1-05-9	9	set 1	-0.5	17.47	0.055
18	I-S1-05-9	9	set 1	-0.5	16.25	0.036
19	W-S1-2-9	9	set 1	-0.5	16.14	0.088
20	I-S1-2-9	9	set 1	-2	14.85	0.025

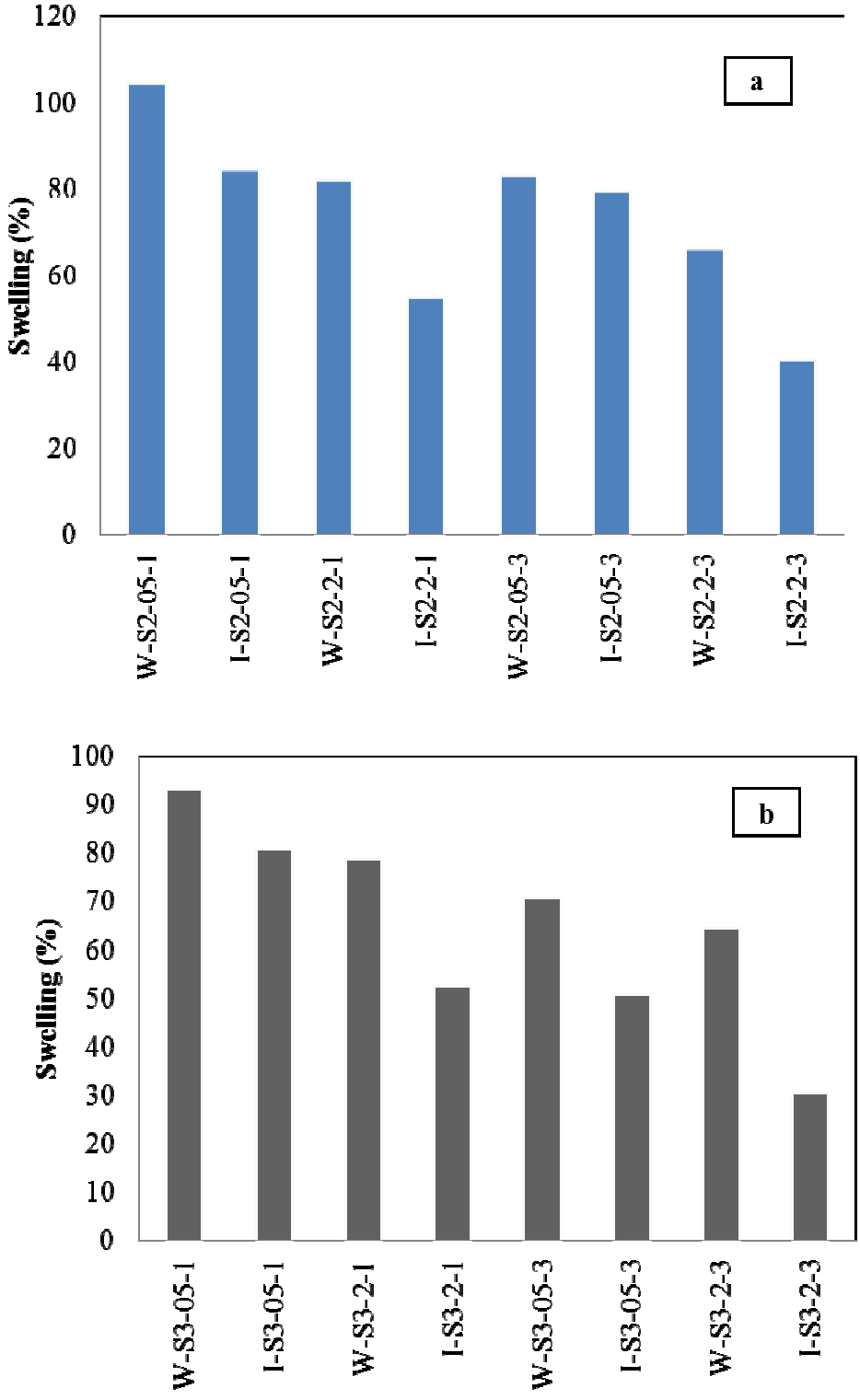
Table 4.7-2 Swelling ratio of cryogels (polymer set 2) with and without incubation

No.	Specimen Code	pH	Use polymer suspension set (1,2 and 3)	Cooling rate during freezing (K/min)	% Swelling	SD.
21	W-S2-05-1	1.2	set 2	-0.5	104.28	0.016
22	I-S2-05-1	1.2	set 2	-0.5	84.54	0.078
23	W-S2-2-1	1.2	set 2	-2	82.21	0.064
24	I-S2-2-1	1.2	set 2	-2	55.14	0.052
25	W-S2-05-3	3	set 2	-0.5	83.25	0.016
26	I-S2-05-3	3	set 2	-0.5	79.69	0.069
27	W-S2-2-3	3	set 2	-2	66.23	0.025
28	I-S2-2-3	3	set 2	-2	40.50	0.018
29	W-S2-05-5	5	set 2	-0.5	44.25	0.013
30	I-S2-05-5	5	set 2	-0.5	38.84	0.037
31	W-S2-2-5	5	set 2	-2	23.62	0.052
32	I-S2-2-5	5	set 2	-2	18.06	0.027
33	W-S2-05-7	7	set 2	-0.5	18.29	0.015
34	I-S2-05-7	7	set 2	-0.5	14.50	0.007
35	W-S2-2-7	7	set 2	-2	18.41	0.054
36	I-S2-2-7	7	set 2	-2	16.79	0.01
37	W-S2-05-9	9	set 2	-0.5	16.58	0.028
38	I-S2-05-9	9	set 2	-0.5	13.48	0.013
39	W-S2-2-9	9	set 2	-2	14.79	0.003
40	I-S2-2-9	9	set 2	-2	11.41	0.011

Table 4.7-3 Swelling ratio of cryogels (polymer set 3) with and without incubation

No.	Specimen Code	pH	Use polymer suspension set (1,2 and 3)	Cooling rate during freezing (K/min)	% Swelling	SD.
41	W-S3-05-1	1.2	set 3	-0.5	93.04	0.021
42	I-S3-05-1	1.2	set 3	-0.5	80.61	0.063
43	W-S3-2-1	1.2	set 3	-2	78.52	0.012
44	I-S3-2-1	1.2	set 3	-2	52.29	0.035
45	W-S3-05-3	3	set 3	-0.5	70.62	0.001
46	I-S3-05-3	3	set 3	-0.5	50.74	0.042
47	W-S3-2-3	3	set 3	-2	64.28	0.028
48	I-S3-2-3	3	set 3	-2	30.22	0.03
49	W-S3-05-5	5	set 3	-0.5	40.83	0.015
50	I-S3-05-5	5	set 3	-0.5	35.24	0.052
51	W-S3-2-5	5	set 3	-2	23.60	0.031
52	I-S3-2-5	5	set 3	-2	16.45	0.008
53	W-S3-05-7	7	set 3	-0.5	18.20	0.011
54	I-S3-05-7	7	set 3	-0.5	14.30	0.031
55	W-S3-2-7	7	set 3	-2	16.96	0.015
56	I-S3-2-7	7	set 3	-2	15.50	0.012
57	W-S3-05-9	9	set 3	-0.5	14.54	0.018
58	I-S3-05-9	9	set 3	-0.5	13.16	0.065
59	W-S3-2-9	9	set 3	-2	13.40	0.088
60	I-S3-2-9	9	set 3	-2	10.33	0.019

For visual comparison, the results for polymer set 2 and set 3 are shown as bar charts in **Figure 4.11**. It can be seen that incubation exhibited a desirable negative effect on the swelling behavior of the cryogel nanocapsules. Obviously, post-freezing incubation induced more crosslinks and possibly higher bond strength.



The last digits 1 and 3 mean pH 1.2 and 3, respectively

Fig. 4.11 Effect of incubation on the swelling properties of the freeze-dried cryogel specimens: (a) Polymer set S2 (b) Polymer set S3

4.4.3 Effect of freezing rate and ratio of Carrageenan to NaCMC on average particle size and zeta potential of thawed-cryogel

Table 4.8 Particle size characteristics of freeze-dried cryogel nanocapsules and zeta potential of thawed cryogel suspended in water with and without post-freezing incubation

No.	Specimen code	Particle size (mean±S.D., nm)	Zeta potential (mean±S.D., mV)	Polydispersity index (mean±S.D.)
1	W-S2-05	79 ± 0.82	31.17 ± 0.90	0.74 ± 0.01
2	I-S2-05	121 ± 0.42	20.17 ± 0.64	0.87 ± 0.07
3	W-S3-05	80 ± 0.77	41.17 ± 0.35	0.75 ± 0.01
4	I-S3-05	83 ± 0.04	7.27 ± 0.18	0.73 ± 0.06
5	W-S2-2	82 ± 0.64	27.97 ± 0.14	0.74 ± 0.07
6	I-S2-2	84 ± 0.86	25.80 ± 0.78	0.76 ± 0.02
7	W-S3-2	75 ± 0.29	21.53 ± 0.71	0.67 ± 0.05
8	I-S3-2	117 ± 0.57	8.89 ± 0.16	0.76 ± 0.04
9	Spray dry-Set2	148 ± 89.38	26.70 ± 1.14	0.50 ± 0.03
10	Spray dry-Set3	269 ± 78.23	15.57 ± 1.32	0.68 ± 0.16
11	Emulsion(O/W)	128 ± 0.20	23.7 ± 0.90	0.49 ± 0.06
12	Carrageenan	536 ± 0.78	-49.40 ± 0.90	1
13	CMC	248 ± 0.35	-51.40 ± 0.90	0.87± 0.12

The combined effect of the concentration ratio of κ-carrageenan to CMC on the size distribution of cryogel nanocapsules and zeta potential was investigated at κ-carrageenan:CMC = 4:6 (polymer set 2) and 6:4 (polymer set 3), with and without post-freezing incubation. The obtained zeta potentials, number-averaged sizes and polydispersity indices are listed in **Table 4.8** and the sizes are also graphically shown in **Figure 4.12**.

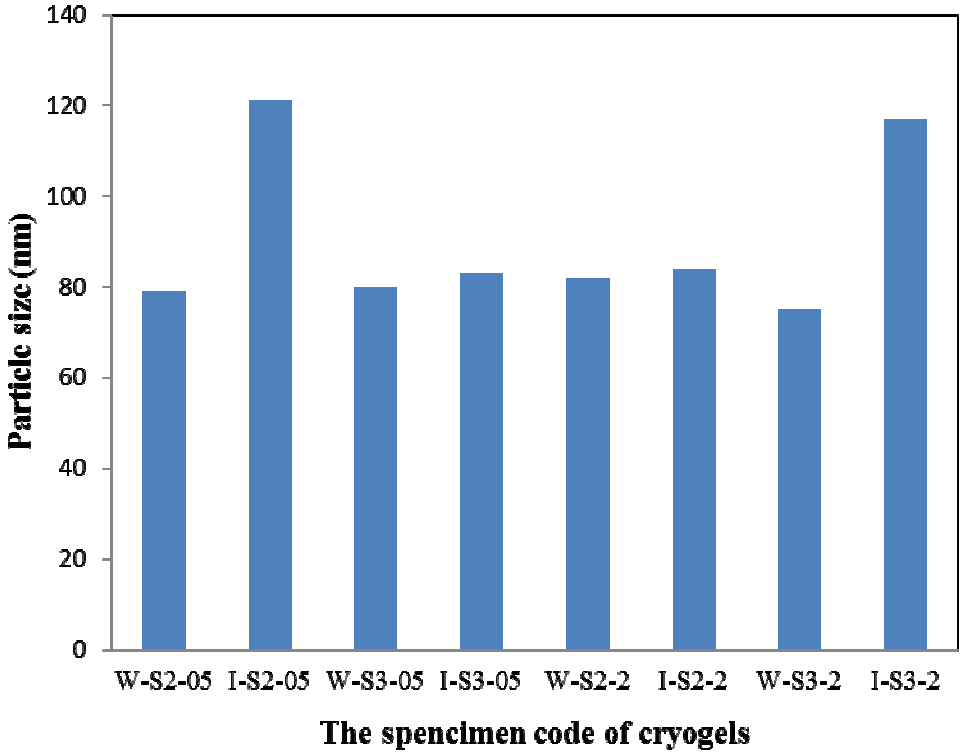


Fig. 4.12 Effect of concentration ratio of carrageenan to CMC with and without incubation on number-averaged sizes of cryogel nanocapsules

Though cryogels that underwent incubation always showed a larger size than their non-incubated counterparts, the effect of incubation was dominant for polymer set 2 at a slower cooling rate of 0.5 ^{o}C/min and for polymer set 3 at 2.0 ^{o}C/min. The results may be attributed to the exposure of amino groups (–NH3+) from the network by the formation of three-dimensional cross-linked network during low-temperature incubation (Bejrapha et al., 2011). Prego et al. (2006) have reported that an increase in particle size indicated the attachment of polymers to the surface of the oil core. Therefore the increase in the average size of the nanocapsules may be considered to indicate that chitosan is located on the surface of the carrageenan due to the

electrostatic interactions described in Section 2.2, which is useful for the stabilization of the oil core. Interestingly, the particle sizes of I-S2-05 and I-S3-2 were significantly larger than their non-incubated counterparts. It may be considered that the freezing rates of -0.5 and -2 ^{o}C/min did not match the polymer set S2 and S3, respectively. As a consequence, the particle size increased significantly and resulted in less dense matrix. This effect can be deduced from the fact that both I-S2-05 and I-S3-2 exhibited a faster release rate and shorter release period than the other two incubated specimens, as shown afterwards in Figures 4.13 and 4.14.

It may be deduced from the zeta potential data in Table 4.8 that the negatively charged carrageenan and carboxymethyl cellulose (CMC) sodium salt were completely stabilized by the positively charged chitosans and the total charge of the cyrogels became positive. Interestingly, post-freezing incubation always resulted in reduction of the positive zeta potentials compared with their non-incubated counterparts. As explained above, incubation increased the electrostatic interactions between the positive-charged amino groups of chitosan and negative-charged sulfate groups (–SO42-) of carrageenan, thereby resulting in more neutralizing effect. Therefore, post-freezing incubation can be cosnidered to be another factor that controls microstructure formation in the cryogel.

When the fine colloidal suspension was spray-dried (with 5% w/v of dextrin added as a stabilizer, using a simple magnetic stirrer) instead of unidirectional freezing and subsequent freeze-drying, micron-size dextrin powder was obtained as seen in **Table 4.8** and **Figure 4.13**, whose number-averaged sizes were 1,863 and 1,869 nm for set 2 and set 3 polymer suspensions, respectively. Without the addition of dextrin, spray drying did not yield any dried powder. Therefore, the dextrin powder

encapsulated multiple core-shell particles. Next the spray dried powders were rehydrated to investigate the dispersed core-shell particles. The measured average sizes were around 148 and 269 nm for set 2 and set 3 suspensions, respectively. These values suggested that the coalescence and/or flocculation of the particle occurred during drying, and the extent was greater for the specimen made from set 3.

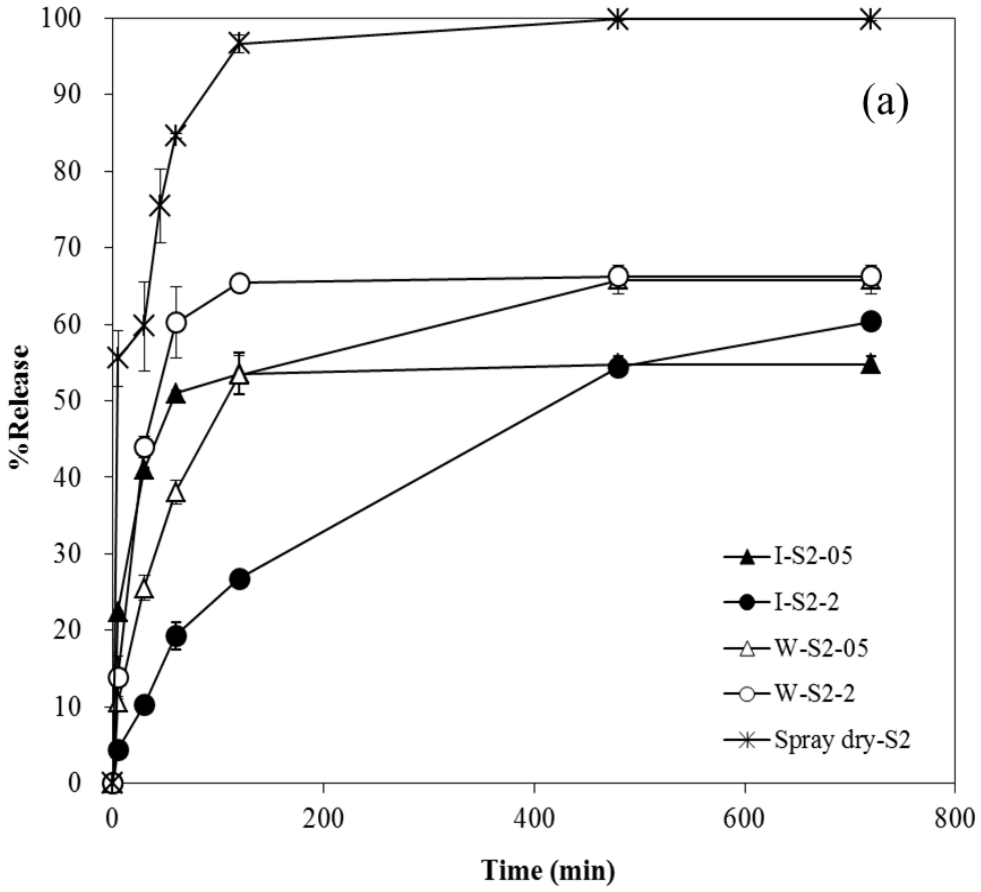
4.4.4 Effect of freezing rate and ratio of Carrageenan to NaCMC on the release behavior of curcumin from freeze-dried cryogels

Figure 4.13 compares the release patterns of encapsulated curcumin (actual and normalized patterns) in the case of polymer set 1 (S1) between freeze-dried cryogels (prepared at cooling rate of -0.5 and -2.0 ^{o}C/min, with and without incubation) and spray-dried microcapsules obtained from a similar colloidal suspension, as described in **Figure 3.3-3.4**. Freeze-dried cryogel specimens W-S2-05, W-S3-05, W-S2-2, and W-S3-2 were prepared without any incubation, whereas I-S2-05, I-S3-05, I-S2-2, and I-S3-2 all underwent incubation. For easier release pattern comparison, the actual release amounts have also been normalized by division with the ultimate release value at 12 hours. **Figure 4.14** is the same as **Figure 4.13** but for the case of polymer set 2 (S2). Generally, spray-dried microcapsules were found to exhibit faster release rates than all of their freeze-dried counterparts.

Between each freeze-dried pair, incubation tended to slow down the release rate. Though spray-drying successfully encapsulated the oil droplets, it did not produce rigid and strong membrane structure on the surface of the droplets. Therefore, curcumin could be released not only very fast but also more completely from the

matrix. The ultimate release at 12 hours was close to 99.9%. When we compared between polymer sets S2 and S3 without incubation at the cooling rate of -0.5 ^{o}C/min, W-S3-05 was found to exhibit a slower release rate than W-S2-05. A similar conclusion may be reached for the case of incubation when comparing I-S3-05 and I-S2-05. In fact the cooling rate -0.5 ^{o}C/min turned out to be more suitable for slow release in the case of polymer set S3 (Carrageenan:CMC ratio of 6:4). On the other hand, when we compared between S2 and S3 at the cooling rate of -2.0 ^{o}C/min, W-S2-2 was found to have a slower release rate than W-S3-2, and a similar conclusion is also valid when comparing I-S2-2 and I-S3-2. Therefore, the cooling rate of -2.0 ^{o}C/min turned out to be more suitable for slow release in the case of polymer set S2 (Carrageenan:CMC ratio of 4:6). When cold incubation was carried out, the release rate became even slower than the corresponding case of no incubation because more crosslinking was enhanced during the incubation period. In addition, the release rate of I-S3-2 and I-S2-2 approached first-order rate.

In short, slow controlled release of curcumin was achieved with set 2 polymer suspension when the cooling rate was sufficiently high at −2 °C/min with post-freezing incubation. It is logical that a higher gel fraction (specimen I-S2-2) leads to a slower release of curcumin (first-order release rate). It is obvious that the specimen I-S2-2 exhibited the slowest release rate and the longest release time. This is consistent with the report that freeze-drying is better than spray-drying for protecting sensitive substances (Abdelwahed et al., 2006). In short, it may be considered that unidirectional freezing followed by low-temperature incubation and freezing drying significantly influenced the formation of nano- and microstructure in the present cryogels and rendered them favorable for controlled release of curcumin.


(a)
%Release
Time (min)
0
10
20
30
40
50
60
70
80
90
100
0
200
400
600
800
I-S2-05
I-S2-2
W-S2-05
W-S2-2
Spray dry-S2

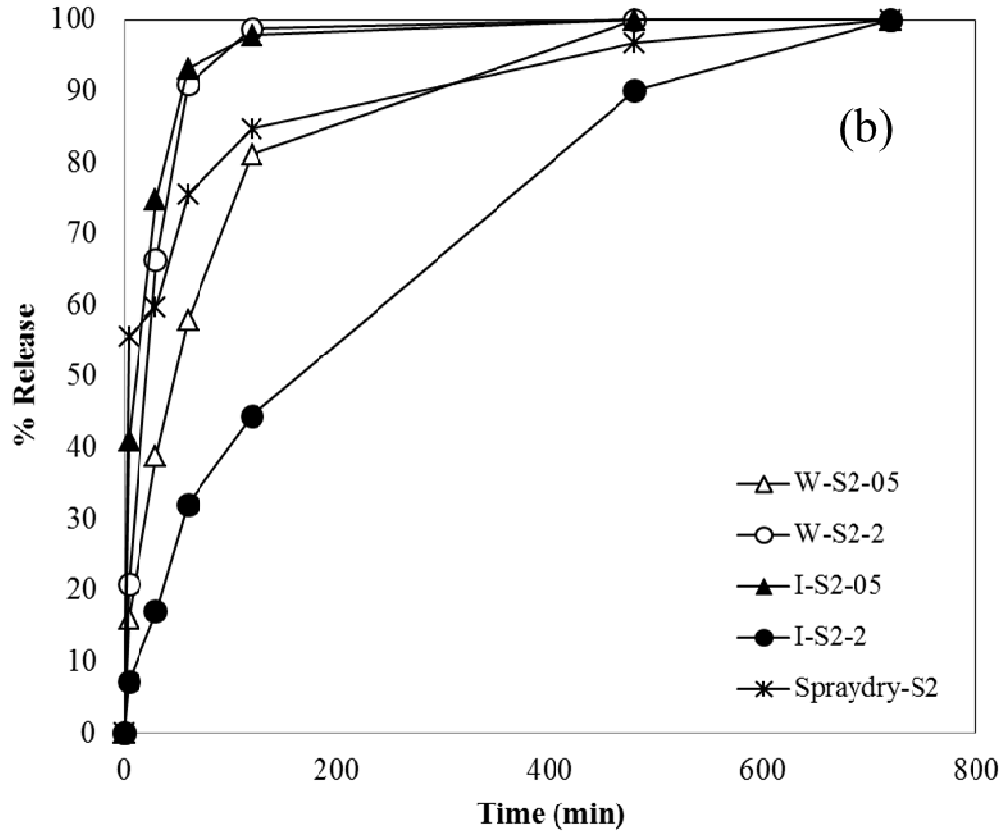
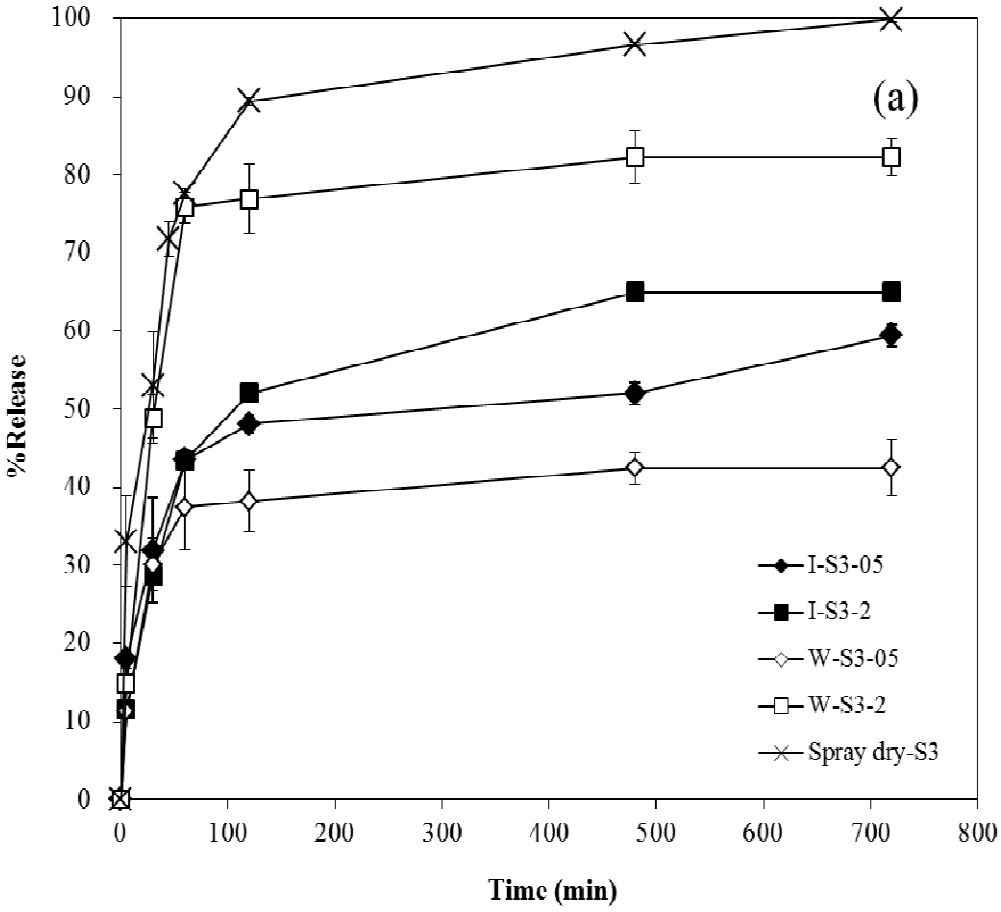


Fig. 4.13 Effect of the drying method on the release of actual and normalized patterns of curcumin from freeze-dried cryogels and spray-dried nanocapsules in a phosphate buffer solution with pH 7.4 for polymer set S2: (a) actual release curve, (b) normalized release curve.


(a)
%Release
Time (min)
I-S3-05
I-S3-2
W-S3-05
W-S3-2
Spray dry-S3
0
10
20
30
40
50
60
70
80
90
100
0
100
200
300
400
500
600
700
800

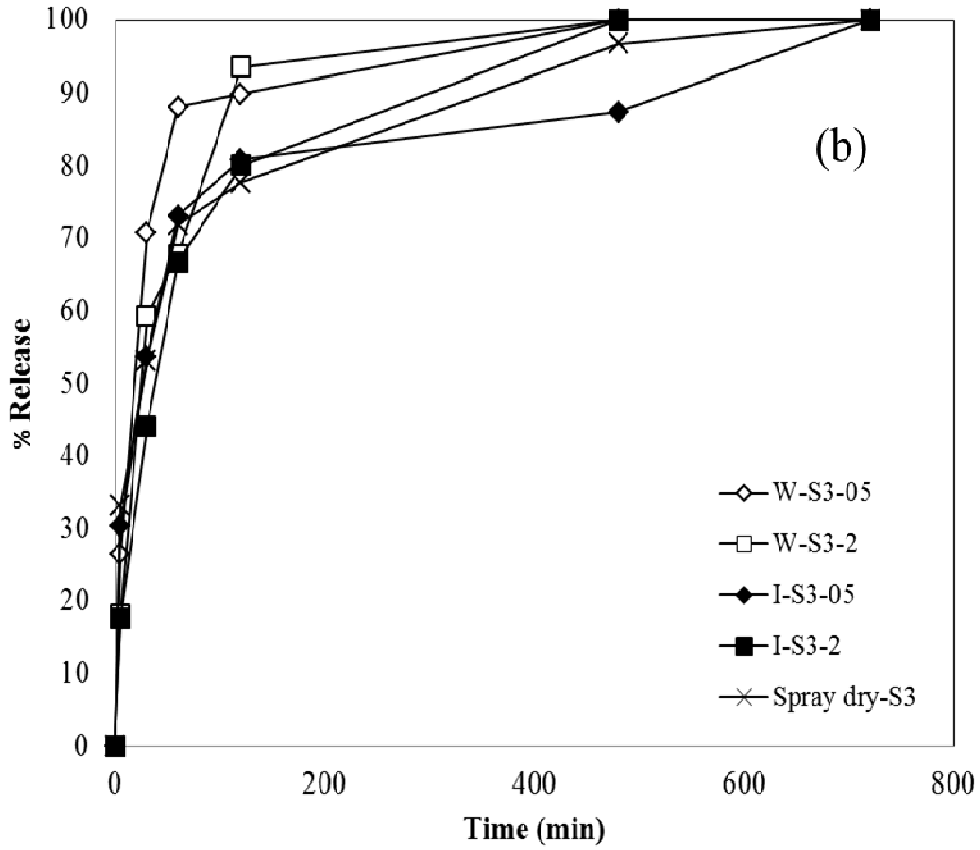


Fig. 4.14 Effect of the drying method on the release of actual and normalized patterns of curcumin from freeze-dried cryogels and spray-dried nanocapsules in a phosphate buffer solution with pH 7.4 for polymer set S3: (a) actual release curve, (b) normalized release curve.

4.5 Spray Drying of Fine Colloidal Suspensions

As shown in **Figure 3.5**, the author thought that it would be interesting to apply high pressure homogenization before spray drying the resulting fine colloidal suspension. However, it was found to be too viscous and the concentrations of O/W emulsion and polymer suspension had to be diluted by 10 times. When the fine colloidal suspension was spray-dried (with 5% w/v of dextrin added as a stabilizer, using a simple magnetic stirrer) instead of unidirectional freezing and subsequent freeze-drying, micron-size dextrin powder was obtained, whose number-averaged sizes were 1,863 and 1,869 nm for set 1 and set 2 polymer suspensions, respectively. Without the addition of dextrin, spray drying did not yield any dried powder. Therefore, it may be considered that the dextrin particles also contain multiple core-shell particles though we could detect only the size of spray-dried dextrin particles.

Figure 4.15 shows a SEM micrograph of the microstructure of the spray-dried microcapsules with and without incubation. Since the number-averaged sizes of core-shell particles in Table 4.8 ranged from 80 to 120 nm, most of them would be too small to be seen at this level of magnification. Nevertheless, it is more likely that the nano-sized particles were embedded in the dried porous matrix.

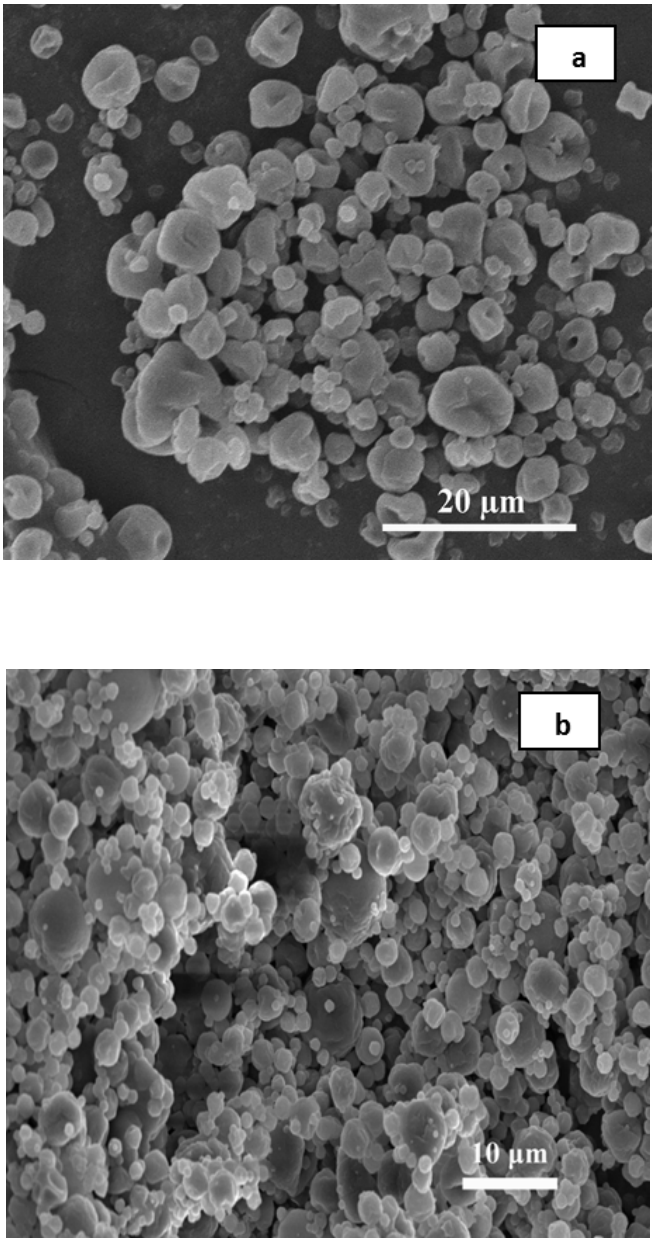


Fig. 4.15 SEM micrograph of spray-dried microcapsules

(a) without incubation, (b) cold incubation

CHAPTER V

CONCLUSIONS AND RECOMMENDATION

4.1 Conclusion

2.5.1 Characteristics of cryogel prepared via unidirectional freezing without incubation

A ternary system of chitosan, κ-carrageenan, and NaCMC based on cryotropic gelation has been developed for encapsulation of nano-emulsion of curcumin and experimentally investigated with respect to the suitable sol-gel formulation, the yield of gel fraction, the yield of encapsulation of the cryogel system, the swelling extent, particle size and zeta potential as well as the release behavior of curcumin from the freeze-dried cryogels. The main conclusions are as follows.

1. Not only the molecular weight of chitosan but the rate of cooling during freezing also had a significant effect on the encapsulation yield of curcumin. Highest MW of chitosan and most rapid cooling rate (-2 ^{o}C/min) resulted in higher yields, the other parameters being the same.

2. Both the rate of cooling and the pH of the buffer media had a significant effect on the swelling ratio. The swelling ratio decreased as either the cooling rate increased or the pH became less acidic.

3. Irrespective of the introduced oil phase composition, the controlled release of curcumin was achievable when the cooling rate was sufficiently high at -2 ^{o}C/min, especially when polymer suspension set 2 was used. Interestingly, either a burst release or a first order release was achievable simply by changing the freezing condition.

5.1.2 Characteristics of cryogel prepared via unidirectional freezing with cold incubation and of nanocapsules prepared via spray-drying

The effects of post-freezing incubation and concentration ratio of carrageenan to NaCMC on the physical properties and release behavior of the curcumin-loaded nanocapsules have been investigated. It can be seen from the experimental results that both of these factors showed significant effects on the yield of gel fraction, swelling extent, particles size and zeta potential as well as the curcumin release pattern, which can be attributed to changes in the nano- and microstructure of the cryogel. Specimen I-S2-02, which was made of polymer suspension set 2 at the most rapid freezing (-2 ^{o}C/min) with incubation, showed the slowest prolonged release rate of curcumin

The author applied to use high pressure homogenization to the colloidal suspension before spray drying. It found that the number-averaged sizes of spray dried powders were 1,863 and 1,869 nm for polymer suspensions set 2 and set 3, respectively. When the author rehydrated the spray dried powders to analyze the dispersed core-shell particles, the sizes were around 148 and 269 nm for set 2 and set 3 suspensions, respectively. For controlled release of curcumin, spray dried powders were found to exhibit faster release rates than all of their freeze-dried counterparts.

Between each freeze-dried pair, incubation tended to slow down the release rate. Though spray-drying successfully encapsulated the oil droplets, it did not produce rigid and strong membrane structure on the surface of the droplets. Therefore, curcumin could be released not only very fast but also more completely from spray-dried powder. The ultimate release at 12 hours was close to 99.9%.

4.2 Recommendations for future work

4.2.1 Suggestion for study on raising the ultimate release quantity of the prepared cryogel

The author has successfully prepared curcumin-containing cryogel using a ternary system of chitosan, κ-carrageenan, and NaCMC based on cryotropic gelation. However, more study and experiments must be carried out to raise the maximum released amount of curcumin significantly above the present 50% of the initial amount. Otherwise, the nano-capsules might not be totally effective because a big portion of the encapsulated curcumin remained trapped and could not be released for a long time. Therefore, the present process requires further development to improve the release profile, for exsample, by changing the kind of polymer suspension and the ratio of polymer suspension.

4.2.2 Suggestion for cosmetic application

To develop the present cryogel for cosmetic application, it is essential to reduce and control the particle size and determine the stability of nano-emulsion in the cryogel and then evaluate *in vitro and in vivo* skin permeation of the release curcumin.

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APPENDIX

PUBLICATIONS

International Journal

1. **Sowasod, N.**, Nakagawa, K., Tanthapanichakoon W., and Charinpanitkul, T. Development of encapsulation technique for curcumin loaded O/W emulsion using chitosan based cryotropic gelation. Materials Science and Engineering C 2012; 32: 790-798.
2. Nakagawa, K., **Sowasod N**., Charinpanitkul, T., Soottitantawat, A., Tanthapanichakoon, W. Encapsulation of curcumin loaded oil droplets by cryotropic gel formation from O/W emulsion. Procedia Food Science 2011; 1: 1973 – 1979.

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1. **Sowasod, N**., Nakagawa, K., Tanthapanichakoon W., and Charinpanitkul, T. Cryogel Based Oil Encapsulation for Controlled Release of Curcumin by Using a Ternary System of Chitosan, kappa- Carrageenan, and Carboxymethylcellulose Sodium Salt. Advanced Materials Research
2. **Sowasod, N.**, Nakagawa, K., Charinpanitkul, T., and Tanthapanichakoon W. Encapsulation of curcumin loaded oil droplets with chitosan based cryogel: Influence of freezing condition on nanocapsule properties. Food Science and Technology Research

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1. Nakagawa, K., **Sowasod N.**, Charinpanitkul, T., Soottitantawat, A., Tanthapanichakoon, W. Encapsulation of curcumin loaded oil droplets by cryotropic gel formation from O/W emulsion. 11th International Conference on Engineering and Food (ICEF), May 22 to 26, 2011, Athens, Greece, pp. 2171-2172, 2011.
2. Nakagawa, K., Nishimoto, N., **Sowasod N.**, Charinpanitkul, T., Soottitantawat, A., Tanthapanichakoon, W. Cryotropic Gel Formation for Food Nutrients Encapsulation-A controllable processing of hydrogel by freezing. 11th International Conference on Engineering and Food (ICEF), May 22 to 26, 2011, Athens, Greece, pp.519-520, 2011.
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4. Nakagawa, K., **Sowasod N**., Charinpanitkul, T., Soottitantawat, A., Tanthapanichakoon, W. Cryogel Based Oil Encapsulation: An attempt to control properties of core-shell nanoparticles by cryo-processing 18th International Symposium on Microencapsulation 2011, September 12 to 14, 2011, Antalya,Turkey. pp.198 - 200, 2011
5. Tanthapanichakoon W., Nakagawa, K., **Sowasod, N.**, and Charinpanitkul, T. Preparation of core-shell microparticles by cryotropic gelation of chitosan based

biopolymers. 2012 AIChE Spring Meeting, April 01 to 05, 2012, Houston, United States of America, 2012.

6. **Sowasod, N.**, Nakagawa, K., Tanthapanichakoon W., and Charinpanitkul, T. Cryogel Based Oil Encapsulation for Controlled Release of Curcumin by Using a Ternary System. International Symposium on Novel Nanotechnology and Biomaterials 2012 (NNB2012), December 5, 2012, Himeji, Japan, 2012.

7. **Sowasod, N**., Nakagawa, K., Tanthapanichakoon W., and Charinpanitkul, T. Cryogel Based Oil Encapsulation for Controlled Release of Curcumin by Using a Ternary System of Chitosan, kappa- Carrageenan, and Carboxymethylcellulose Sodium Salt. 2013 3rd International Conference on Key Engineering Materials (ICKEM 2013), March 8 to 9, 2013, Kota Kinabalu, Malaysia, 2013.

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