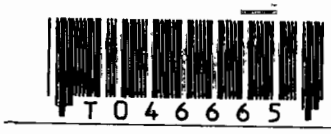
PREPARATION MICROSPHERE FROM PROTEIN SERICIN OF THAI SILK



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Special Project Title	The preparation of microsphere from protein sericin of Thai silk	
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Program	Polymer Science and Technology	
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Abstract

The objective of this project was preparation of microspheres from protein sericin of Thai silk. Protein sericin solutions were extracted from 3 types of cocoon; Nangnoi, Danchang and Kaw suphan, by using autoclave at 105 ^{0}C for 1 hr. The extracted sericin solution was frozen and then liophlization. Suspension and Emulsion technique were used for preparation microsphere. Several factors such as types of surfactant and oil, amount of glutaraldehyde, temperature and acid condition were investigated.

Microspheres received from each experiment were analyzed by using optical microscope. It was found that the suitable condition for forming microsphere was double emulsion technique. The optimum conditions were sericin 1 g, vegetable oil 5 ml with drug 0.282 g (piroxicam), Span 80 surfactant 0.1 ml and performed at 150 ^{0}C. Moreover, only sericin extracted from Nangnoi can prepared the microspheres under these conditions. Picture from Scanning Electron Microscope (SEM) indicated that surface of microspheres were compared of many small pores and non smooth. The average particle size of microsphere were around 200-800 μm.

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Chapter 1
Introduction

1.1 Introduction [1-6]

Protein-based materials afford many advantages over synthetic biodegradable polymers in applications of tissue engineering or wound healing. In tissue engineering applications, biomaterials are often intended to be seeded in-vitro with cells and maintained in aqueous culture medium for extended periods of time. During this time polymeric materials sensitive to hydrolysis will begin breaking down and an immediate change in physical and mechanical properties will result. Water stable protein-based materials may avoid physical change during processing while still providing for ultimate resorption when implanted into the body. The degradation products of protein-based implants are natural amino acids and peptides which cause no known toxicity. Additionally, proteins can be engineered to include amino acid sequence components that mediate cell adhesion and other cellular differentiation functions. We describe the physical and biological properties of synthetically engineered protein polymers in forms such as injectable gels, porous sponges, and durable coatings. The protein designs include protein structural elements from natural silk and elastin proteins. Protein polymers can be designed to resorb over various time periods after implantation by adjusting their amino acid sequences. Inclusion of amino acid sequences serving as protease cleavage sites can increase their specific proteolytic degradation by 10 fold. Implant studies have been used to evaluate material biocompatibility, resorption, and cell and tissue integration.

Silk sericin is a natural macromolecular protein derived from silkworm Bombyx mori. During the various stages of producing raw silk and textile, sericin can be recovered for other uses. Also, sericin recovery reduces the environmental impact of silk manufacture. Sericin protein is useful because of its properties. The protein resists oxidation, is antibacterial, UV resistant, and absorbs and releases moisture easily. Sericin protein can be cross-linked, copolymerized, and blended with other macromolecular materials, especially artificial polymers, to produce materials with improved properties. The protein is also used as an improving reagent or a coating material for natural and artificial fibers, fabrics, and articles. The materials modified with sericin and sericin composites are useful as degradable biomaterials, biomedical materials, polymers for forming articles, functional membranes, fibers, and fabrics.

Controlled drug delivery technology represents one of the frontier areas of science, which involves multidisciplinary scientific approach, contributing to human health care. These delivery systems offer numerous advantages compared to conventional dosage forms, which include improved efficacy, reduced toxicity, and improved patient compliance and convenience. Such systems often use macromolecules as carriers for the drugs. By doing so, treatments that would not otherwise be possible are now in conventional use. This field of pharmaceutical technology has grown and diversified rapidly in recent years. Understanding the derivation of the methods of controlled release and the range of new polymers can be a barrier to involvement from the non specialist. Of the different dosage forms reported, nanoparticles and microparticles attained much importance, due to a

tendency to accumulate in inflamed areas of the body [4-6]. Nano and microparticles for their attractive properties occupy unique position in drug delivery technology. Some of the current trends in this area will be discussed.

The term "microcapsule" is defined, as a spherical particle with the size varying in between 50 nm to 2 mm containing a core substance. Microspheres are in strict sense, spherically empty particles. However, the terms microcapsules and microspheres are often used synonymously. In addition, some related terms are used as well. For example, "microbeads" and "beads" are used alternatively. Sphere and spherical particles are also employed for a large size and rigid morphology. Due to attractive properties and wider applications of microcapsules and microspheres, a survey of the applications in controlled drug release formulations is appropriate.

Motivation

1. Nobody know about the valuable of sericin, though in Japan and China use sericin as an additive in cosmetic.

2. From many literatures and journals say that sericin has many advantages, valuable and useful. Therefore, the aim of this project is to modify sericin from Thai silk.

3. Increase value added for protein sericin.

4. Open a new option of protein sericin utility by recovery protein sericin from waste water of silk process.

1.2 Objective

To determine and find out a suitable condition for preparation microsphere particle of protein sericin from Thailand silk.

1.3 Scope

1.3.1. Preparation protein sericin powder by water extraction in autoclave and then freezing dryer.
1.3.2. Preparation of microsphere from 3 kinds of cocoon in Thailand such as Kaw Suphan, Danchang, Nangnoi.
1.3.3. Characterization on properties of sericin and microsphere.

1.4 Expected Result

1. To know about methods and technique for extraction sericin from silk cocoon
2. To know about methods and technique for preparation microsphere particle from protein sericin.

3. To know about suitable condition for preparation microsphere particle from protein sericin.

Chapter 2
Theory

2.1 Silk [7]

2.1.1 History of silk

Since it was first processed in China, silk thread has been used in embroidery for more than 5,000 years. Silk is an animal fiber made from the viscous fluid of the silkworm. Originally, silkworm cocoons were collected from trees. In 2640 B.C., a Chinese Empress discovered that if a silk cocoon was placed in hot water to soften the natural glue or sericin (holds the cocoon together), then a silk cocoon could be unwound and stretched into a long filament. Silk rapidly became a popular luxury fabric in the many areas accessible to Chinese merchants, because of its texture and lustre. Because of the high demand for the fabric, silk was one of the staples of international trade prior to industrialization .**[8]**

The silk spinnerets of *Bombyx mori* produce a delicate twin thread of silk and surround it with a protective cover of sericin during the spinning procedure. This principle, designed by mother nature and used successfully for millions of years, is now being copied and perfected to provide an innovative active ingredient for the cosmetic industry. Sericin is in fact a unique biopolymer with a unique structure leading to unique performance. The high hydroxyl amino acid content of sericin (approx. 46%) is of particular importance for the water-binding capacity which regulates the skin's moisture content.**[9]**

On the other hand, it has a unique carbohydrate moiety and a unique repetitive amino acid sequence which give sericin its high affinity for proteins resulting in a tightening, anti-wrinkle effect. The uniform film formed after the application of sericin results from its very high molecular weight. This substantive semiocclusive film persists even after washing, and protects the skin against harmful environmental influences. The sericin film leaves the skin with a smooth, silky feeling and substantiates several appropriate cosmetic effects. Its versatility makes sericin a very valuable natural ingredient for a wide range of cosmetic products.

2.1.2 Worm cycle of Bombyx mori - the silkworms' blog **[7]**

This is a photo-intensive, blow-by-blow description of my silkworm rearing

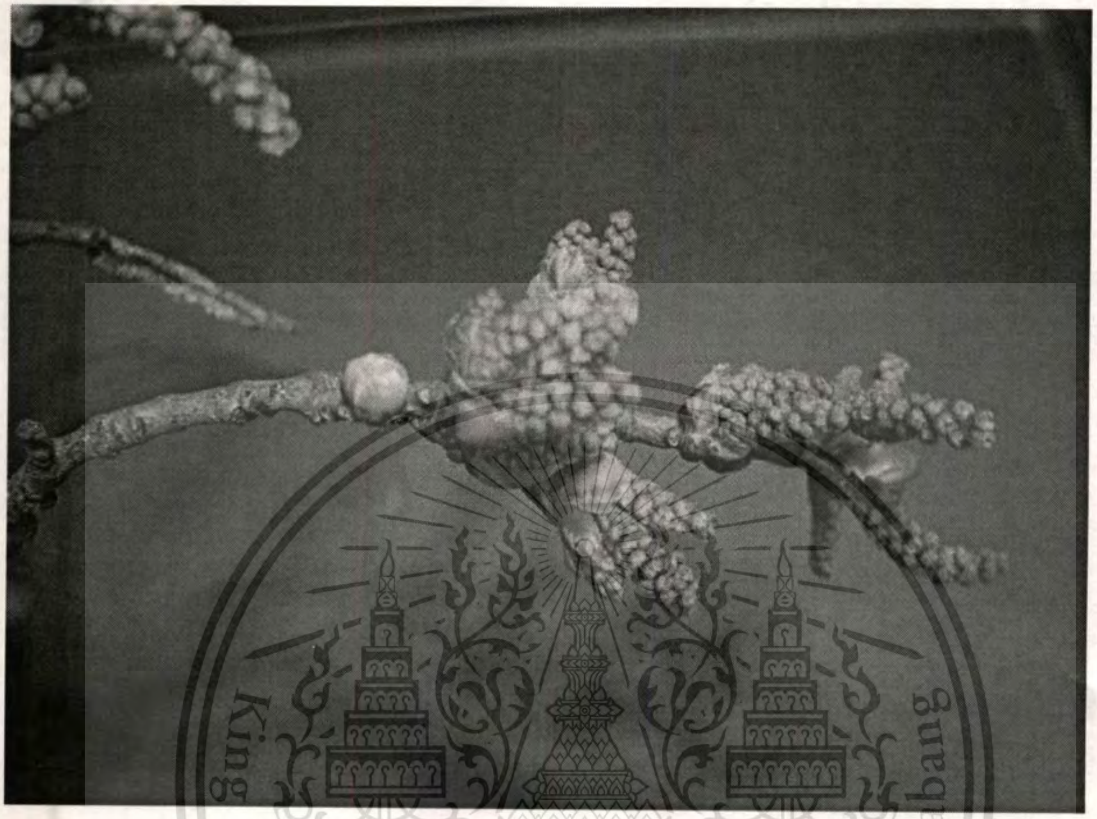


Figure 2.1 Taking the worm egg from mulberry tree [7]

When they growth ,worms have begun to spin. The worms stop eating and go into a rest, like when they are going to shed, but instead they dump out the undigested food in their gut, and start to wander and spin silk thread everywhere they go. The worm in the front has changed over and started to spin; the worm behind it hasn't yet.

Note how much smaller the worm in front is - they lose a lot of size when they make the change. They also develop a translucent yellowish shade that the camera can't quite pick up - they look a little waxy, and the greenish hue (caused by the mulberry leaves inside!) that they usually have, disappears.

Figure 2.2 Silkworm [7]

Figure 2.3 Silkworm start to spin up [7]

The worms will wander around, and find a space to spin. Ideally, they want a close environment; they will spin up in the leaves, if you let them. Their second choice is in the lid of the top of the tray. Either of these locations tends to lead to slightly irregular cocoons; it's best to put them into a dedicated space to spin. Using toilet paper tubes, this worm has begun spinning the "flossing" of his cocoon - this forms the framework that will support the cocoon, sort of like a hammock. This silk is slightly thicker than the reel able filament, and is not continuous. It is used in making spun silk thread, and is called **cocoon stripping, blaze, or keba.**

The worm will spin non-stop for up to three days before shiing its final skin and becoming a pupa. The worm waves back and forth to spin the flossing, but once it gets the framework set up, it will make a slow, deliberate figure 2.4 motion with its head, thousands and thousands of times.

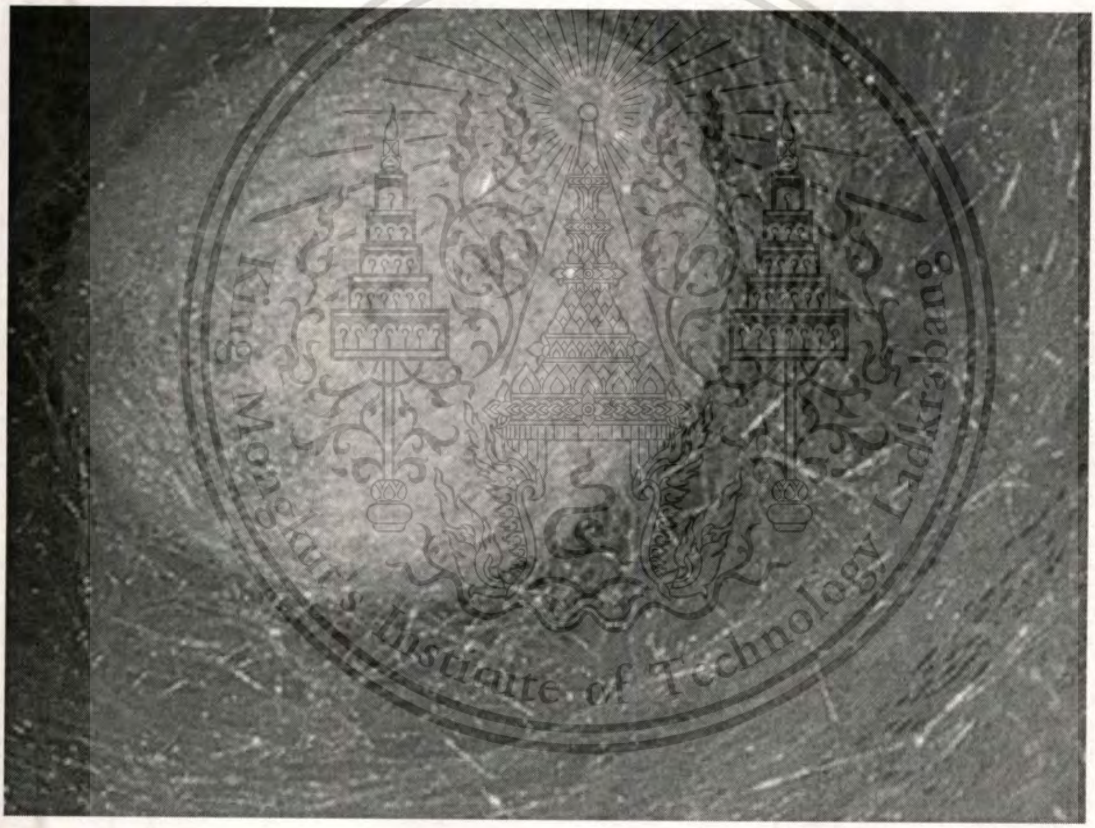


Figure 2.4 After a couple of hours, the cocoon is dense enough that you can no longer easily see the worm at work [7]

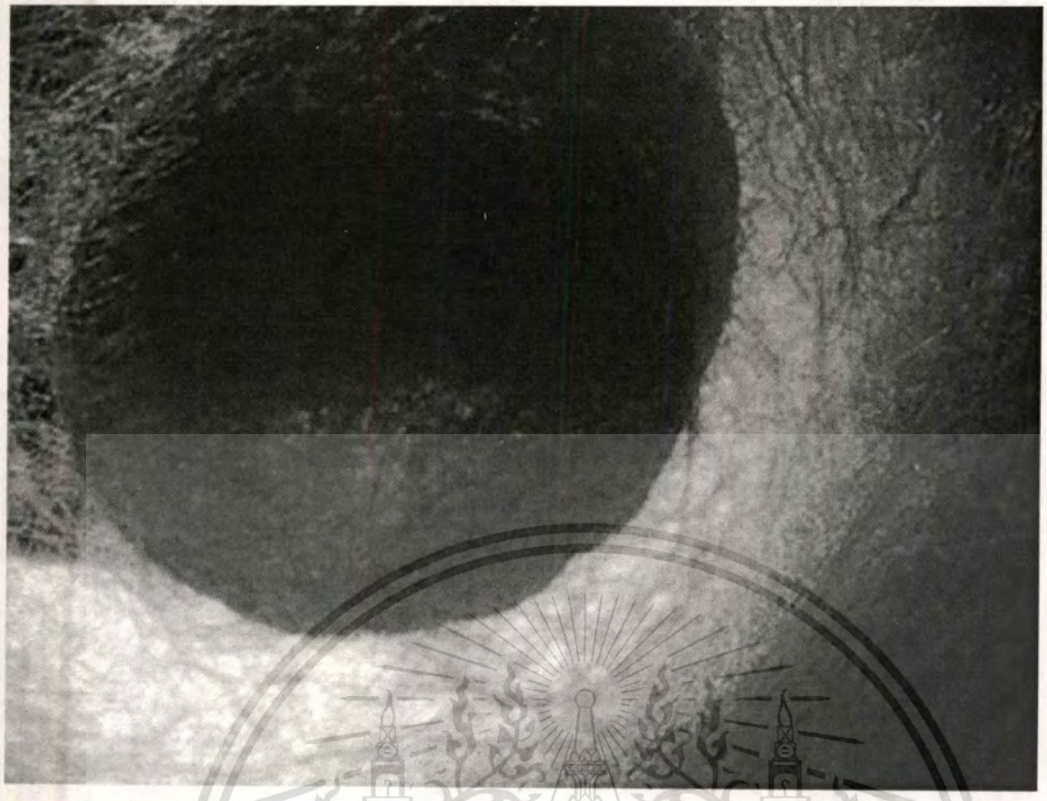


Figure 2.5 When you hold the cocoon up to a strong light, you can see through the cocoon. [7]

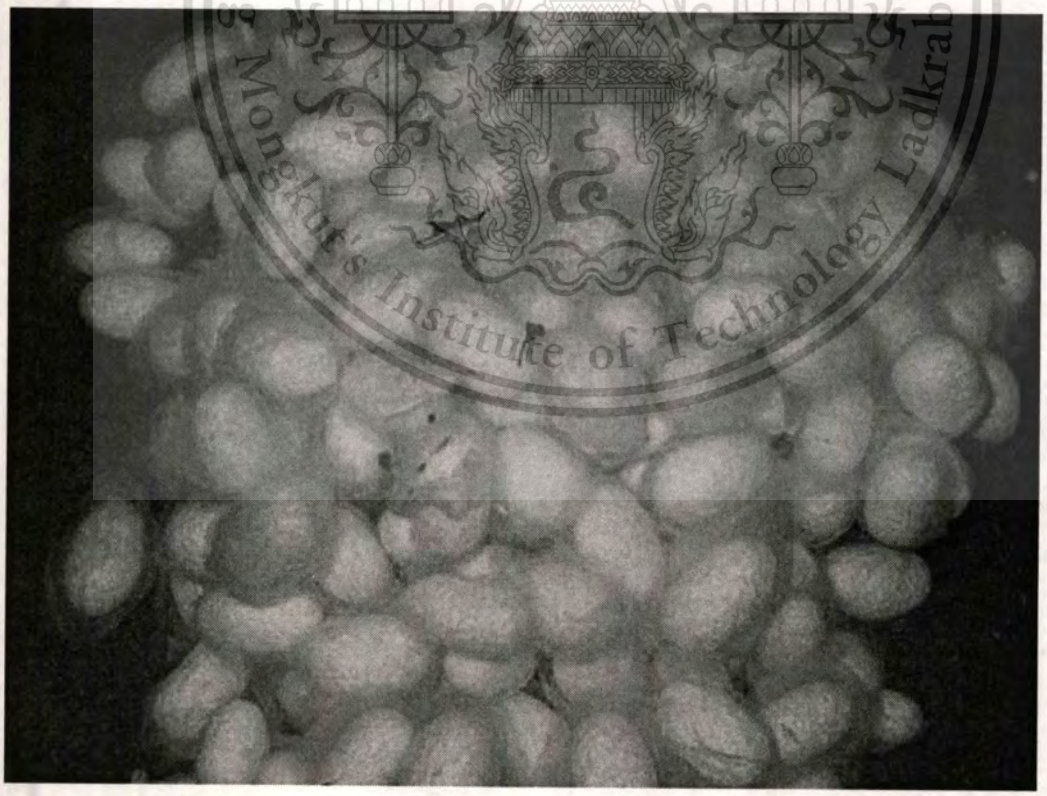


Figure 2.6 At least for several hour, it becomes totally opaque. [7]

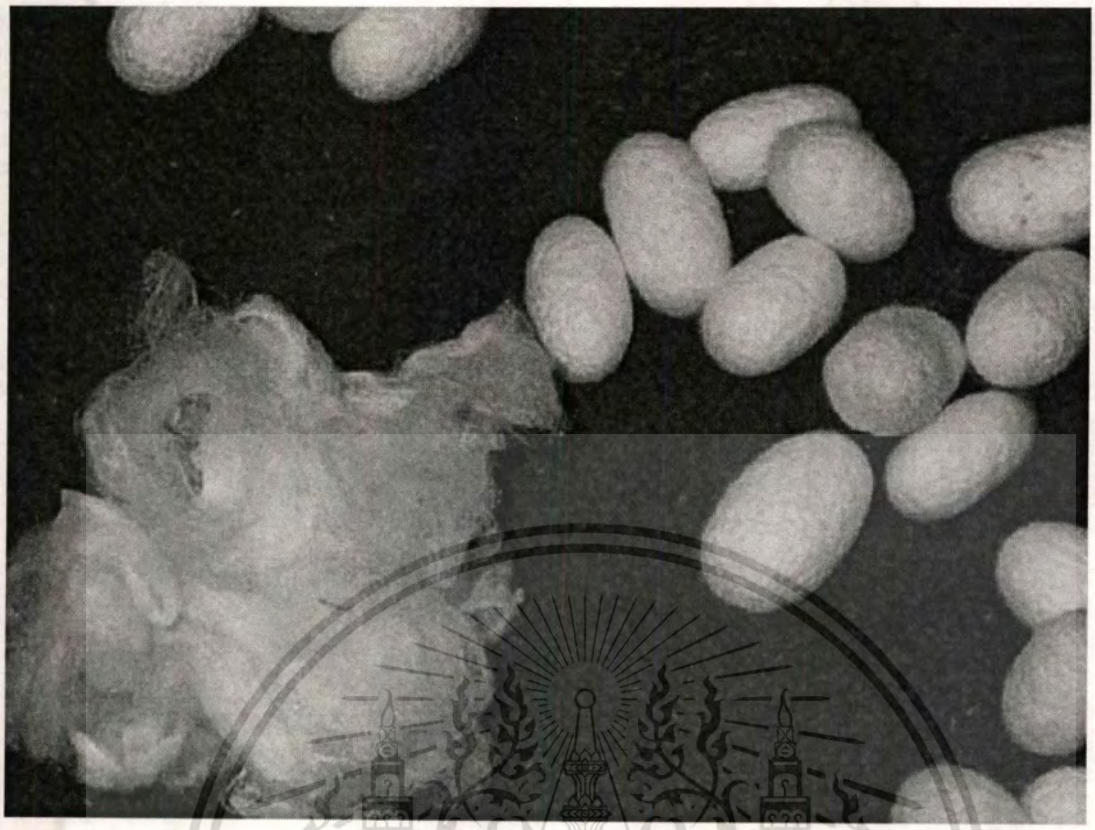


Figure 2.7 Reeling silk and cocoon [7]

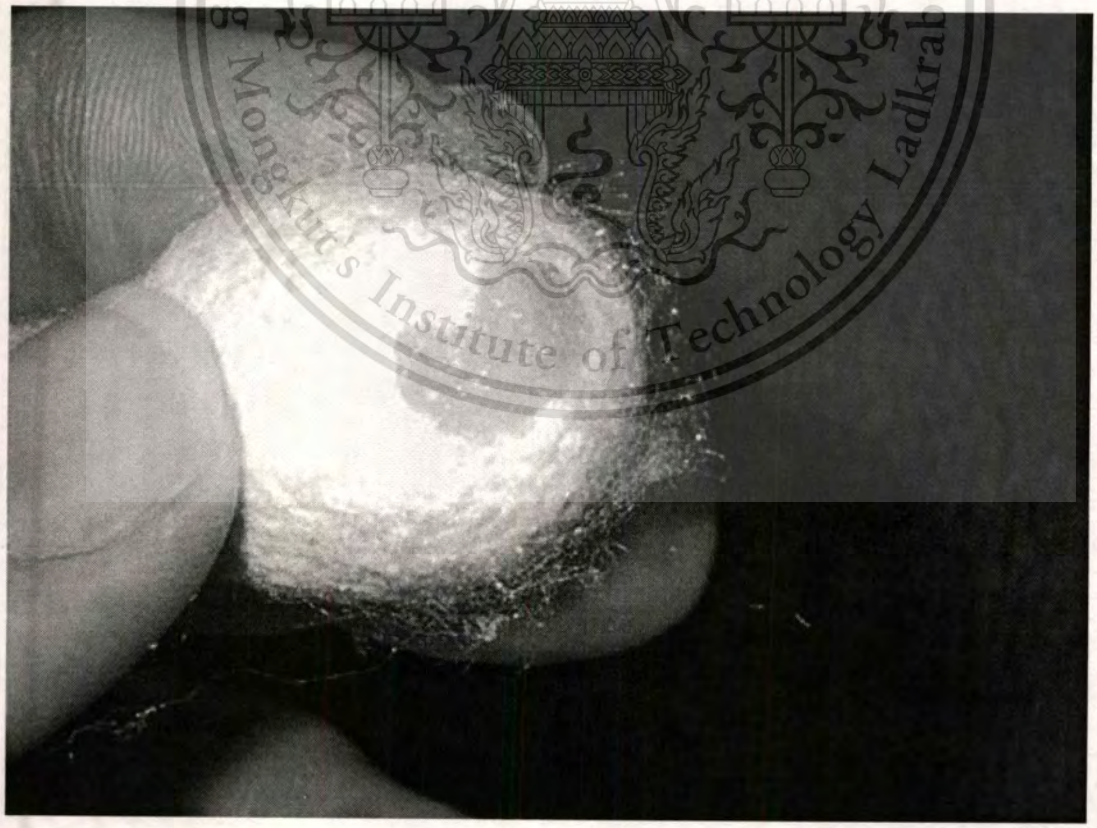


Figure 2.8 Finally cocoon from silk worm [7]

2.1.3 Silk Protein structure [10]

Silk is a kind of consecutive protein fibre, which is composed of two bunches of fibroin and sericin that covering outside.

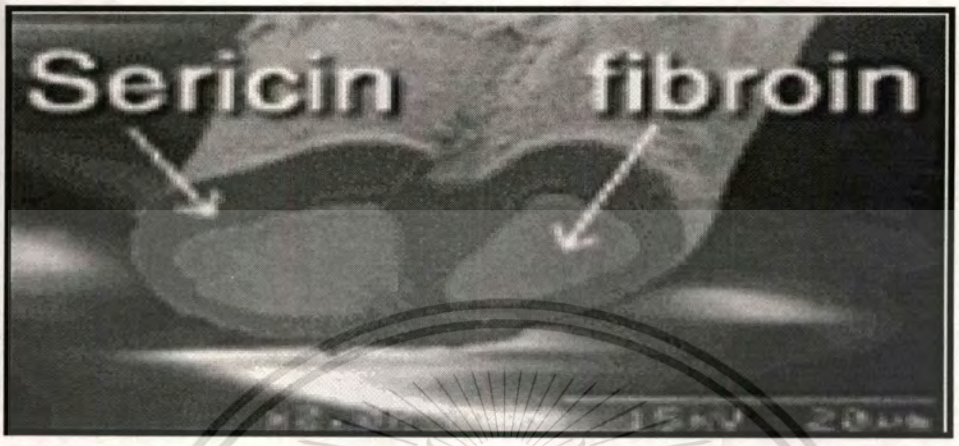


Figure 2.9 Show about 2 types of protein in silk [10]

Silk Principal Component Analysis:

- Fibroin	70—80 %
- Sericin	20—30 %
- Cere	0.4—0.8 %
- Hydrocarbon	1.2—1.6 %
- Pigment	0.2 %
- Ash	0.7 %

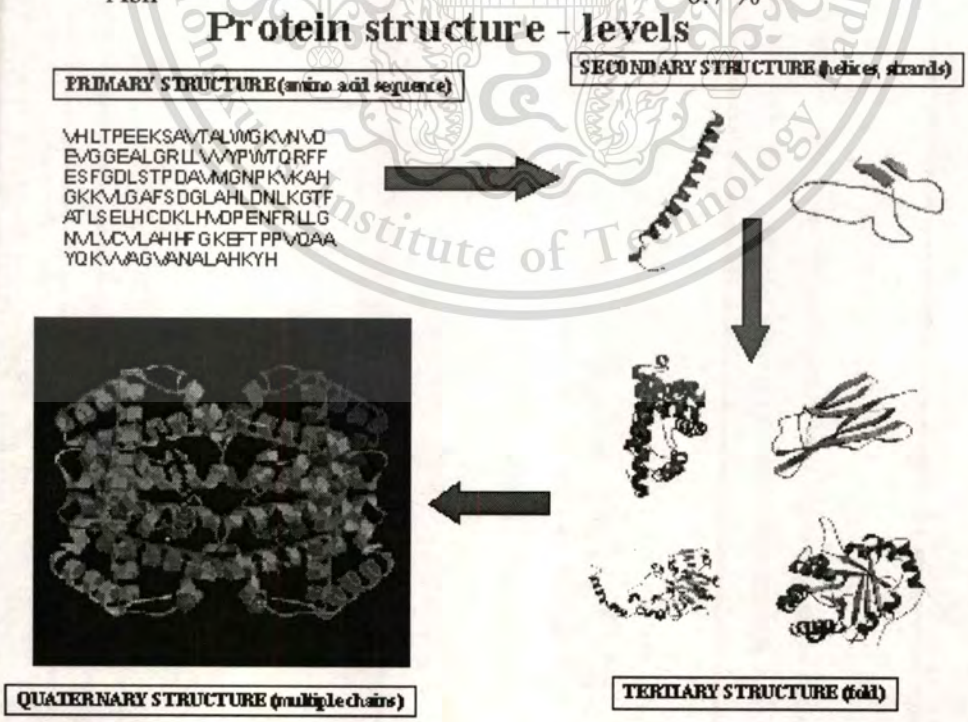


Figure 2.10 Protein structure **[11]**

Silk Structure

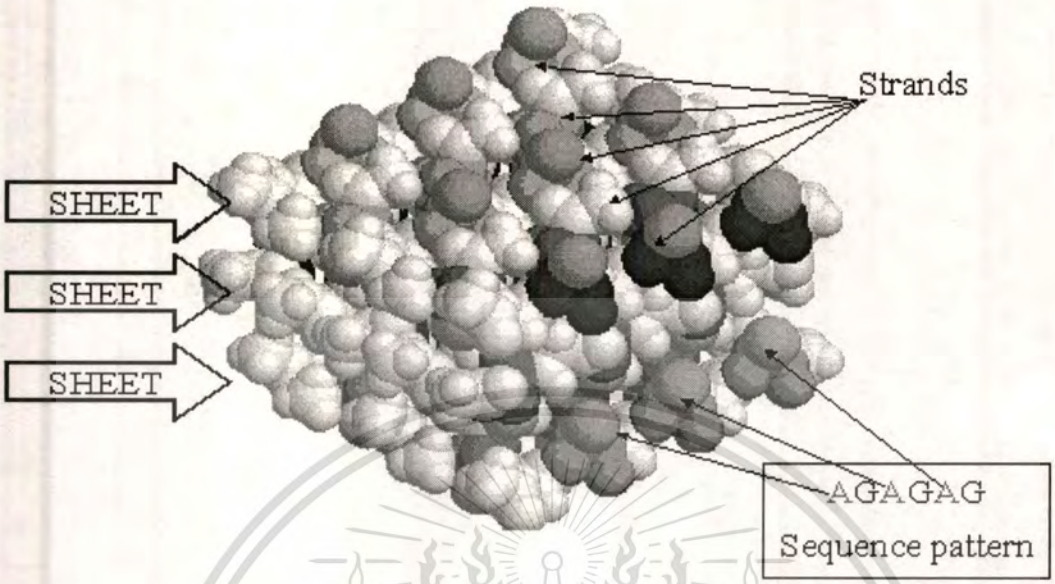


Figure 2.11 Silk structure **[12]**

2.1.4 Utilizable Character are :**[10]**

1. It is made from degummed silk by biotechnology, the total content of alanine, glycin and serine is above 85%. It's high nutritional material of obvious function.

2. Not only can glycine and serine be used as the essential materials of synthesizing protein in organism, but also as the predecessor of synthesizing the glutathione (GSH, SSG), which
plays a certain preventive function for heart disease and cancer. Meanwhile, the glycin also has the detoxication effects for some aromatics which can cause cancer. Its rich alanine also help promoting the metabolism of alcohol in human body and protecting liver. Tyrosine also have obvious effect on preventing the old from Alzheimer disease.

3. Silk amino acid is very easy to permeate the derma layer of skin and absorbed easily, nurturing skin very well. Due to its action in the enzyme metabolism, silk amino acid can keep and adjust wet, prevent skin from ultraviolet radiation, restrain splash generation, anti-aging for skin.

2.2 Protein sericin [13]

Sericin is a high molecular weight, water-soluble glycoprotein isolated from silk. The raw material for the manufacture of sericin is a top-quality silk grège or raw silk yarn ,obtained exclusively from the domesticated mulberry silkworm (*Bombyx mori*). The manufacturing process, which is carried out in accordance with GMP standards, is carefully designed to preserve this versatile biopolymer in its native state as far as possible. One characteristic feature of sericin is its opalescence, which results from its light scattering effect.

Sericin has a unique affinity with other proteins which allows it to bind very effectively to the keratin of skin and hair, to form a multifunctional protective film.

2.2.1The efficiency of silk sericin

a. Skin moisturizing

The moisturizing effect of sericin has been determined on the inner forearm of six human volunteers by measuring the transient thermal transfer (TTT). This highly sophisticated technique makes it possible to evaluate the degree of hydration within the epidermis. One hour after a single application, the moisture content had been increased by 7%. After two hours the moisture content had been increased by 6% and after three hours by 5%.

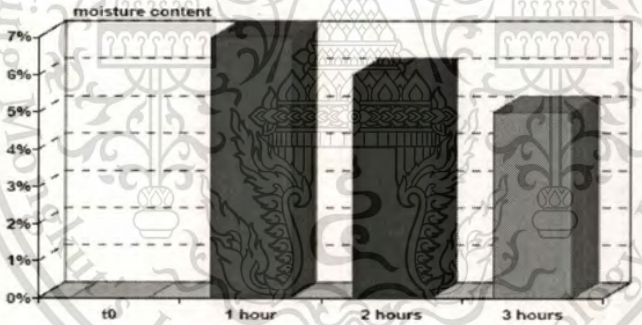


Figure 2.12 Moisturizing effect of sericin [13]

b. Anti-irritant properties

The anti-irritant effect of a single application of a gel containing 5% sericin has been evaluated on the SLS-treated inner forearm of ten human volunteers. The skin irritation was assessed using the laser Doppler technique. Sericin has been shown to have very good anti-irritant activity. Four hours after the application, the sericin treated skin areas approached the base line level and the skin irritation index was 82% lower than that with the placebo.

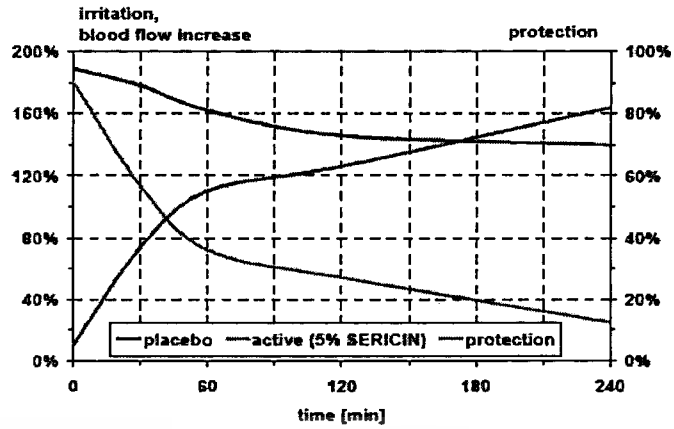
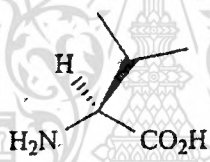


Figure 2.13 The anti-irritant effect of a single application of a gel containing 5% sericin [13]

2.2.2 Advantages of essential amino acids [14]

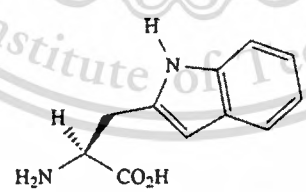
1. Valine



[15]

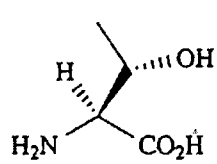
-Abundant in muscles, energy source in muscles; deficiency may affect the myelin covering of the nerves; very high levels of valine can cause symptoms such as a crawling sensation on the skin, as well as hallucination .

2. Tryptophan



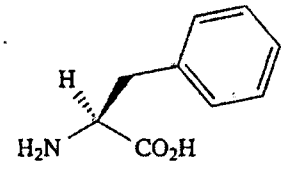
-Precusor of niacin, serotonin, melatonin (from serotonin)

3. Threonine



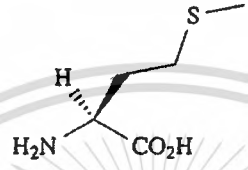
-Abundant in heart, skeletal muscles and central nervous system ; assist for collagen and elastin formation , immune system.

4. Phenylalanine



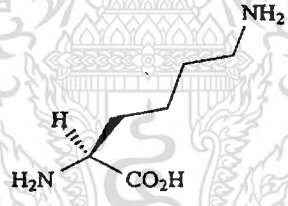
-Used in elevating the mood since it is so closely involved with the nervous system; helps with memory and learning.

5. Methionine



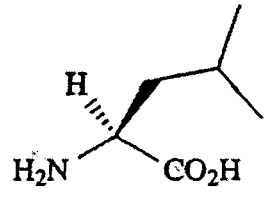
-Lipotropic that important in the process of methylation, breakdown of fats and thereby prevents the build up of fat in the arteries; precursor for cysteine; needed by the body to manufacture creatine monohydrate, a compound essential for energy production and muscle building.

6. Lysine



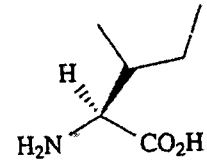
-Required for growth and bone development; need to produce antibodies, hormones, enzymes, collagen formation as well as repair of tissue.

7. Leucine



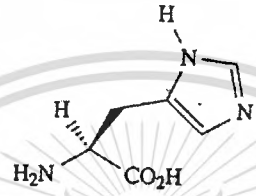
-Helps with the regulation of blood sugar levels, the growth and repair of muscle tissue such as bones , skin and muscles ,growth hormone production, wound healing as well as energy regulation.

8. Isoleucine



-Promote muscle recovery; needed for the formation of hemoglobin; assists with regulation of blood sugar levels.

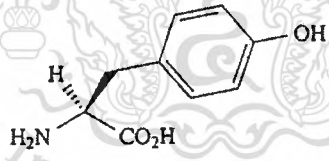
9. Histidine



-Precursor of neurotransmitter histamine; need for growth and for the repaired of tissue, as well as the maintenance of the mylin; required for the manufacture of both red and white blood cells, and helps to protect the body from damage caused by radiation and in removing heavy metals from the body.

2.2.3 Advantages of non-essential amino acids [14]

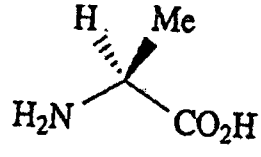
1.Tyrosine



[15]

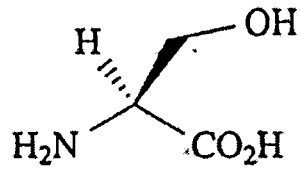
-Abundant in insulin, enzyme papain; precursor-phenylalanine; precursor of epinephrine, dopamine, thyrosine. Suppresses the appetite and reduces body fat, produces skin and hair pigment, maintains the proper functioning of the thyroid as well as the pituitary and adrenal glands.

2.Alanine



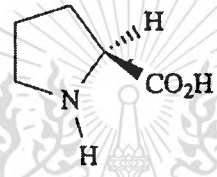
-Required for the metabolism of glucose and tryptophan.

3.Serine



-Required for the metabolism of fat, tissue growth and the immune system as it assists in the production of immunoglobulins and antibodies. It is a constituent of brain proteins and nerve coverings and is also important in the formation of cell membranes, involved in the metabolism of purines and pyrimidines, and muscle synthesis. It is also used in cosmetics as a skin moisturizer.

4.Proline

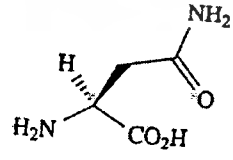


-Improves skin texture and aids collagen formation and helps contain the loss of collagen during aging; gelatine contains 25% praline.

5.Cystine

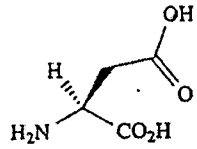
- Required for proper vitamine B6 utilization and is also helpful in the healing of burns and wounds.

6.Asparagine



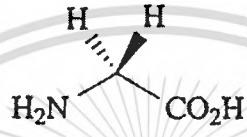
-Required by the nervous system to maintain equilibrium.

7.Glutamic acid



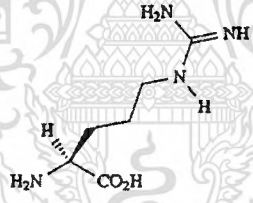
-Brain fuel; precursor of GABA(gamma aminobutyric acid) neurotransmitter; used in the body to balance the acid / alkaline level and is also the basis or building blocks of RNA and DNA.

8.Glycine



-Required to build protein in the body and synthesis of nucleic acids, the construction of RNA as well as DNA.

9.Arginine



-Extremely useful in enhancing the immune system, and it increases the size and activity of the thymus gland; it is used to release in sulin and in the pituitary gland it is a component of human growth hormone, increases HGH levels in the blood; is used in sexual stimulants; vasodilator; most "natural Viagra" formulations.

Table 2.1 Amino acids analysis of Silk Protein (g/100g) **[16]**

Amino Acid Name	Fibroin	Sericin	Structural Character
Aspartic [Asp]	2.60	17.35	Acidity Amino Acid, Polar Side Chains
Threonine [*Thr]	1.23	8.75	Neutral Amino Acid, Polar Side Chains
Serine[Ser]	13.74	30.14	Neutral Amino Acid, Polar Side Chains
Glycine[Gly]	40.55	8.15	Neutral Amino Acid
Alanine[Ala]	32.52	3.06	Neutral Amino Acid
Glutamic[Glu]	2.32	5.55	Acidity Amino Acid, Polar Side Chains
Valine[* Val]	3.33	3.08	Neutral Amino Acid
Isoleucine [* Ile]	0.92	0.67	Neutral Amino Acid
Leucine [* Leu]	0.68	1.37	Neutral Amino Acid
Tyrosine[Tyr]	11.78	4.85	Neutral Amino Acid
Phenylalanine[*Phe]	1.41	0.43	Neutral Amino Acid
Lysine [*Lys]	0.57	3.29	Alkaline Amino Acid, Polar Side Chains
Histidine [His]	0.38	1.54	Alkaline Amino Acid, Polar Side Chains
Arginine [Arg]	0.96	4.61	Alkaline Amino Acid, Polar Side Chains
Proline [Pro]	0.41	0.44	Neutral Amino Acid
Cystine [Cys]	0.26	0.39	Neutral Amino Acid
Methionine [* Met]	0.15	0.08	Neutral Amino Acid
Tryptophan [* Trp]	0.41	0.56	Neutral Amino Acid

Note that (*)Essential Amino Acids (Polar Side Chains Structure)

Table 2.2 Component of amino acid in Protein Sericin **[16-17]**

Amino acid	Ab	Content(g/100g) % *	Content(g/100g) % **
1.Serine	Ser -	30.14	33.40
2.Aspartic	Asp-	17.35	16.70
3.Glutamic	Glu -	5.55	4.40
4.Glycine	Gly -	8.15	13.50
5.Threonine	Thr-	8.75	9.70
6.Lysine	Lys -	3.29	3.30
7.Tyrosine	Tyr -	4.85	2.60
8.Arginine	Arg -	4.61	3.10
9.Alanine	Ala -	3.06	6.00
10.Valine	Val -	3.08	2.80
11.Histidine	His -	1.54	1.30
12.Leucine	Ieu -	1.37	1.10
13.Isoleucine	Ileu -	0.67	0.70
14.Phenylalanine	Phe -	0.43	0.50
15.Tryptophan	Trp -	0.56	0.20
16.Proline	Pro -	0.44	0.70
17.Cystine	Cys -	0.39	0.20
18.Methionine	Met -	0.08	0.04

Ref* **[16]** http://www.hth.com.cn/tiansi/html/eng/egsjj.htm

Ref** **[17]** http://www.smiss.cn/smissEnglish/silk%20derivatives/silkraw.asp

Table 2.3 Component of amino acid in Protein Sericin from **Kaw suphan [18]**

Amino acid	**Ab**	**Content(g/100g) %**
1.Serine	Ser -	22.47
2.Aspartic	Asp-	0.10
3.Glutamic	Glu -	5.43
4.Glycine	Gly -	8.40
5.Threonine	Thr-	8.19
6.Lysine	Lys -	3.32
7.Tyrosine	Tyr -	0.06
8.Arginine	Arg -	4.49
9.Alanine	Ala -	3.43
10.Valine	Val -	2.92
11.Histidine	His -	1.37
12.Leucine	Leu -	1.29
13.Isoleucine	Ileu -	0.73
14.Phenylalanine	Phe -	0.12
15.Tryptophan	Trp -	0.02
16.Proline	Pro -	0.49
17.Cystine	Cys -	0.99
18.Methionine	Met -	16.26

Table 2.4 Component of amino acid in Protein Sericin from **Danchang [19]**

Amino acid	**Ab**	**Content(g/100g) %**
1.Serine	Ser -	16.01
2.Aspartic	Asp-	0.07
3.Glutamic	Glu -	5.06
4.Glycine	Gly -	6.48
5.Threonine	Thr-	5.92
6.Lysine	Lys -	3.20
7.Tyrosine	Tyr -	0.00
8.Arginine	Arg -	3.42
9.Alanine	Ala -	2.96
10.Valine	Val -	2.92
11.Histidine	His -	1.24
12.Leucine	Leu -	1.19
13.Isoleucine	Ileu -	0.73
14.Phenylalanine	Phe -	0.08
15.Tryptophan	Trp -	0.02
16.Proline	Pro -	0.56
17.Cystine	Cys -	0.94
18.Methionine	Met -	12.15

Table 2.5 Component of amino acid in Protein Sericin from **another research [20-21]**

Amino acid	**Ab**	**Kirimura , 1972***	**Sole Silk****
1.Serine	Ser -	30.10	30.31
2.Aspartic	Asp-	16.80	17.50
3.Glutamic	Glu -	10.10	5.69
4.Glycine	Gly -	8.80	8.55
5.Threonine	Thr-	8.50	8.40
6.Lysine	Lys -	5.50	3.36
7.Tyrosine	Tyr -	4.90	4.92
8.Arginine	Arg -	4.20	4.63
9.Alanine	Ala -	4.00	3.10
10.Valine	Val -	3.10	3.09
11.Histidine	His -	1.40	1.56
12.Leucine	Leu -	0.90	1.33
13.Isoleucine	Ileu -	0.60	0.65
14.Phenylalanine	Phe -	0.60	0.41
15.Tryptophan	Trp -	0.02	0.02
16.Proline	Pro -	0.50	0.42
17.Cystine	Cys -	0.30	0.30
18.Methionine	Met -	0.10	0.07

Ref * **[20]** http://www.fao.org/docrep/x2099e/x2099e-03.htm

Ref** **[21]** http://www.silkmelody.com/news_details.asp?nid=15

2.3 Glutaraldehyde [22]

GA (1,5-pentanedial $OCH(CH_2)_3CHO$) was synthesised about 1908. Health concerns about formaldehyde in the 1970s led to increasing GA usage. It is widely used as a; - hardener in X-ray developer solution, (acts on the gelatine in the film);cold sterilant for endoscopes in hospital theatres; in dentistry and by vets; for farm housing at a 50% solution, e.g. chicken sheds;

Before 1962 the only satisfactory fixative for electron microscopy was buffered osmium tetroxide. This preserves cellular structure by combining with lipids, especially in membranes, and by insolubilizing some proteins without coagulation, but it is expensive and toxic, penetrates tissues extremely slowly, and extracts much protein and RNA. With the introduction of glutaraldehyde **[23-24]** electron microscopists had a more rapidly penetrating fixative that thoroughly insolubilized proteins and was cheap enough to deliver by vascular perfusion.

Glutaraldehyde has fairly small molecules, each with two aldehyde groups, separated by a flexible chain of 3 methylene bridges. It is $HCO\text{-}(CH_2)_3\text{-}CHO$. The potential for cross-linking is obviously much greater than with formaldehyde because it can occur through both the -CHO groups and over variable distances. In aqueous solutions, glutaraldehyde is present largely as polymers of variable size. There is a free aldehyde group sticking out of the side of each unit of the polymer molecule (Fig. 2.14), as well as one at each end. All these -CHO groups will combine with any protein nitrogens with which they come into contact, so there is enormous potential for cross-linking, and that is just what happens (Fig. 2.15). There are also many left-over aldehyde groups (not bound to anything) that cannot be washed out of the tissue.

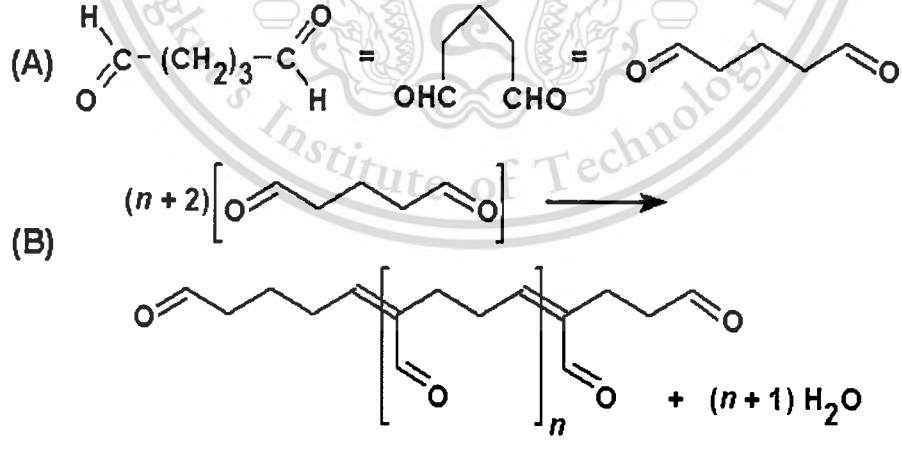


Figure 2.14 **(A)** Three representations of a molecule of monomeric glutaraldehyde. **(B)** Polymerization reaction of glutaraldehyde, showing an aldehyde side-chain on each unit of the polymer **[22].**

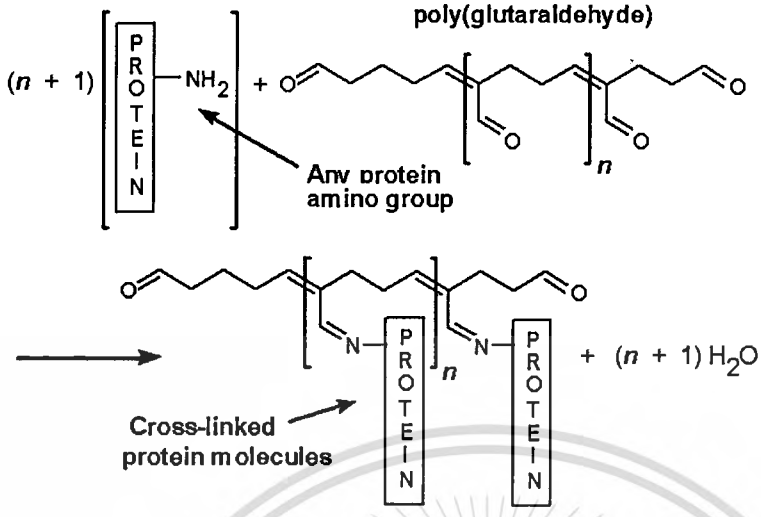


Figure 2.15. Reaction of poly(glutaraldehyde) with amino groups of proteins **[22].**

Practical aspects of glutaraldehyde fixation **[24]**

Five important points must be remembered when using glutaraldehyde as a fixative for light or electron microscopy.

1. If it's to be any use as a fixative, especially for electron microscopy, the glutaraldehyde solution must contain the monomer and low polymers (oligomers) with molecules small enough to penetrate the tissue fairly quickly. This means you must buy an "EM grade" glutaraldehyde (25% or 50% solution), not a cheaper "technical" grade. The cheaper stuff, which is for tanning leather, consists largely of polymer molecules too large to fit between the macromolecules of cells and other tissue components.

2. The chemical reaction of glutaraldehyde with protein is fast (minutes to hours), but the larger molecules, especially the oligomers, penetrate tissue slowly. A rat's brain left overnight in a buffered glutaraldehyde solution and sliced the next day shows a colour change and harder consistency to a depth of 2-3 mm. Objects fixed for a few hours in glutaraldehyde are no longer osmotically responsive.

3. The free aldehyde groups introduced by glutaraldehyde fixation cause various problems. These include non-specific binding of proteinaceous reagents, notably antibodies, and a direct-positive reaction with Schiff's reagent). The free aldehydes must be removed or blocked by appropriate histochemical procedures, as

described in textbooks **[25-26],** before attempting immunohistochemistry, lectin histochemistry, the Feulgen reaction of periodic acid-Schiff staining on glutaraldehyde-fixed material.

4. The thorough cross-linking of a glutaraldehyde-fixed specimen impedes the penetration of fairly large paraffin wax molecules. This makes for difficult cutting and peculiar differential shrinkage artifacts within the specimen. You can stain mitochondria nicely in cells surrounded by obviously abnormal spaces. This is an exaggeration of the inadequacy of formaldehyde and osmium tetroxide as fixatives to precede paraffin, and it also highlights the shortcomings of predominantly coagulant fixatives, which preserve the micro-anatomy well but destroy or displace little things like organelles. Fortunately, plastic monomers penetrate glutaraldehyde-fixed tissue adequately. It has been shown that they do not enter every crevice **[27],** but there is enough support to allow the cutting of ultrathin sections for electron microscopy.

5. Immunohistochemistry, which requires as many intact amino acid side-chains as possible, is severely impaired by glutaraldehyde fixation. Nevertheless, clever people have generated antibodies to individual amino acids, that are glutaraldehyde-bound to protein. These allow the detection of soluble amino acid neurotransmitters such as glutamate, GABA and even glycine in presynaptic axon terminals in glutaraldehyde-perfused central nervous tissue **[28-29].** Extensive cross-linking also results in the loss or severe reduction of most histochemically demonstrable enzymatic activities, though several are retained after brief fixation **[22-29].**

2.4 Microsphere particles [30-31]

"microspheres" is generally employed to describe colloidal particles which are substantially spherical and have a diameter in the range 10 nm to 2 mm. Particles having a diameter of less than 1 nm are sometimes called "nanoparticles". Microspheres made from a very wide range of natural and synthetic polymers have found use in a variety of biomedical applications. They can be labelled with markers (labels or sensing devices) and transported through various media both in-vitro and in-vivo. The labels may be chemical fluorescent, magnetic or radioactive and thus they may, by appropriate sensing equipment, be observed when in use. The sort of applications for which microspheres have been used are diagnostic screening, cell separation, immunoassays, studies of phagocytosis and blood flow, studies of cell motility, haemoperfusion and extracorporeal therapy, drug delivery devices, targeted drug delivery, cell encapsulation and endovasular embolisation.

An important property which microspheres must possess for biomedical applications is biocompatibility. They should be as resistant as possible to attack from the immune system in-vivo. Further, for many applications it is important that the microspheres be biodegradable and/or resorbable in the body once their function has been discharged. Also, in other cases, they should be small enough for easy introduction into the body.

For these reasons, naturally occurring polymer materials such as proteins have

been the subject of much study for the preparation of microspheres. Nanoparticles as small as 100 nm can be prepared from, for example, albumin using certain preparation techniques and this is very useful for, among other things, injectable preparations.

Because of their biocompatability some proteins have been used in making coatings for artificial prostheses which will be introduced into the human body and therefore in contact with body fluids. As with microspheres any such coating should be as resistant as possible to attack from the immune system and furthermore should not be thrombolytic i.e. should cause only minimal platelet activation.

A number of methods are known for preparing protein microspheres and films and protein coatings for prostheses but certain drawbacks are associated with them all. For example, a well-known method of preparing protein microspheres is suspension cross-linking. In this process an aqueous solution of protein is added to an immiscible liquid or oil phase. Droplets of protein are dispersed by high speed stirring and then hardening or stabilization of the droplets to form microspheres is brought about by heating of the suspension to, for example, a temperature above 80°C. or alternatively by chemical cross-linking employing a cross-linking agent such as glutaraldehyde. Various methods of preparation of albumin microspheres by the suspension cross-linking technique.

A disadvantage of preparing microspheres by the suspension cross-linking technique is that it is difficult to produce microspheres less than 500 nm in size, although nanospheres of about 100 nm diameter have been prepared using high power ultra-sonication. A further disadvantage is that the cross-linking agents used are often toxic which is not conducive to biocompatability.

2.5 Emulsion based preparation [32]

Emulsion stabilization, also called "emulsion crosslinking", is a frequently used method for the preparation of protein- a polysaccharide-based microcapsules and microspheres. This method can be used for the encapsulation of soluble or insoluble liquids, or solid agents. Release of encapsulants is mainly by diffusion, erosion or dissolution.

The main advantages of this technology are the flexibility in controlling the degree of stabilization and the small particle size that can be obtained. Disadvantages include the costs and effort related to removal of oil phase and the loss of encapsulant during processing.

Spray drying of emulsions is another technology for production of microcapsules with emulsions as a starting point. More details about the technology of spray drying can be found in the corresponding Tech Center.

Emulsion stabilizations are a technique based on single or double emulsions. Since many biopolymers are generally water-soluble, two systems can be distinguished when biopolymers are used as wall material:

Hydrophilic active ingredients single water-in-oil (w/o)-emulsions are employed if the active ingredient is water-soluble. In this case an aqueous biopolymer solution containing the active ingredient is emulsified in a hydrophobic phase like vegetable oil or organic solvent (Figure 2.16). When the desired droplet size is obtained, the matrix material is stabilized by crosslinking. Then, the oil phase is removed by washing with solvents like hexane and the particles are isolated. The particles can either be dried to obtain a powder, or used as slurry.

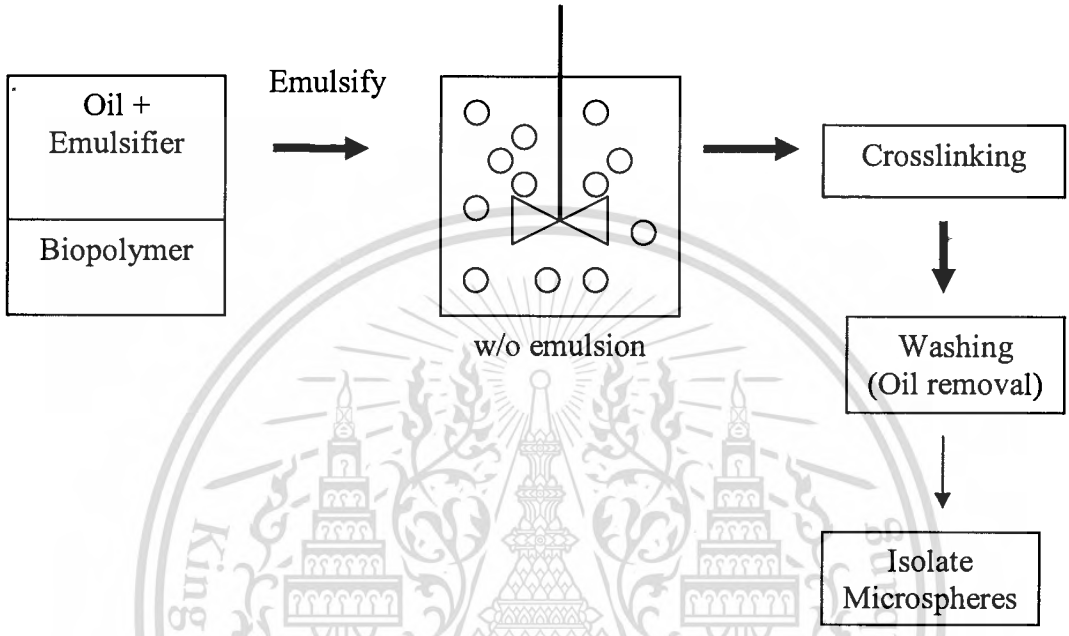


Figure2.16 Processing scheme for microsphere-preparation by single emulsion technique **[32]**.

Hydrophobic active ingredients double oil-water-oil (O/W/O)-emulsions can be used if the active ingredient is hydrophobic. The active ingredient is first added to an oil phase. This oil phase is then emulsified in the aqueous biopolymer phase to form an O/W/O-emulsion. Common challenge with emulsion stabilisation for hydrophobic ingredients is retention of the core; often lost occur during the encapsulation process (Figure 2.17).

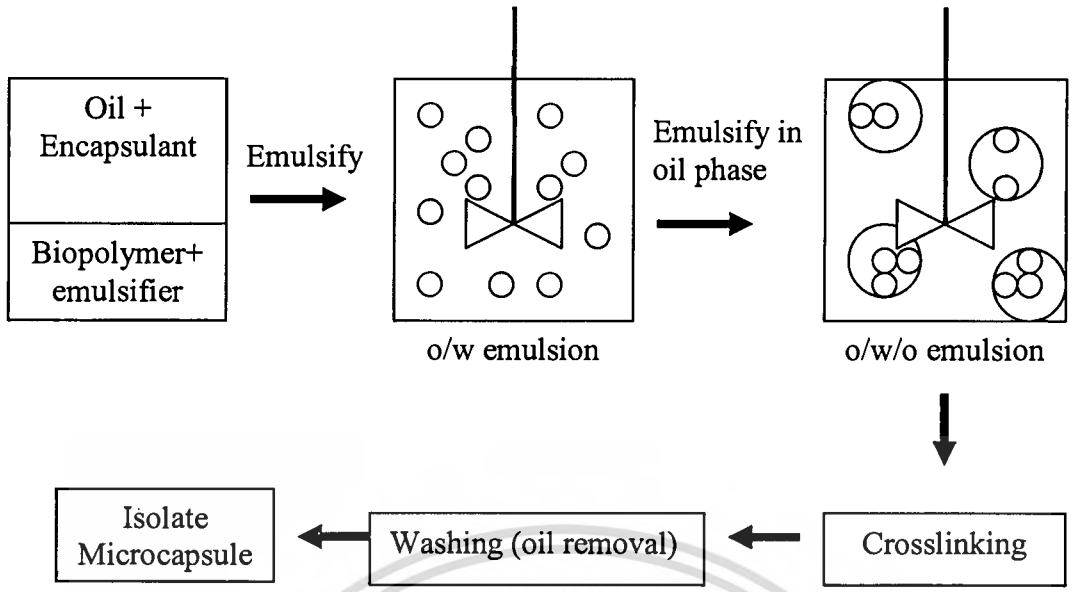


Figure 2.17 Processing scheme for microspere-preparation by double emulsion technique [32].

Control parameters biopolymers are usually stabilized in order to keep the particles intact (e.g. prevent the particles from dissolving in water), and controlling the release rate. Different methods can be applied such as chemical, thermal, or enzymatic crosslinking. Both nano- and microparticles can be formed, depending on several parameters:

- Ratio droplet phase/suspension medium(R)
- Stirring speed (N)
- Viscosity of the suspension medium(v_m)
- Viscosity of the droplet phase(v_d)
- Surface tension(γ)
- Surface concentration(C_x)
- Vessel diameter(D_v)
- Stirrer diameter(D_s)

In the equation below, the different parameters on the final particle size are given:

$$d \propto K \frac{D_v R_v d_\gamma}{D_s N_{v_m} C_x}$$

Where d is the diameter of the particle, and K a constant depending on equipment parameters

Different biopolymers can be used for the preparation of microparticles by emulsion stabilization. Polysaccharides such as dextran, derivatives of starch and cellulose, chitosan, and different carbohydrate-based hydrocolloid are frequently used. They have the advantage of being abundant, relatively, and biodegradable. They

possess some functionality for derivatization. An emulsifier is required to emulsify the aqueous phase in the oil phase.

Proteins are also abundant, relatively inexpensive, and biodegradable, but moreover they have extremely good emulsifying properties and a lot of functional groups available for modification. In general, proteins are suitable for microsphere preparation if they are (partly) soluble in water. Besides the soluble proteins (e.g. gelatin, albumin casein, and whey protein), also less water-soluble proteins have shown to be suitable for microsphere preparation under certain conditions, including soluble wheat gluten, zein, soy protein, pea protein, and potato protein.

Albumin is by far the most investigated natural polymer in microparticles preparation by emulsion stabilization.

Applications

- Drug delivery systems
- Encapsulation of fragrances for laundry applications
- Encapsulation of ingredients for food applications
- Encapsulation of pesticides

2.5.1 Comparision between Emulsion and Suspension Polymerization [33]

1. Emulsion Polymerization

   -*Emulsion Polymerization* is a process in which polymer is generated inside micelles formed by a surfactant (anionic, cationic or neutral) and initiated by a water-soluble initiator. Monomer transfers from monomer droplets to micelles by diffusion. The final product is a latex with a very fine and narrow particle size (<1 μm^3). Nanocomposites can be obtained by ion exchange of cationic surfactant (located in polymer particle surface) into clay layers dispersed in water.

2. Suspension Polymerization

   -*Suspension Polymerization* is a process in which equilibrium between breakup and coalescence/agglomeration of droplets/particles is established using strong agitation and suspending agents. Agitation provides (a) shear stress needed for the droplet/particle breakup and (b) flow needed for homogeneous distribution of droplets/particles in the polymerization mixture. Suspending agents have two functions: (a) reduce the interfacial tension at the droplet surface, and (b) create a protective layer around the droplet/particle to protect against coalescence/agglomeration. Several types of suspending agents are used, including polyvinyl alcohol, cellulose ethers and finely divided inorganic powders. The final product is a suspension of grains from 10 to 300 μm in diameter with a porous internal structure determined by suspending agents and agitation. Nanocomposites can be obtained by using as stabilizing

agents clays previously exchanged with organic cations containing reactive groups (e.g. azo or vinyl).

2.6 Microcapsules [34]

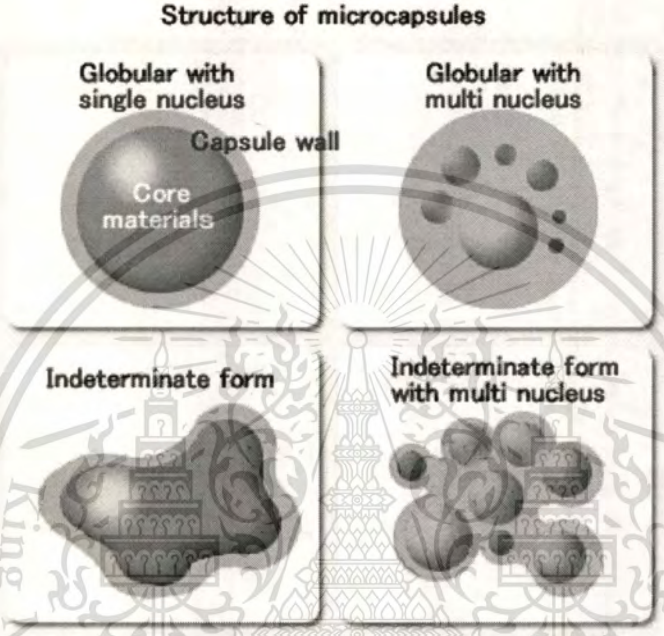


Figure 2.18 Structure of microcapsules **[34]**

In microencapsulation, the "core material" for a particular purpose is sealed in minute shells, measuring anywhere from a few μm to a few hundred μm, and then dispersed in a matrix. Thus the core material is protected against contaminants, damage, deterioration, and chemical reaction with other materials. The material is discharged either by breaking the capsule wall all at once or by letting the material gradually penetrate through the wall.

Microencapsulation is a remarkable technology, which is easy to handle and offers several advantages with respect to protecting the environment. As it comes into increasing use, it can be expected to contribute to the development of new technology and new products.

Microcapsules have four main functions

1. Preventing the active ingredient from deterioration

The membrane of the capsule protects the active ingredient it contains---the core material---against degradation, by insulating it from external moisture, light, and oxygen.

2. Improvement of workability

Because the membrane segregates its contents, capsules can contain and mix different, mutually reactive ingredients, and preserve them for a long time. Even liquids can be prepared in what appears to be a powder or particles. This makes a material easily workable.

3. Release the core materials when needed

One method of releasing the core material is to break the membrane by applying pressure or heat, so that the core material is released all at once. This method is appropriate for pressure-sensitive copying paper, adhesives, and aroma impregnated printing.

4. Gradual release (Gradual release of the core materials)

Another method is to have the core material penetrate the membrane so that it is released moderately and gradually, at a speed that can be controlled. This method is useful for medicines, aromatic substances, and agricultural chemicals such as fertilizers. For the last of these, microencapsulation makes it possible to use less of the core material, and disperse it more infrequently, thus saving resources.

2.6.1 Types of Microencapsulation Technology [34]

The microencapsulation technologies most widely used can be roughly categorized in three different types or "formulations," each of which includes a wide variety of methods. They are listed in the table below. Three Bond has products that apply three of these methods: in situ polymerization, phase separation from water solution, and (in prototype) surface polymerization.

Table 2.6 Type of Microcapsules technology [35]

	Formulation	Overview of formulation	Sample wall material	Sample use
Chemical formulation	Surface polymerization	Polymerizing the core material on a dispersion intermediary surface	Polyurethane, polyamide, and polyurea	Thermal-sensitive papers, pressure-sensitive copying papers, adhesives, and agricultural chemicals
	In Situ polymerization	Supplying monomer from either inside or outside of the core material and polymerizing it on the surface of the core material	Urea, PVA, and melamine	Cosmetics, pressuresensitive copying papers, inks, adhesives, and perfumes
	Submerged curing coating	Dripping a polymer solution containing the core	Alginic acid and gelatin	Medications and perfumes

	method	material from an orifice into a bath of the curing agent		
Physical and chemical formulation	Phase separation from a water solution	Separating the phases of a polymer solution containing the core solution by applying an electric charge	Gelatin	Pressure-sensitive copying papers, perfumes, adhesives, medications, and display materials
	Phase separation from organic solvent	Separating the phases of a polymer organic solvent containing the core material by adding nonaqueous solvent	Ethylcellulose	Medications and enzymes
	Submerged drying	Enclosing drops of a water solution containing the core material by a polymer solution, and drying by a method such as heat or decompression	Polystyrene, gelatin, and PVA	Medications
	Dissolution decentration cooling	Coating the core material with a substance that dissolves with heat but solidifies at room temperature	Paraffin and polyethylene	Medications, perfumes, and feed
Mechanical formulation	Spray drying	Dispersing or spraying the core material into a polymer solution and making it into particles, and then drying the solution	Gelatin, starch, PVA, and cellulose	Food, medication, agricultural chemicals, and cosmetics
	Air-suspension coating	Suspending the core material on a fluidized bed and coating it by spraying the material of capsule wall on it	Polymers, alumina, and carbon	Medications and agricultural chemicals
	Dry blending	Physically energizing the particles of core material and raw wall material, and bonding the wall material to the particles of the core material	Nylon and acrylic materials	-

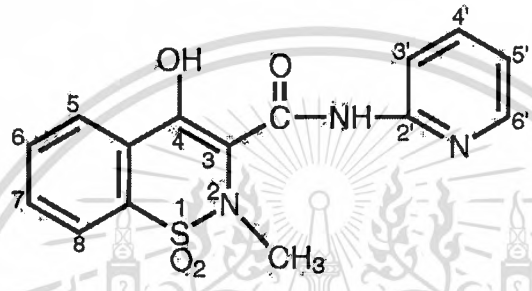
Reference[35]:: Seminar text "Encapsulation technology of fine particles and its application and development" (2001)

2.7 Piroxicam [36]

The chemical name for piroxicam is 4-hydroxyl-2-methyl- *N* -2-pyridinyl-2 *H* -1,2-benzothiazine-3-carboxamide 1,1-dioxide.

Piroxicam occurs as a white crystalline solid, sparingly soluble in water, dilute acid and most organic solvents. It is slightly soluble in alcohol and in aqueous solutions. It exhibits a weakly acidic 4-hydroxy proton (pKa 5.1) and a weakly basic pyridyl nitrogen (pKa 1.8).

The molecular weight of piroxicam is 331.35. Its molecular formula is $C_{15}H_{13}N_3O_4S$ and it has the following structural formula:



[36]

2.8 Literature Surveys

- **Yamada and Fuwa [37]** prepared a membrane from sericin. This membrane was capable of resolving racemic mixtures. Sericin solution (10% sericin) was mixed with diglycidyl ether (0.38 M), diethylenetriamine (0.14 M), and distilled water, and the mixture was cast on a plate and allowed to stand for 48 h at room temperature. Then the temperature was raised to 85 ^{0}C for 2 h. The sericin membrane obtained was immersed in a mixture of glutaraldehyde (0.1%), sulfuric acid (1%), and sodium sulfate (20%) for 24 h. The resulting cross-linked filter membrane resolve racemic mixtures. This ability was apparently associated with the chiral conformation of the amino acids residues in sericin.

- **Yoshikawa et al [38]** produced the gel material by mixing agar or agarose with sericin of 20 kDa average molecular weight can separate ether–alcohol mixtures. To form the gel, sericin powder is mixed with agar or agarose, sodium azide, and deionized water at 60 ^{0}C, and the solution is cast on a plate for 24 h at room temperature. The agar/agarose-sericin film is a porous gel film that absorbs water. The film contains from 0.1% to 60% sericin and can withstand pressures of 0.01–2 kgf/cm^2. The agar/agarose-sericin gel film can be used for separating methyl butylether (MTBE) from a mixture of MTBE and alcohol. Blended gel films made of agar/agarose and sericin or polyoxyethylene and hydroxyalkyl cellulose are also good separating materials for ether–alcohol mixtures, especially mixtures of MTBE and Methanol.

- **Kwang Yong Cho and coworkers [39]** use silk sericin reacted with activated poly(ethylene glycol) (PEG) to obtain self-assembled SS nanoparticles. The aliphatic and aromatic hydroxyl groups of serine and tyrosine residues as the reaction sites in SS were clarified by amino acid analysis and ^{1}H NMR spectroscopy, respectively.

- **Jose Maria Bielza De Ory [40]** prepared water in oil nano-emulsions by condensation (low-energy) methods. These nano-emulsions are formed with a surfactant or surfactant mixture, an oil component consisting of hydrocarbons, esters or their mixtures and an aqueous component. The surfactant concentration can vary from 3 to 15wt% while the oil component ranges from 50 to 95 wt% and the aqueous phase from 2 to 25 wt%. The droplet diameters of these nano-emulsions are comprised between 60 and 100 nm and some of them show stability higher than one year. These nano-emulsions can be prepared by condensation methods such as stepwise addition of the oil component to the water-surfactant mixture applying mild agitation (manually) or with vortex stirrer at about 3000 rpm. They can be also prepared by stepwise addition of water to the oil-surfactant mixture applying the same type of mild agitation.

- **Wheatley et al [41]** studied a mild alginate/polycation microencapsulation process, as applied to encapsulation of bioactive maromolecules such as proteins. The protein drugs were suspended in sodium alginate solution and sprayed into 1.3% buffered calcium chloride to form cross-linked microcapsules, large (up to 90%) losses of encapsulation species were encountered, and moderate to strong protein-alginate interactions caused poor formation of capsules. As a result, a diffusion-filling technique calcium alginate microcapsules were formed by spraying 10 ml of the sodium alginate solution into 250 ml of buffered 2-[4-(2-hydroxyethyl)piperazin-1-

yl]ethane sulfonic acid (HEPES) chloride (13 MM HEPES, 1.3% $CaCl_2$ pH 7.4) from a 20 ml plastipack syringe through a 22 G needle.

- **Goosen and coworkers [42]** attempted to mimic the properties of PPL by extending the length of the cationic spacer arm on the chitosan main chain. In the chemical modification, chitosan was first reacted with a-bromoacylbromide followed by reaction with an amine. The major problem in this procedure was the competing hydrolysis reaction of the bromoacylbromide. The presence of two amino groups in this side chain may even enhance membrane-forming properties. Chemical modification of poly(vinyl alcohol) (PVA) by a similar procedure may also produce a-polyamine with membrane-forming properties similar to that of PPL. All alkylation products were characterized by solution ^{1}H-and ^{13}C-NMR and by solid-state CP-MAS ^{13}C-NMR. These microcapsules were prepared by extrusion of a solution of blue dextran in sodium alginate into a solution containing calcium chloride and the membrane polymer. Measuring the elution of the blue dextran from the capsules, spectrophotometrically, assessed membrane integrity and permeability.

- **Rojas and coworkers [43]** optimized the encapsulation of β-lactoglobulin (BLG) within PLGA microparticles prepared by the multiple emulsion solvent evaporation method. The role of the pH of the external phase and the introduction of the surfactant Tween 20, in the modulation of the entrapment and release of BLG from microparticles. Better encapsulation of BLG was noticed on decreasing the pH of external phase to a value close to the PI of BLG, however, a larger burst release effect. In contrast, the addition of Tween 20 increased the encapsulation efficiency of BLG and considerably reduces in the burst release effect. In addition. Tween 20 reduced the number of aqueous channels between the internal aqueous droplets .

- **Yao and coworkers [44]** reported chitosan/gelatin network polymer microspheres for controlled release of cimetidine. The drug loaded microspheres were prepared by dissolving chitosan,gelatin (1:1 by weight) and cimetidine in 5% acetic acid. A certain amount of Tween 80 and liquid paraffin at water to oil a ratio of 1:10 was added to the chitosan/gelatin mixture under agitation at 650 rev. per min at 30 ^{0}C. A suitable amount of 25% aqueous glutaraldehyde solution was added to the inverse emulsion and maintained for 2 h. Finally, the liquid paraffin was vaporized under vacuum to obain microspheres.

- **Kidchob, T and cowokers [45]** prepared microcapsules from $[Glu(OME)]_m$ $(Sar)_n$ (m 21, n 19) and $[Lys(z)]_m$ $(Sar)_n$ (m 27, n 15), and were chemically modified to obtain a pH-responsive releasing membranes. One mrmbrane was prepared by partially deprotecting the ester groups of $[Glu(OMe)]_m$ $(Sar)_n$. The other membrane was prepared by connecting of poly (Glu) to side chain amino groups that were generated by a partial deprotection of $[Lys(z)]_m$ $(Sar)_n$. Both microcapsules showed pH-responsive release of FITC-dextran encapsulated in the microcapsules. The release rate was observed to be slower in the medium at pH 3.0 than pH 7.5. $[Glu(OMe)]_m$ $(Sar)_n$ microcapsules swelled more at pH 7.5 than at pH 3.0.

- **Katti and Krishnamurti [46]** prepared albumin microspheres by suspension crosslinking in the absence of any surfactant using paraffin oil as the dispersion medium and formaldehyde as the crosslinking agent. They characterized the microspheres by SEM and found to be spherical having a particle size distribution in the range of 50-400 μm.

- **Thakkar H and coworkers [47]** prepared albumin microspheres by emulsion polymerization technique A weighed amount of BSA (30% wt/wt) was dissolved in distilled water. Tween-80 was added at a concentration of 2% wt/wt. Celecoxib was finely titurated in a mortar and passed through sieve no. 400. Ten milligrams of finely powdered celecoxib was added to the above solution and sonicated to obtain a uniform dispersion. One milliliter of this dispersion was injected into a mixture of 20 mL of heavy liquid paraffin and 0.5 ml of span-85, while stirring at 2500 rpm. Stirring was continued for 10 minutes to obtain a water/oil (w/o) emulsion. One milliliter of glutaraldehyde was added into the emulsion to cross-link the albumin present in the internal phase of the emulsion. Microspheres formed were then separated by centrifugation and washed with 30 ml of petroleum ether to remove the liquid paraffin. The microspheres were then suspended in 10 ml of 5% wt/vol sodium bisulphite solution and stirred on a magnetic stirrer for 10 minutes to remove the residual glutaraldehyde. Finally, the microspheres were washed with 100 ml of water until they were free from residual glutaraldehyde; then they were dried at room temperature and stored in a desiccators until further use.

- **Payam Chini and coworkers [48]** prepared albumin microspheres by 47.5 ml of cellulose acetate buterate (2 w/v%) was added to 2.5 ml of a solution of bovine serum albumin (30 w/v%) in a flask, and this solution was stirred at 1250 rpm for 20 minutes. The next step was the addition of the cross-linking agent, glutaraldehyde. (Three separate batches of microspheres were prepared at different GTA concentrations.) After addition of GTA, the solution was stirred at 600 rpm for 3 hours. After 2 hours had passed, 50 ml of acetone was added to the mixture to compensate for some of the acetone evaporation. Results suggest that microspheres capped with glycine have significantly better loading properties than uncapped microspheres or those capped with other amino acids.

Chapter 3
Experiments

3.1 Materials

3.1.1 Cocoon
- 3.1.1.1. Kaw Suphan (white cocoon) from Suphan Buri
- 3.1.1.2. Danchang (yellow cocoon) from Suphan Buri
- 3.1.1.3. Nangnoi (yellow cocoon) from Burirum

3.1.2 Distilled water

3.1.3 25% Glutaraldehyde: $OCH(CH_2)_3CHO$ 1 l = 1.06 kg MERCK for synthesis. specification Assay (hydrogensulfit methode) 23-27% Density (d_{20}).

3.1.4 Sulfuric acid: MW 98.08 BDH Laboratory.

3.1.5 $NaHCO_3$: Fluka chemika.

3.1.6 Vegetable oil (brand name อร่อย)

3.1.7 Paraffin oil: Fisher chemicals code P103201171.

3.1.8 Span 80: Fluka .

3.1.9 Surfactant: Sunlight Unilever.

3.1.10 Johnson soap

3.1.11 Hexane: (Analytical reagent) LAB-SCAN UN-NO 1208

3.1.12 Methylene blue $C_{16}\ H_{18}\ CIN_3\ S$ aq
mr 319.86 +aq .: Fluka AG

3.1.13 Drug: Piroxicam

3.1.14 Acetone ($CH_3CO\ CH_3$): MW 58.08 Fisons Analytical Reagent A/0600/17 batch 35201313 Net 2.5 Lt.

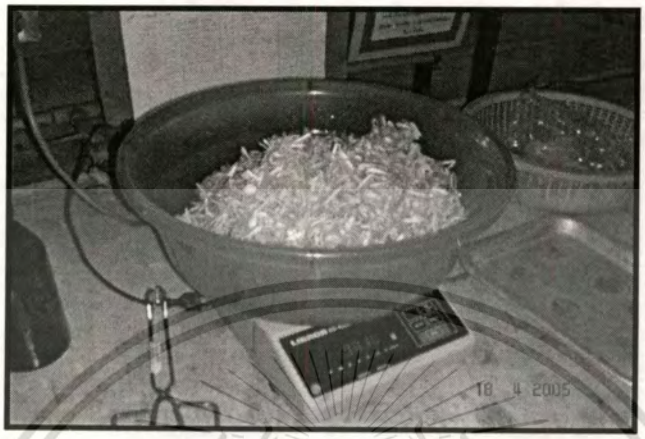
3.1.15 Diethyl ether $(C_2H_5)_2\ O$ MW 74.12 BDH Laboratory supplies (Anala R®).

3.2. Apparatus

3.2.1 Beakers
1000 ml – Beakers: (Pyrex ®), (Schott ®), (Duran®)
500 ml – Beakers: (Pyrex ®), (Schott ®), (Duran®)
250 ml – Beakers: (Pyrex ®), (Schott ®), (Duran®)
3.2.2 Graduated Cylinders
500 ml: (Pyrex ®)
100 ml: (Simax Kavaliek Stabil)[Csn]
3.2.3. Glass rod
3.2.4. Gloved
3.2.5. Dropper
3.2.6. Filter paper: 5C 70 mm Toyo Roshi Kaisha , Ltd.
3.2.7. Test tubes
3.2.8. Filtration flash
3.2.9. Buchner funnel
3.2.10. Aluminum tray
3.2.11. PP Bottom
3.2.12. Petri-dish : (Pyrex ®)
3.2.13. Plastic bags
3.2.14. Aluminum foil
3.2.15. Pipette
3.2.16. Hot plate
3.2.17. Distilled bottom
3.2.18. Thermometer: W-Germany

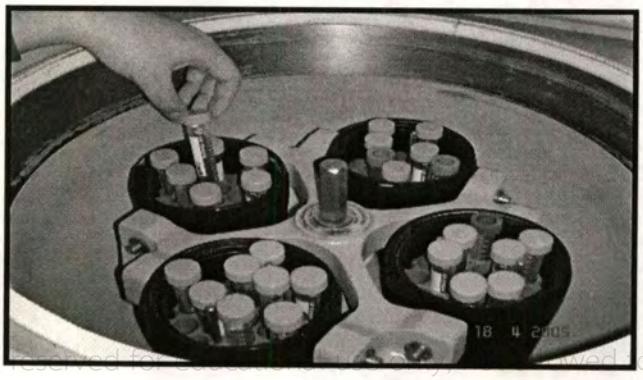
3.3. Equipments

3.3.1 Balance: "LIBROR" EB – 4000H SHIMADZU.



3.3.2 Freezer at – 81°C : Ultra-low temperature freezer "SANYO" .

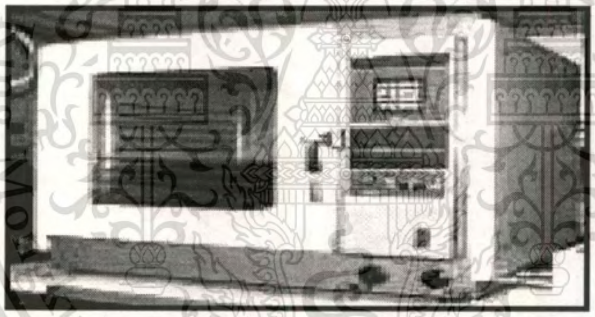
3.3.3 Refrigerated Centrifuge : "FALCON" 6/300



3.3.4 Suction and Filter : "GAST" MTG .Corp BENTON HARBOR ,Mj U.S.A. GE motors V220-250 PH 1 RPM 1425 .

3.3.5 Oven

Type	ULM:	400
Voltage		230 v
Temperature		220°C

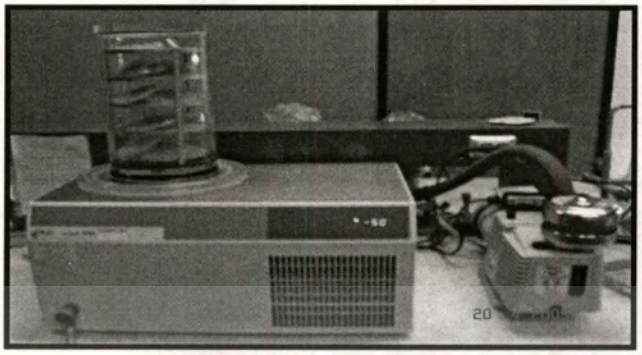


3.3.6 Autoclave

Hirayama manufacturing corporation

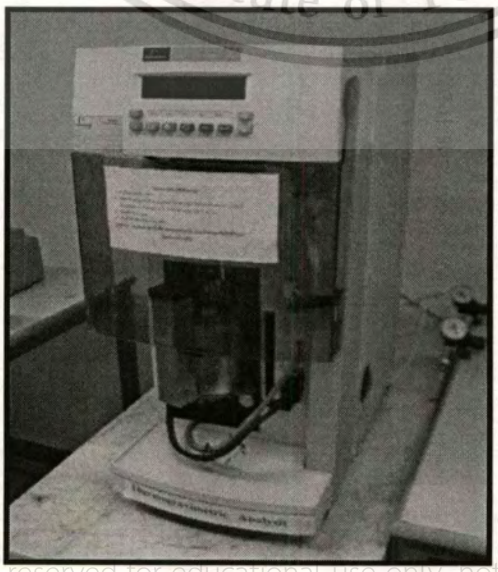
Type	HA-300MIT
Chamber capacity	0.0521 m^3
Maximum pressure	1.9 kg/cm^3
Electric power	2.0 kw
Voltage	200 v

3.3.7 Freezer dryer : Heto Lyolab 3000

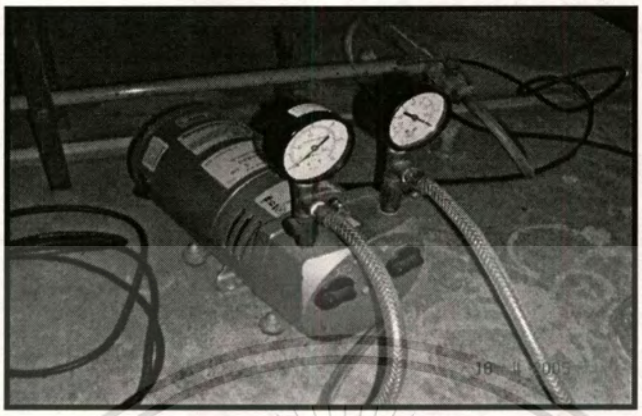


3.3.8 Fourier Transform Infrared Spectroscopy FTIR
Type FTIR spectrum GX , PekinElmer.

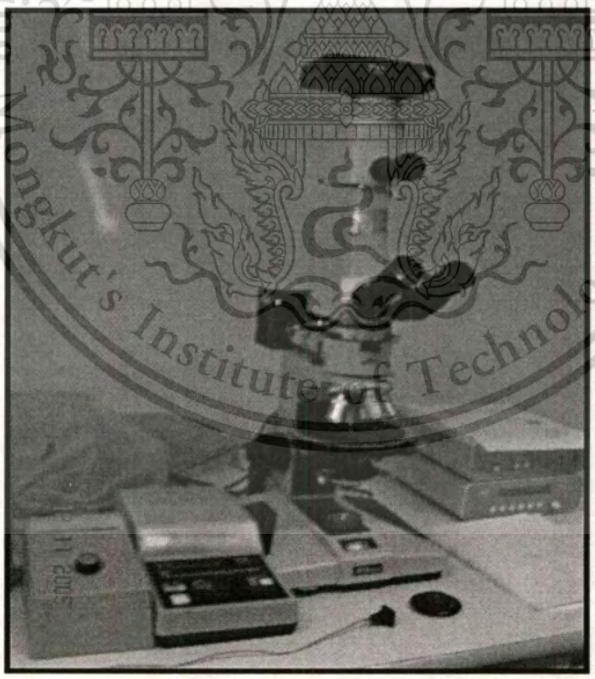
3.3.9 Thermogavimetric Analyzer
Type Pyris 1 TGA , PerkinElmer.



3.3.10 Vacuum



3.3.11 Polarize Microscope
Optiptiot-pol Nikon
Nikon FX-35WA



3.3.12 Scanning Electron Microscope[SEM]

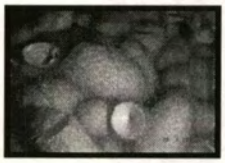
"LEO" 1455 VP , LEO Electron Microscopy Ltd
Clifton Road Cambridge CB1 3QH England

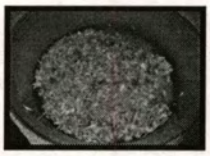
3.4 Methodology

Procedure

3.4.1 Preparation of sericin powder.

Cut cocoon to small size





Weight 30 g of cocoon

Clean cocoon 3-4 times by water

Put into PP bottom and add 300 ml distilled water

Autoclave at 121 ^{o} C for 60 minutes

Filter

Fiber

(fibroin)

Yellow water(solution)

(sericin)

Centrifuge

1cm pour into Petri-dish

Freeze at -80 ° C

Freeze dry over night

Sericin powder keep in plastic bag

3.4.2 Preparation of microspheres

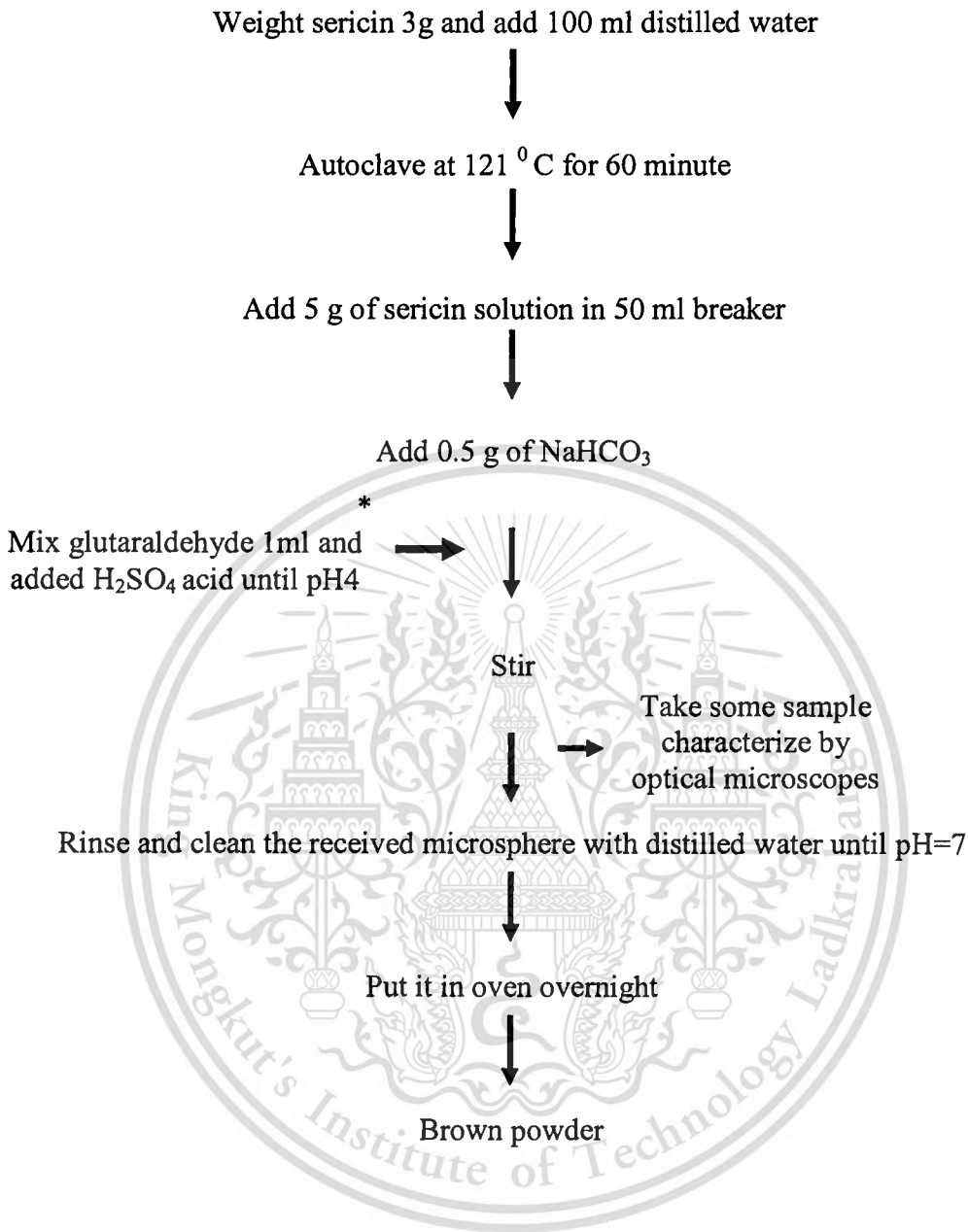
Technique used for preparation of microspheres in this work divided into 2 main techniques; suspension technique and emulsion technique. The influence of the amount of $NaHCO_3$ and 25% glutaraldehyde were studied under suspension techniques. On the other side, several parameters such as type of oil, surfactant, the amount of glutaraldehyde and acid were investigated under emulsion technique

Suspension technique

Section1. Preparation of microspheres by varying $NaHCO_3$

* Repeat this step by changing $NaHCO_3$ from 0.5g to 1g and 2g

Section2. Preparation of microspheres by varying the amount of glutaraldehyde



* Repeat this step by using 2 ml and 3ml of this solution for other samples

Table 3.1 Preliminary study the effect of the amount of glutaraldehyde and $NaHCO_3$

Batch	3% Sericin [g]	$NaHCO_3$ [g]	25% Glutaraldehyde [ml]	98% H_2SO_4 [ml]
A	5	0.5	2	1
B	5	1.0	2	1
C	5	2.0	2	1
D	5	0.5	1	1
E	5	1.0	2	1
F	5	2.0	3	1

Mixed each component into beaker then stirred at 900 rpm by using magnetic stirrer at 50 ^{0}C . After 20 minutes, washing microsphere bubbles with hexane several times and filtering. Optical polarized microscopy was used for determination microspheres characteristic in each batches.

Emulsion technique

Section I. Preparation microspheres by the single emulsion crosslinking technique

Mix 5ml of oil with 0.1ml of surfactant

Stir at 900 rpm | 10 min

Add 5ml of 3% of Sericin

Stir at 900 rpm | 10 min

Add 2ml of mixing glutaraldehyde 1ml and added H_2SO_4 acid until pH4

Stir at 900 rpm | 2-3 min → Take some sample characterize by optical microscopes

Washed by organic solvent

Dry at room temperature

Optical Microscope

Repeat all the step by changing the condition as shown in the table3.2

Table 3.2 Single emulsions

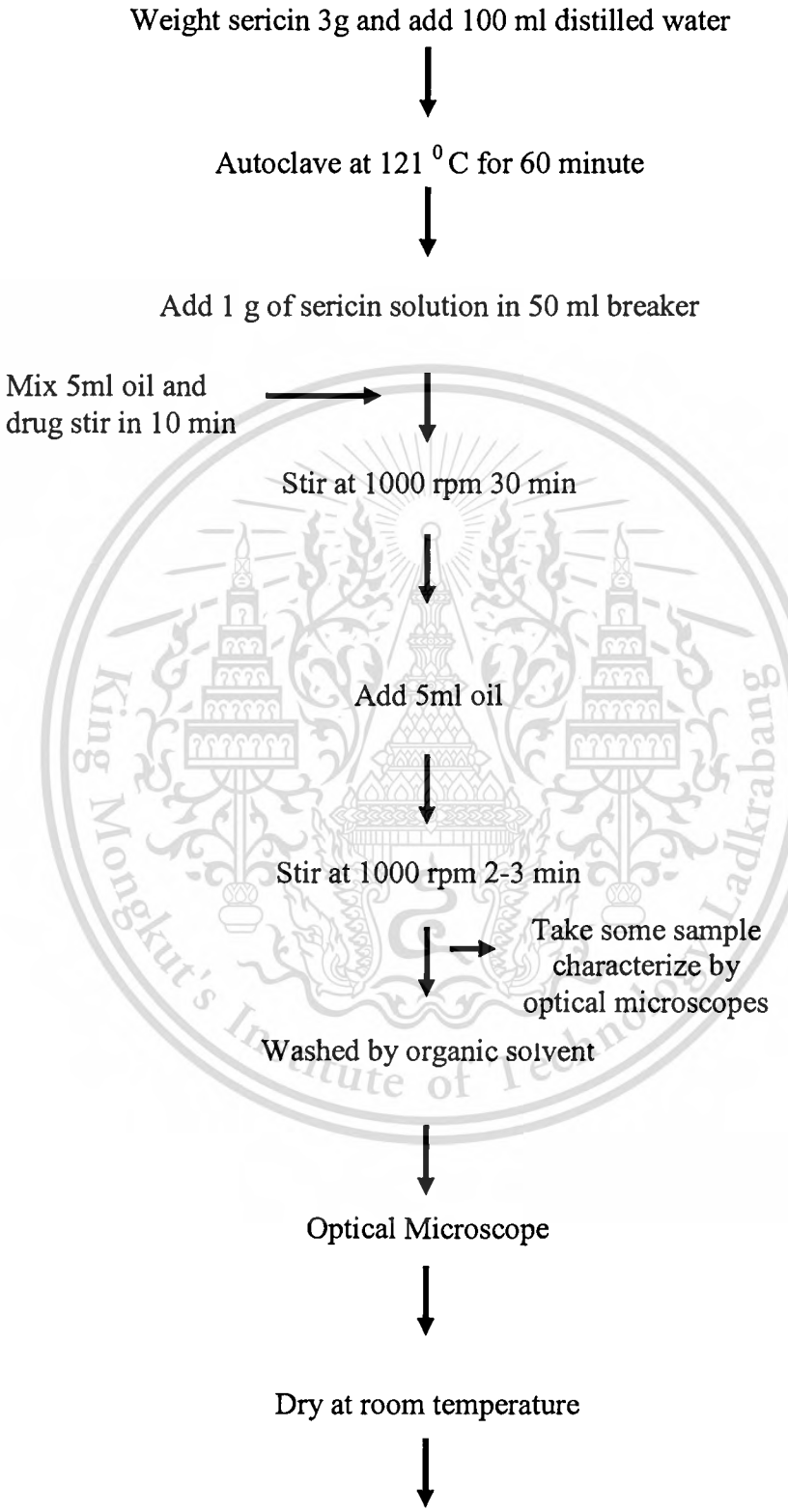
Condition	3%Sericin solution [ml]	Vegetable Oil [ml]	Paraffin Oil [ml]	Surfactant * [ml]	25% Glutaraldehyde [ml]	98% Acid(H^+) [ml]
1	5	5	-	0.1	2	1
2	5	-	5	0.1	2	1
3	5	-	5	0.1	4	1
4	5	5	-	0.1	1	-
5	5	5	-	0.1	4	1
6	5	5	-	Span 80 0.1	4	1
7	5	5	-	-	2	1
8	5	5	-	Span 80 0.1	2	1
9	5	5	-	Soap solution 0.3	2	1
10	5	5	-	0.3	2	1
11	5	5	-	0.5	2	1
12	Kaw Suphan 5	5	-	0.1	2	1
13	Danchang 5	5	-	0.1	2	1
14	5	5	-	0.1	2	-

* Surfactant used sunlight

An ingredient Sunlight: Linear alkylbenzene sulfonate 15.23%
Sodium lauryl ether sulphate 2.67%
Cocamido propyl betaine (CAPS) 0.1%

Section II. Preparation microspheres by the double emulsion crosslinking technique

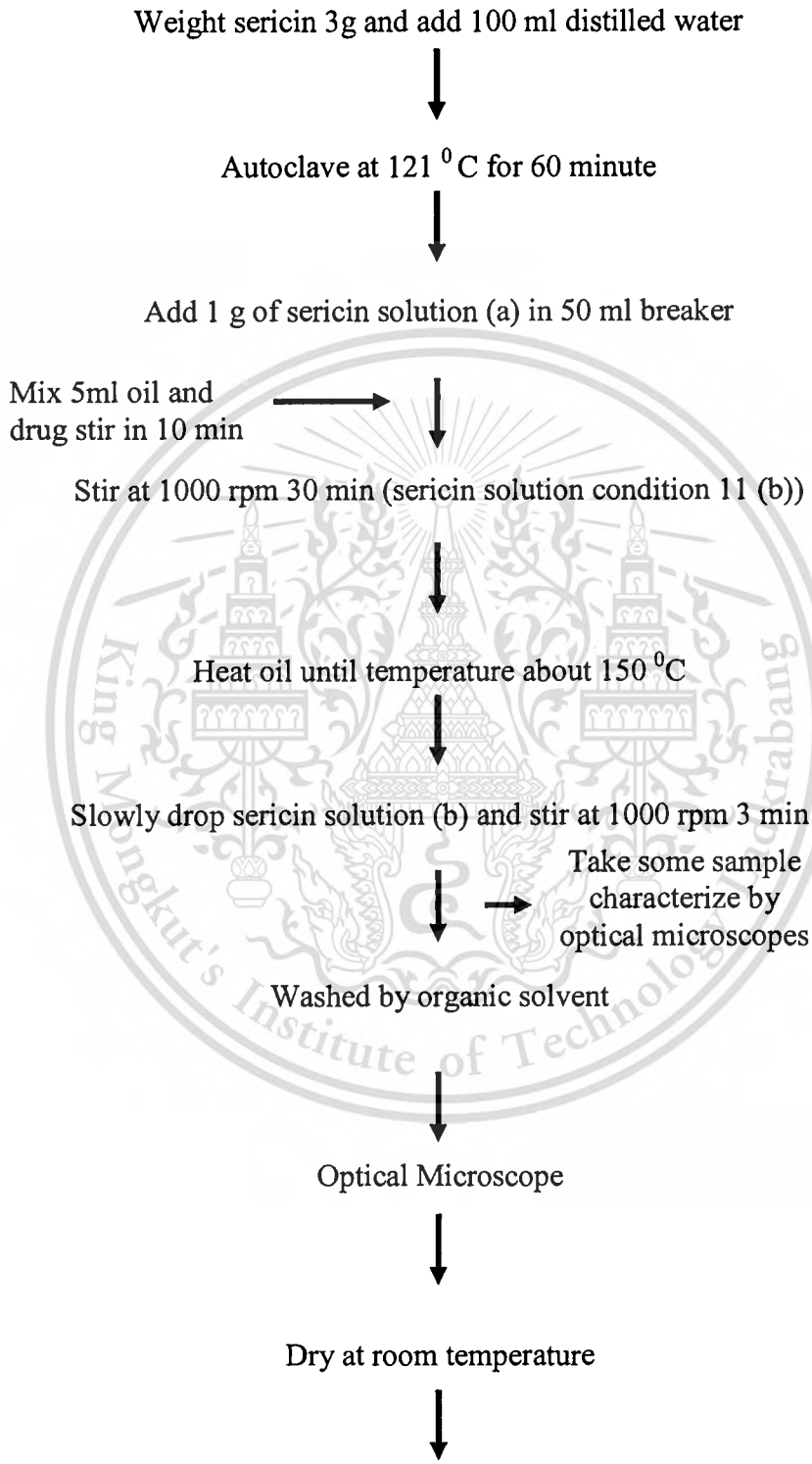
The following procedures represent the reaction do at room temperature and at 90 ^{0}C



Repeat all the step by changing the condition as shown in the table 3.3

Section III. Preparation microspheres by the double emulsion (hot oil) crosslinking technique

The following procedures represent the reaction do at 150 ^{0}C



Repeat all the step by changing the condition 12 and 13as shown in the table 3.3

Table 3.3 Double emulsions

Condition	3%Sericin solution* [g]	Vegetable Oil [ml] with drug	Surfactant * [ml]	Vegetable Oil [ml] after stir 30 min	Drug(g)	25% Glutaraldehyde [ml]	Temperature (^{0} C)
1	1	5	0.2	5	0.275	-	90
2	1	10	0.2	10	0.278	-	90
3	1	5	0.2	5	0.265	2	90
4	1	5	0.2	5	0.272	2	-
5	1	5	0.1	5	0.282	-	90
6	1	5	0.4	5	0.268	-	90
7	1	5	Sunlight 0.1	5	0.284	-	90
8	1	5	Sunlight 0.2	5	0.262	-	-
9	1	5	0.1	5	-	-	90
10	Nangnoi 1	5	0.2	20	0.2.75	-	150
11	Danchang 1	5	0.2	20	0.273	-	150
12	Kaw suphan 1	5	0.2	20	0.278	-	150

* Surfactant used Span 80
* Sericin used sericin from Nangnoi

Do the experiment in each formulas as shown in Figure 2.17 Polarized microscope was used for characterization morphology of microcapsules.

Chapter 4
Results and Discussion

4.1 FT-IR Spectrophotometry and Thermogravimetric Analysis.

Sericin powders from 3 types of cocoon; Nangnoi, Danchang, and Kaw suphan, were analyzed by using FT-IR and TGA.

A. FT-IR of 3 types of sericins.

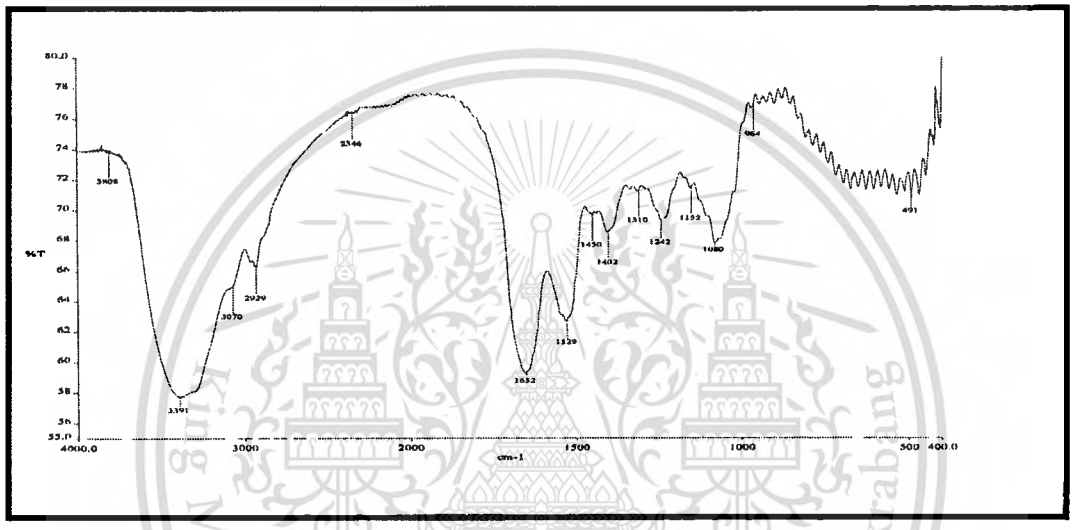


Figure 4.1a FT-IR spectrum of sericin Nangnoi

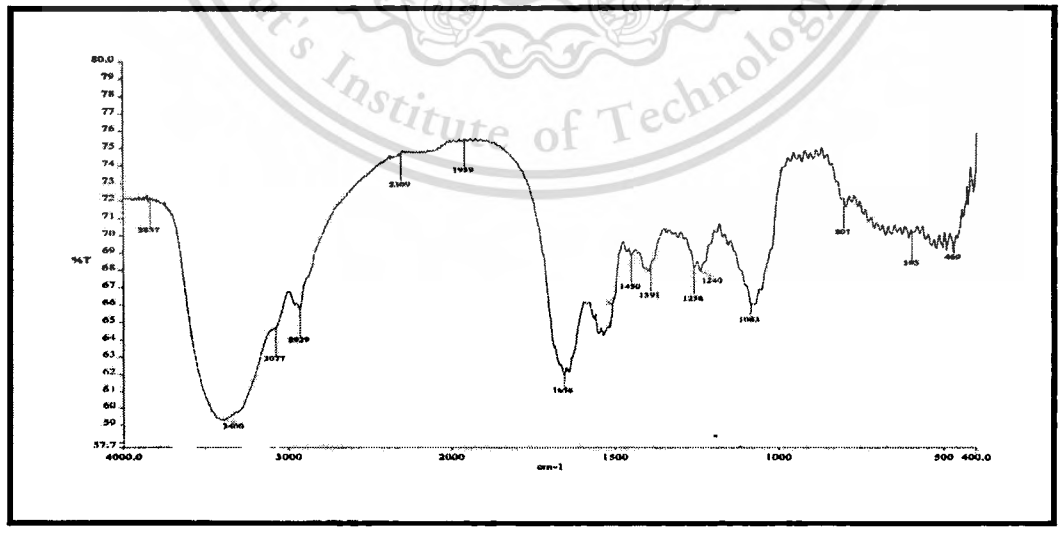


Figure 4.1b FT-IR spectrum of sericin Danchang

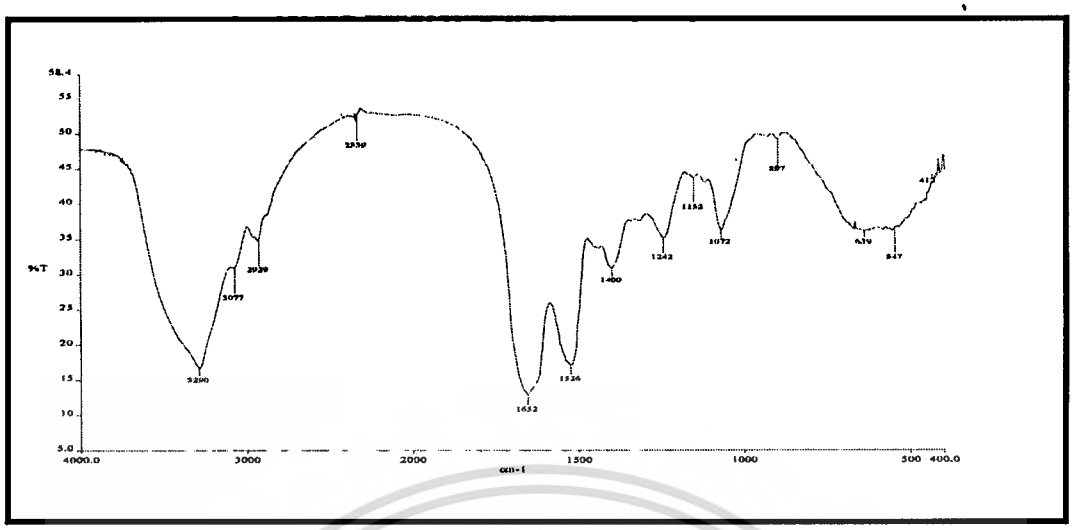


Figure 4.1c FT-IR spectrum of sericin Kaw suphan

The results from FT-IR indicated that all kinds of sericins exhibited absorption band at 3391(NH-stretching), 1652 (amide I), 1529 (amide II) and 639 cm^{-1} (amide V) which are characteristic of random coil conformation. This FT-IR spectra confirmed that powder received from hot water extracted cocoon is polyamide which corresponding to sericin as reported in the previous literatures.(Appendix A)

B. Thermogravimetric Analysis

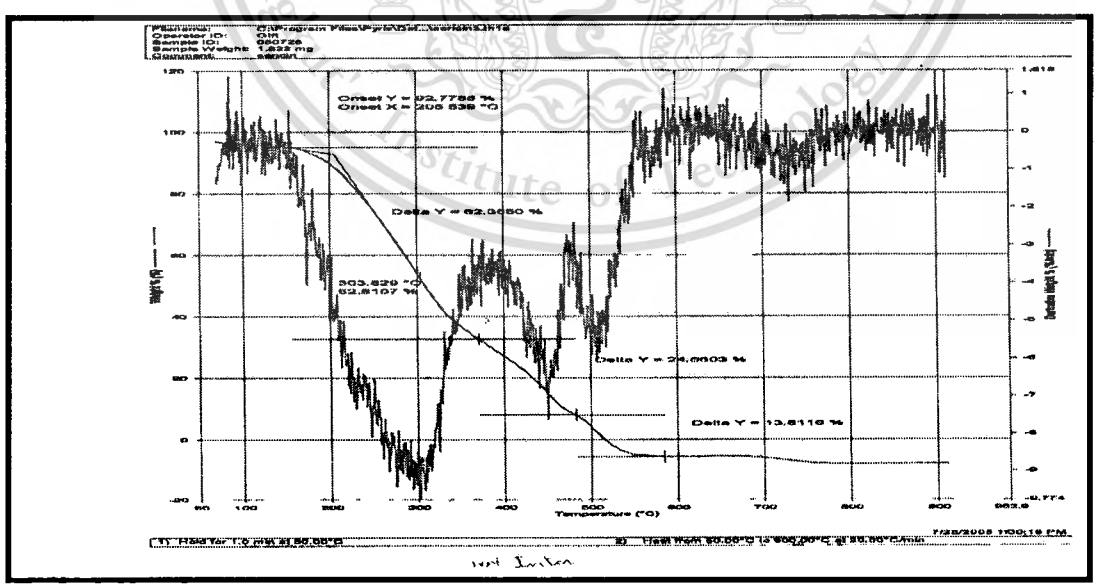


Figure 4.2a TGA of sericin Nangnoi

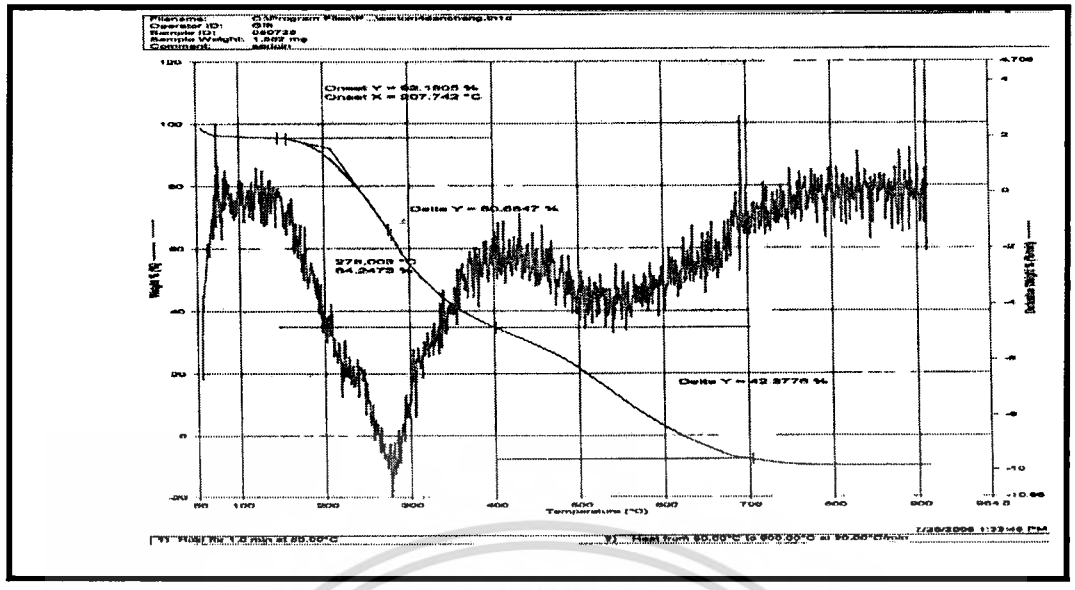


Figure 4.2b TGA of sericin Danchang

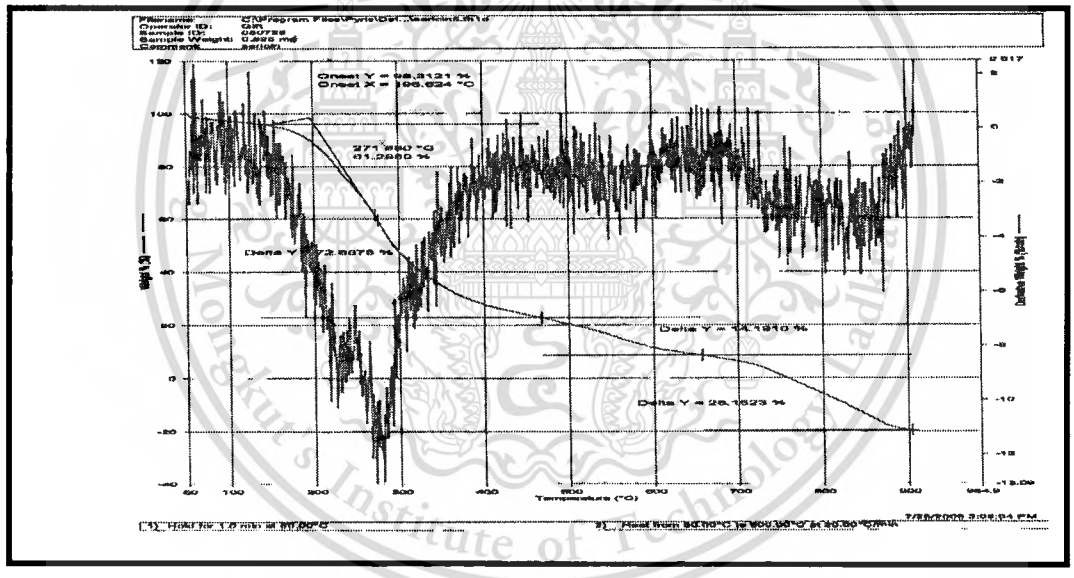


Figure 4.2c TGA of sericin Kaw suphan

A TGA analysis revealed the differences of molecular structure for each type of sericin. TGA thermograms of Nangnoi, Dangchang and Kaw suphan show decomposition temperature near 303 ^{0}C, 279 ^{0}C and 271 ^{0}C, respectively. Derivative thermograms suggested that sericin Nangnoi contained 4 fractions of different sequences of amino acid. Sericin Dangchang composed of 2 fractions and sericin Kaw suphan composed of 3 fractions.

The amino acid sequences of sericin depends on the species of silkworm. However, this work did not focus on amino acid analysis.

4.2 Suspension technique

In case of suspension technique, several parameters such as $NaHCO_3$, glutaraldehyde, and acid were studied. The role of $NaHCO_3$ and glutaraldehyde were determined. $NaHCO_3$ used for providing bubbles and 25% glutaraldehyde was used as protein crosslinking agent .

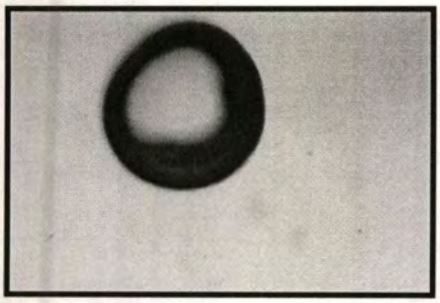
Section I : Effect of $NaHCO_3$

To determine the effect of $NaHCO_3$, 3% of sericin Kaw suphan 5 g was added in to the mixture of 25% glutaraldehyde 2 ml and 98% sulfuric acid. Then, in each batch the amount of $NaHCO_3$ from 0.5 g to 2.0 g as shown in Table 4.1 were added. The solution mixture was stirred vigorously by magnetic stirrer for 2-3 min. Addition of $NaHCO_3$ raised the pH of the solution towards neutral pH. As the amount of the added $NaHCO_3$ increased, the pH of the mixture became high. However, such a high pH is not desirable, since acidic pH is necessary for the decomposition of $NaHCO_3$. The small amount of suspension solution was taken and determined the size of microbubbles by using optical microscope (magnification 10x10). Sericin Nangnoi and Danchang were performed in the same method.

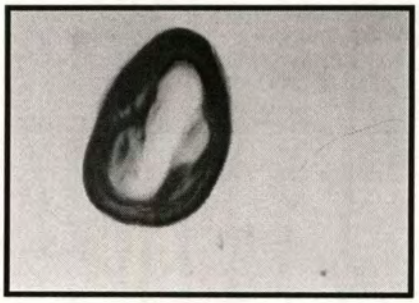
Table 4.1 Study on the effect of $NaHCO_3$ in suspension technique.

Batch	3% Sericin [g]	$NaHCO_3$ [g]	25% Glutaraldehyde [ml]	98% H_2SO_4 [ml]
A	5	0.5	2	1
B	5	1.0	2	1
C	5	2.0	2	1

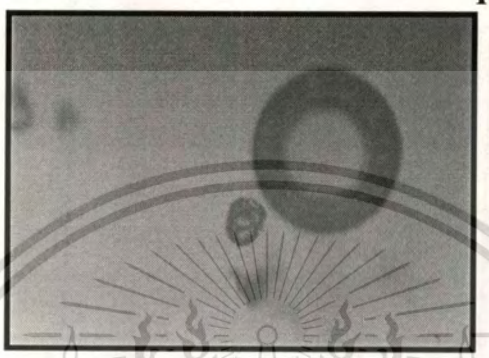
The optical micrograps of microbubbles under condition A, B and C showed in the following figure.



Nang noi



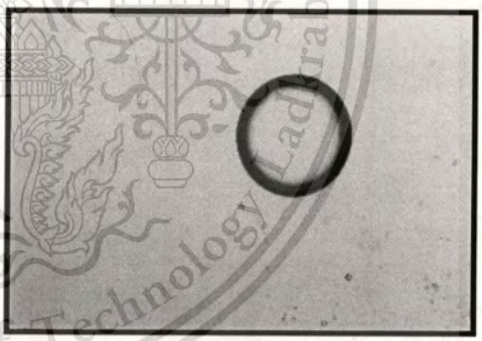
Kaw Suphan



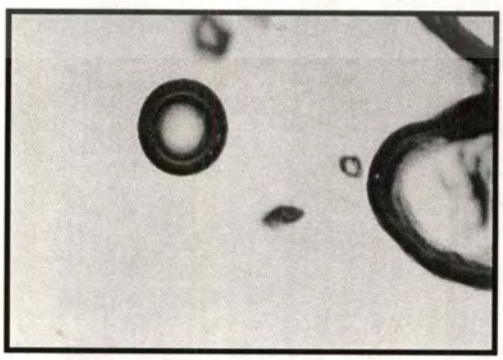
Danchang

Figure 4.3a Optical micrographs of microbubbles from condition A.

Nang noi

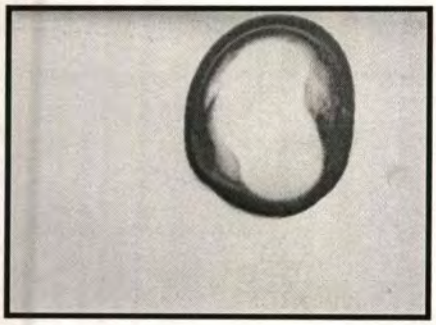


Kaw Suphan

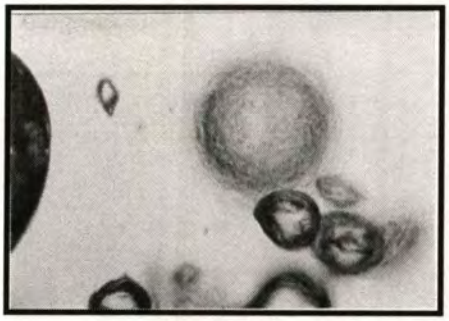


Danchang

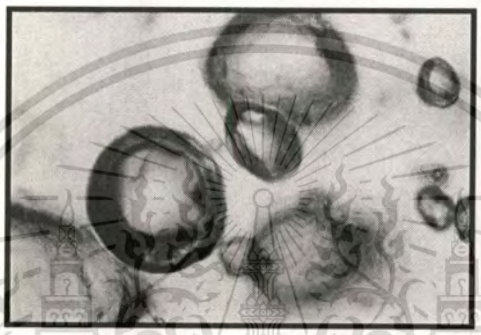
Figure 4.3b Optical micrographs of microbubbles from condition B.



Nang noi



Kaw Suphan



Danchang

Figure 4.3c Optical micrographs of microbubbles from condition C.

Under suspension technique it was found that number of microbubbles and wall thickness increased with increasing the amount of $NaHCO_3$. On the other hand the size of microbubbles was deceased. However, these types of microbubbles can not isolated due to its unstable.

Section II: Effect of glutaraldehyde.

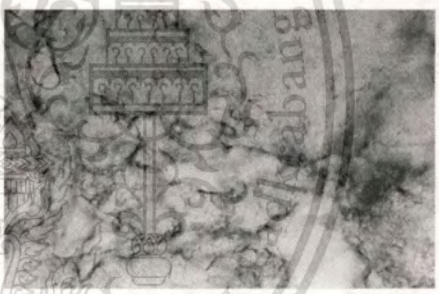
To determine the effect of glutaraldehyde, 3% of sericin Kaw suphan 5 g and 98% sulfuric acid were mixed with various amount of 25% glutaraldehyde as shown in Table 4.2 $NaHCO_3$ 0.5 g was added last in each batch. The solution mixture was stirred vigorously by magnetic then stirrer for 2-3 min. After that filtered the suspension solution, rinsed with hexane and dried in oven. Morphology of the precipitate were characterized by using optical microscope(magnification 10x10). Sericin Nangnoi and Danchang were performed in the same method.

Table 4.2 Study on the effect of glutaraldehyde in suspension technique.

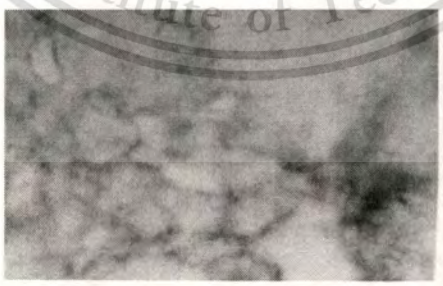
Batch	3% Sericin [g]	$NaHCO_3$ [g]	25% Glutaraldehyde [ml]	98% H_2SO_4 [ml]
D	5	0.5	1	1
E	5	0.5	2	1
F	5	0.5	3	1

The effect of glutaraldehyde concentration on morphology of precipitate which received under condition D, E and F showed in the following pictures.

Condition D



Condition E



Condition F

Figure 4.4 The effect of glutaraldehyde from condition D, E and F in suspension technique.

Suspension technique seemed to be not suitable for forming stable microbubbles. The emulsion technique was selected for further study.

4.3 Emulsion technique.

Two system of emulsion technique namely single emulsion and doubble emulsion technique were investigated. In the emulsion technique oil is combined with a solution of surfactant and protein crosslinking agent. Acid was used as catalyst.

Section I : Single emulsion.

The influences of oil, glutaraldehyde, surfactant, types of sericin and acid were studied. Among three types of sericin, only sericin Nangnoi was selected for finding the suitable parameters.

a. Effect of oil

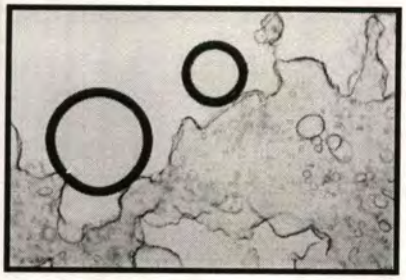
To compare the effect of paraffin oil and vegetable oil, 3% of sericin 5 ml was directly added in a beaker containing the mixture of oil 5 ml and 0.1 ml of surfactant. While stirring with magnetic stirrer, 2 ml of 25% glutaraldehyde and 1 ml of acid were added. Stirring was continued for 2-3 minutes and the emulsion was formed. The small amount of emulsion solution in each batch was taken and characterized by using optical microscope (magnification 10x10).

Table 4.3 : Summary of all reaction conditions based on varied oil .

Condition	3%Sericin solution [ml]	Vegetable Oil [ml]	Paraffin Oil [ml]	Surfactant * [ml]	25% Glutaraldehyde [ml]	98% Acid(H^+) [ml]
1	5	5	-	0.1	2	1
2	5	-	5	0.1	2	1
3	5	-	5	0.1	4	1

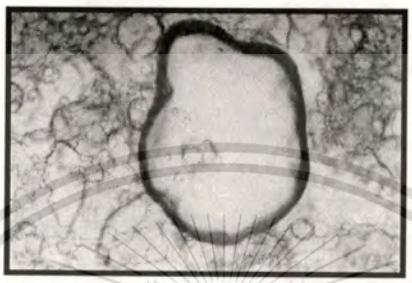
*Note: surfactant used is **Sunlight**.

The pictures from optical microscope showed in the following.



Condition 1

Condition 2



Condition 3

Figure 4.5 Optical micrographs of microbubbles in single emulsion of all reaction conditions based on varied oil .

The results from optical microscope showed that vegetable oil provided better microbubbles formation. Under single emulsion technique the influence of oil types was observed. It was found that vegetable oil gives spherical shape and regular surface of microbubbles. On the other hand paraffin oil did not provide regular shape of microsbubbles. Moreover, the solid microbubbles can not separated from this reaction.

b. Effect of glutaraldehyde

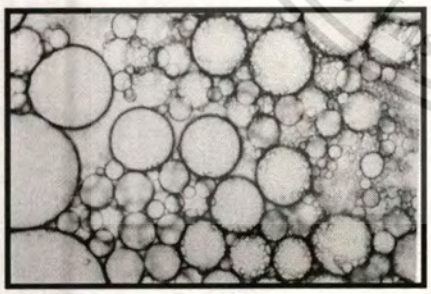
The component used for study the effect of 25% glutaraldehyde are listed in Table 4.4 3% of sericin 5 ml, vegetable oil 1 ml, surfactant 0.1 ml and acid were mixed and stirred vigorously. In the beginning tiny bubbles were formed during stirring. As glutaraldehyde was added dropwise, less bubbles were formed and the volume of emulsion decreased due to the bubbles diminish. The emulsion mixture was stirred for 2-3 minutes. The small amount of emulsion solution in each batch was taken and characterized by using optical microscope (magnification 10x10).

Table 4.4: Summarized all reaction conditions based on varied glutaraldehyde.

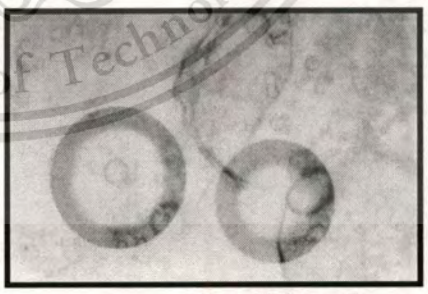
Condition	3%Sericin solution [ml]	Vegetable Oil [ml]	Surfactant [ml]	25% Glutaraldehyde [ml]	98% Acid(H^+) [ml]
1	5	5	0.1	2	1
4	5	5	0.1	1	-
5	5	5	0.1	4	1

The Figures from optical microscope showed in the following.

Condition 1



Condition 4



Condition 5

Figure 4.6 Optical micrographs of microbubbles in single emulsion of all reaction conditions based on varied glutaraldehyde .

Figure 4.6 illustrated that addition of 25 % glutaraldehyde inhibits the forming of microbubbles. The results suggested that increasing 25 % glutaraldehyde from 1.0 ml to 4.0 ml decreased number of microbubbles, and the spherical edge was more thicker. Stable spherical shape and regular surface was found in 2 ml of glutaraldehyde. However, the microbubbles disappear during washing by solvent.

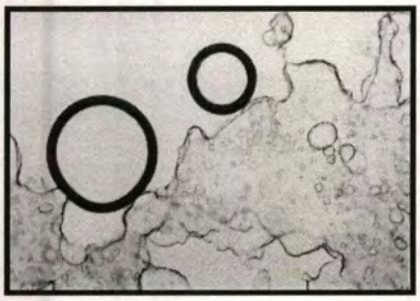
c. Effect of surfactant.

The viscosity of the solution was determined by changing surfactant as shown in Table 4.5 The amount of surfactant were varied from 0.1, 1.0, and 3.0 ml. Surfactant used in these conditions are sunlight, Span80 and soap solution. The solution mixture of all components in each batch was stirred vigorously by magnetic stirrer for 2-3 minutes. The small amount of emulsion solution in each batch was taken and characterized by using optical microscope (magnification 10x10).

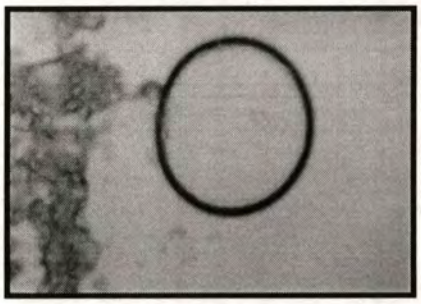
Table 4.5 : Summary of all reaction conditions based on varied surfactant .

Condition	3%Sericin solution [ml]	Vegetable Oil [ml]	Surfactant * [ml]	25% Glutaraldehyde [ml]	98% Acid(H^+) [ml]
1	5	5	0.1	2	1
6	5	5	Span 80 0.1	4	1
7	5	5	-	2	1
8	5	5	Span 80 0.1	2	1
9	5	5	Soap solution 0.3	2	1
10	5	5	0.3	2	1
11	5	5	0.5	2	1

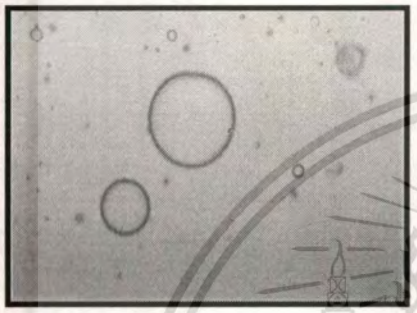
The figures from optical microscope showed in the following.



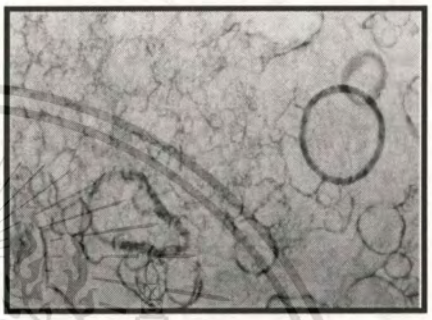
Condition 1



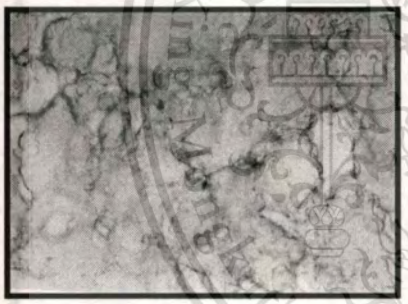
Condition 6



Condition 7

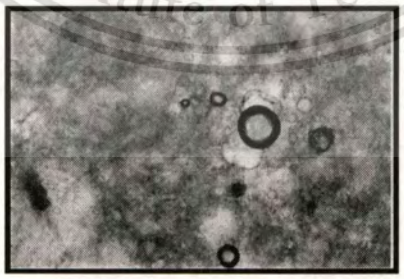


Condition 8



Condition 9

Condition 10



Condition 11

Figure 4.7 Optical micrographs of microbubbles in single emulsion all reactionconditions based on varied of surfactant.

It was found that sunlight and Span80 were the suitable for forming microbubbles in single emulsion technique. The figures illustrated that regular surface shape and spherical shape including thick edge of microbubbles can observed. On the other side, microbubbles were not appeared in the soap solution

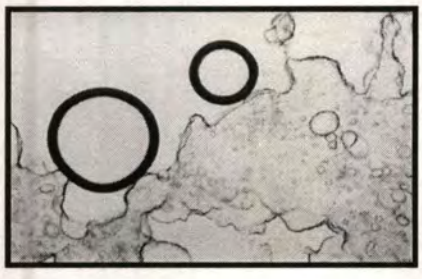
d. Effect of different sericins.

To investigate the effect of protein structure, three types of sericin; Nangnoi Kaw Suphan and Danchang, were compared. The solution mixture of all components in each condition as listed in Table 4.6 was stirred vigorously by magnetic stirrer for 2-3 minutes. The small amount of emulsion solution in each batch was taken and characterized by using optical microscope (magnification 10x10).

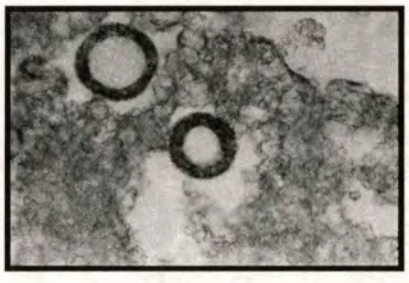
Table 4.6 Summary of reaction conditions based on varied types of sericins

Condition	3%Sericin solution [ml]	Vegetable Oil [ml]	Surfactant * [ml]	25% Glutaraldehyde [ml]	98% Acid(H^+) [ml]
1	Nangnoi 5	5	0.1	2	1
12	Kaw Suphan 5	5	0.1	2	1
13	Danchang 5	5	0.1	2	1

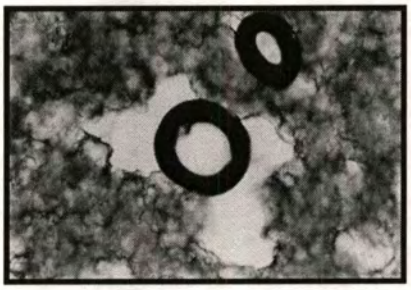
The figure from optical microscope showed as following.



Condition 1



Condition 12



Condition 13

Figure 4.8 Optical micrographs of microbubbles in single emulsion all reaction conditions based on varied type of sericins.

It was found that the similar morphology of microbubbles were obtained. Nangnoi provided larger size and stable spherical shape than the others.

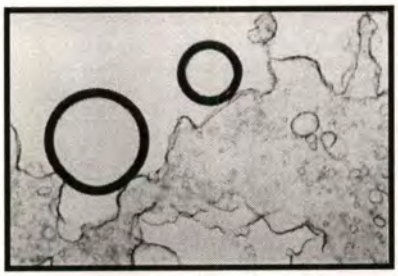
e. Effect of acid

The influence of acid in single emulsion system was studied. The solution mixture of all components in condition 14 was stirred vigorously by magnetic stirrer for 2-3 minutes. The small amount of emulsion solution in each batch was taken and characterized by using optical microscope (magnification 10x10). The figure was compared to the condition 1.

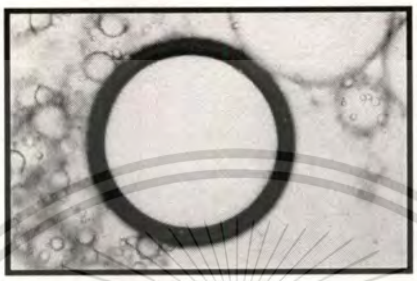
Table 4.7 : Summary of reaction conditions based on varied acid

Condition	3%Sericin solution [ml]	Vegetable Oil [ml]	Surfactant * [ml]	25% Glutaraldehyde [ml]	98% Acid(H^+) [ml]
1	5	5	0.1	2	1
14	5	5	0.1	2	-

The figure from optical microscope as following.



Condition 1



Condition 14

Figure 4.9 Optical micrographs of microbubbles in single emulsion all reaction conditions based on varied acid

The results showed that the similar microbubbles were occur both in condition 1 (with acid) and in condition 14 (without acid).

The optical micrographs from condition 1 and 14 suggested that preparation of microspheres from single emulsion system was not possible. Microbubbles can not collected and unstable.

From result of single emulsion ,When we stirred air can penetrated into sericin solution and air bubbles can struck inside sericin solution phase , like "a foaming liquid" due to viscosity of solution higher than air. So we can not isolated air bubbles from sericin solution phase.

Section II : Double emulsions

Double emulsion consists of two steps, first preparation emulsion and the second addition of excess oil. The role of oil, surfactant, glutaraldehyde, acid and reaction temperature were investigated under double emulsion system. Pyroxicam drug was used as the microspheres core and sericin Nangnoi was selected as outer shell microcapsules.

a. Effect of oil

1 g of 3% sericin was directly added into the mixture of oil, drug and surfactant while stirring. After 30 min, excess hot oil was added and stirring was continue for 2-3 min at temperature 90 ^{0}C. The microspheres in each batch was taken and characterized by using optical microscope (magnification 10x10).

Table 4.8 Summary of reaction conditions based on varied the amount of oil.

Condition	3%Sericin solution [g]	Vegetable Oil [ml] with drug	Surfactant * [ml]	Vegetable Oil [ml] after stir 30 min	Drug(g)	Temperature (^{0} C)
1	Nangnoi 1	5	0.2	5	0.275	90
2	Nangnoi 1	10	0.2	10	0.278	90

*Note : Surfactant used is **Span80**

The figure from optical microscope showed as following.

Condition 1 **Condition2**

Figure 4.10 Optical micrographs of microspheres in all reaction conditions of double emulsion based on varied the amount of oil.

Similar morphology of microspheres was observed in both condition reactions. It is interesting to note that morphology of microspheres prepared in the double emulsion system was different from the previous conditions.

b. Effect of glutaraldehyde and temperature.

1 g of 3% sericin was directly added into the mixture of oil, drug and surfactant while stirring. After 30 min, excess hot oil and glutaraldehyde was added and stirring was continued for 2-3 min at temperature 90 ^{0}C. The microspheres in each batch was taken and characterized by using optical microscope (magnification 10x10).

Table 4.9 : Summary of all reaction conditions based on varied glutaraldehyde and temperature.

Condition	3%Sericin solution [g]	Vegetable Oil [ml] With drug	Surfactant [ml]	Vegetable Oil [ml] after stir 30 min	Drug(g)	25% Glutaraldehyde [ml]	Temperature (^{0} C)
1	1	5	0.2	5	0.275	-	90
3	1	5	0.2	5	0.265	2	90
4	1	5	0.2	5	0.272	2	-

The figure from optical microscope showed as following.

Condition 1

Condition 3

Condition 4

Figure 4.11 Optical micrographs of microspheres in all reaction conditions of double emulsion based on varied glutaraldehyde.

The results suggested that microspheres was obtained from the reaction without glutaraldehyde.

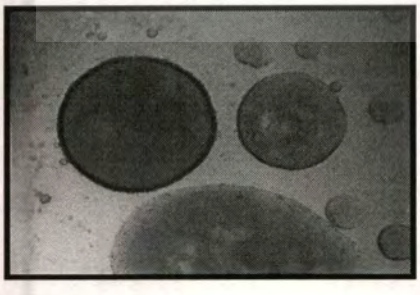
c. Effect of surfactant and temperature

Table 4.10 showed the amount of each component in all conditions. 1 g of 3% sericin was directly added into the mixture of oil, drug and surfactant while stirring. After 30 min, excess hot oil and glutaraldehyde were added and then stirring for 2-3 min at temperature 90^0C. The microspheres in each batch were taken and characterized by using optical microscope (magnification 10x10).

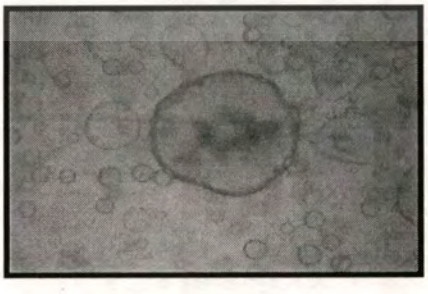
Table 4.10 : Summary of reaction conditions based on varied surfactant and temperature.

Condition	3%Sericin solution [g]	Vegetable Oil [ml] with drug	Surfactant [ml]	Vegetable Oil [ml] after stir 30 min	Drug(g)	Temperature (^{0} C)
1	1	5	0.2	5	0.275	90
5	1	5	0.1	5	0.282	90
6	1	5	0.4	5	0.268	90
7	1	5	Sunlight 0.1	5	0.284	90
8	1	5	Sunlight 0.2	5	0.262	-

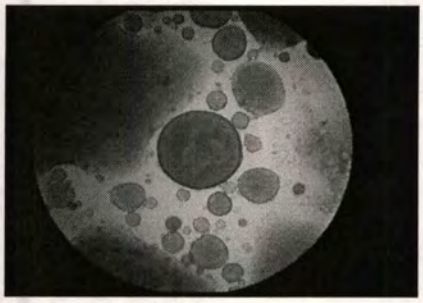
The figure from optical microscope showed as following.



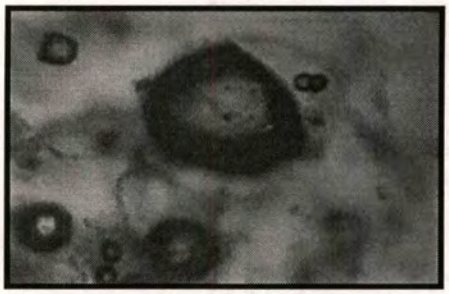
Condition 1



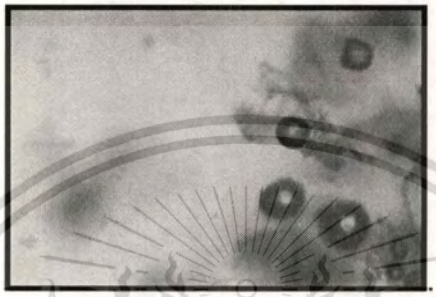
Condition 5



Condition 6



Condition 7



Condition 8

Figure 4.12 Optical micrographs of microspheres in all reaction conditions of double emulsion based on varied surfactant.

It was found that Span80 was suitable surfactant in double emulsion technique. The spherical shape and thick edge of microspheres was observed.

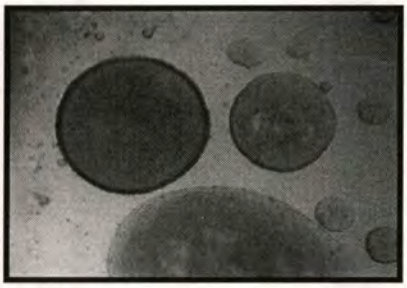
d. Effect of drug

The influence of drugs on microspheres formation between condition 1 (with drugs) and condition 9 (without drugs) was studied. 1 g of 3% sericin was directly added into the mixture of oil and Span80 surfactant while stirring. After 30 min, excess hot oil was added and stirring was continued for 2-3 min at temperature 90 ^{0}C. The microspheres in each batch was taken and characterized by using optical microscope (magnification 10x10).

Table 4.11 : Summary of all reaction conditions based on drug.

Condition	3%Sericin solution [g]	Vegetable Oil [ml] with drug	Surfactant * [ml]	Vegetable Oil [ml] after stir 30 min	Drug(g)	Temperature (^{0} C)
1	1	5	0.2	5	0.275	90
9	1	5	0.1	5	-	90

The figure from optical microscope showed in following.



Condition 1

Condition 9

Figure 4.13 Optical micrographs of microspheres from condition 1and condition 9.

The figure indicated that drugs as well as Span80 surfactant had enhanced microspheres formation. Without containing drugs, the microspheres was not formed. However, the microsphers of condition 1 swell to non sphereical shape after contact with diethyl ether. Diethyl ether used for washing oil from surface of microsphere

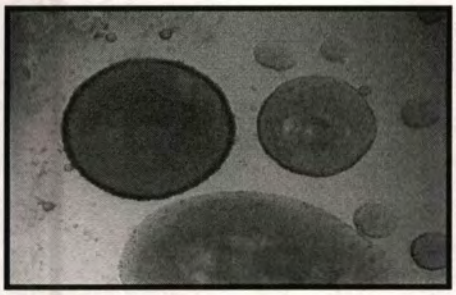
e. Effect of temperature

Changing temperature of the reaction was investigated for improving the stability of microspheres. The components in each condition in accordance with Table 4.12 were performed similarly as previous experiments. The difference between condition 1 and 10 were the amount of surfactant and temperature. Temperature of excess vegetable oil which used after forming emulsion was raised to 150°C for denature protein sericin.

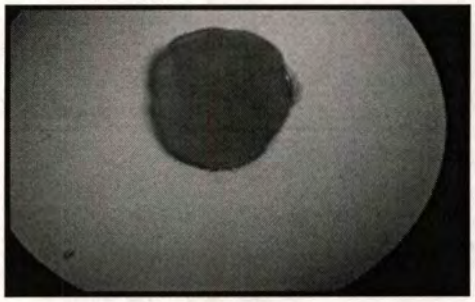
Table 4.12 Summary of all reaction conditions based on varied temperature

Condition	3%Sericin solution [g]	Vegetable Oil [ml] With drug	Surfactant * [ml]	Vegetable Oil [ml] after stir 30 min	Drug(g)	Temperature (^{0} C)
1	Nangnoi 1	5	0.2	5	0.275	90
10	Nangnoi 1	5	0.2	20	0.275	150

The figure from optical microscope showed as following.



Condition 1



Condition 10

Figure 4.14 Optical micrographs of microspheres from condition 1and 10

The increase of reaction temperature to 150 ^{0}C resulted in more stable microsphere. The solid microspheres was collected by filtration and then washed by hexane. This means that the enough external heat is necessary for the condition that was at 150 ^{0}C protein sericin denaturized to formed solid surface which stable after contact with hexane.

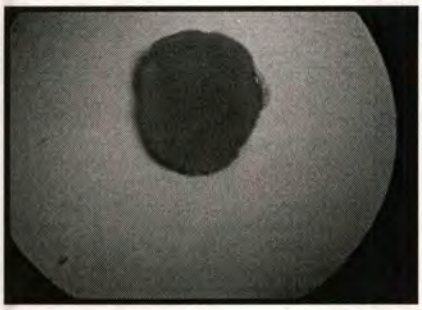
f. Effect of different types of sericins

The condition 10 should be the suitable parameters for double emulsion technique. To determine the role of molecular structure, sericin Danchang and Kawsuphan were used procedure in condition 10 for preparation of microspheres.

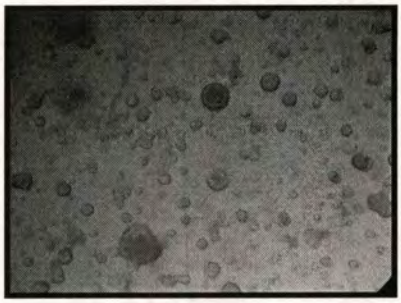
Table 4.13 : Summarized all reaction conditions based on 3 types of sericins

Condition	3%Sericin solution* [g]	Vegetable Oil [ml] With drug	Surfactant [ml]	Vegetable Oil [ml] after stir 30 min	Drug(g)	Temperature (^{0} C)
10	Nangnoi 1	5	0.2	20	0.275	150
11	Danchang 1	5	0.2	20	0.273	150
12	Kaw suphan 1	5	0.2	20	0.278	150

The figure from optical microscope showed as following.



Condition 10



Condition 11

Condition 12

Figure 4.15 Optical micrographs of microspheres in condition 10,11 and 12

Optical micrographs obviously illustrated that the molecular structure of Nangnoi which composed of 4 fractions of amino acid sequences is good for microsphere formation. On the other hand, sericin Dangchang and Kawsuphan were not suitable.

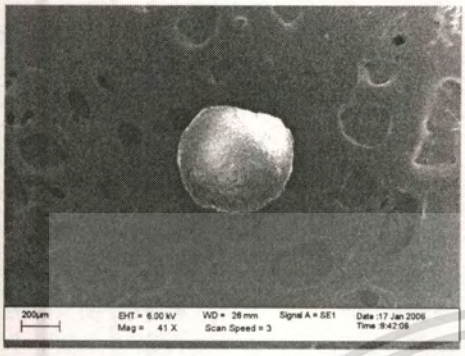
From result of double emulsion, can explain that drug (Piroxicam) can combined with molecule of Protein by Hydrogen bonding. Because their have the similar functional group called peptide bond (CONH). Hydrogen bond could be fulfilled as interior cohesion, resulting in droplets of microsphere. After that when we pour this into hot oil. It's become solidification. The droplets of microsphere more dense than oil and unfavorable interaction between the oil and the droplets microsphere.

The floating of droplets microsphere depends on 2 forces, the repulsion force and the gravity force. If the force of gravity is strong enough. it will prevail and droplets of microsphere will sink. (density greater than oil). If the gravitational force is less than replusion force then the droplets of microsphere will float on the surface of oil.

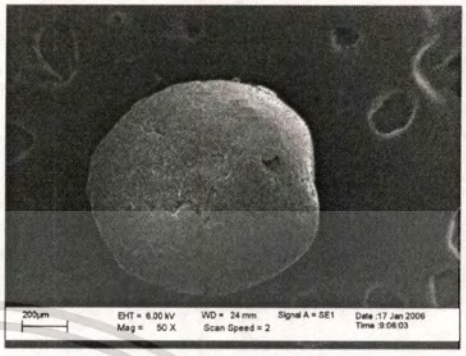
In ours case, the droplets of microsphere more dense than oil. They float on hot oil due to the continuous stirring. If we stop stirring, the droplets will sink.

4.4 Scanning Electron Microscope (SEM).

Three microspheres particles received from condition 10 were characterized by using Scannig Electron Microscope. The micrograms of SEM showed as following.



a) magnification 41x

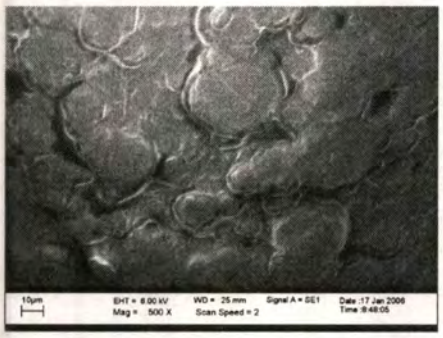


b) magnification 50x

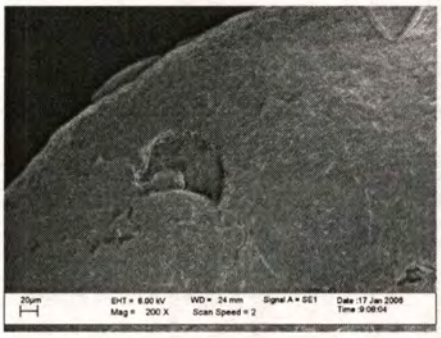
c) magnification 50x

Figure 4.16 showed size and shape of microsphere particles at different magnification of a) particle 1 b) particle 2 c) particle 3

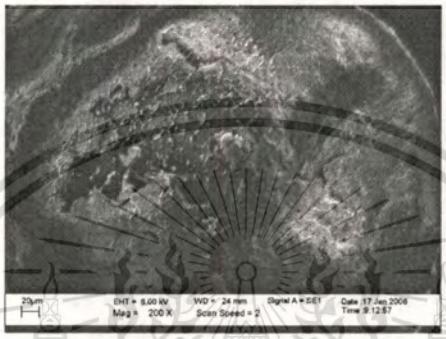
The average particles size of microspheres were around 200-800 µm. The following SEM pictures focused on the surface of each particle and different magnifications.



a) magnification 500x

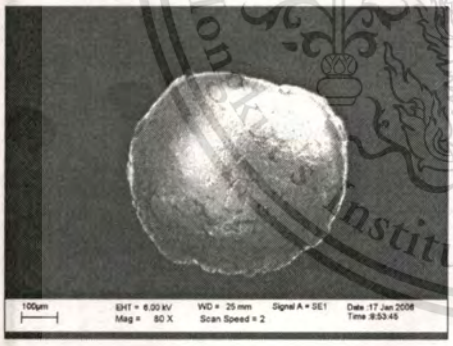


b) magnification 200x

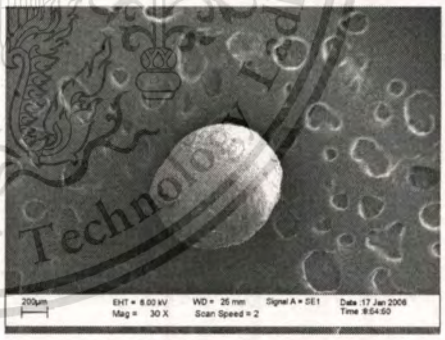


c) magnification 200x

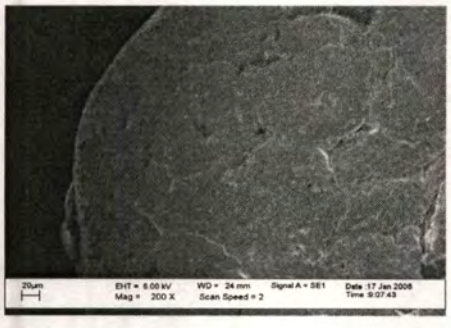
Figure 4.17 Surface of microsphere particles at different magnification of a) particle 1 b) particle 2 c) particle 3



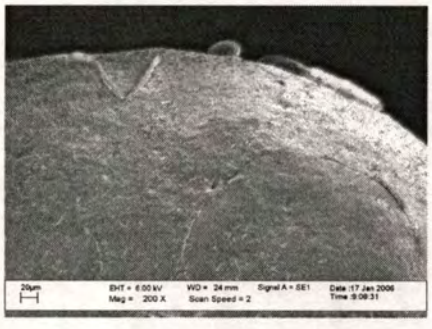
a) magnification 80x



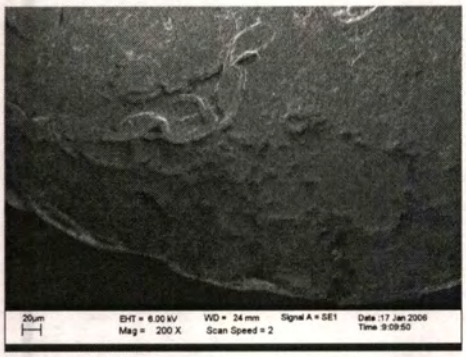
b) magnification 30x



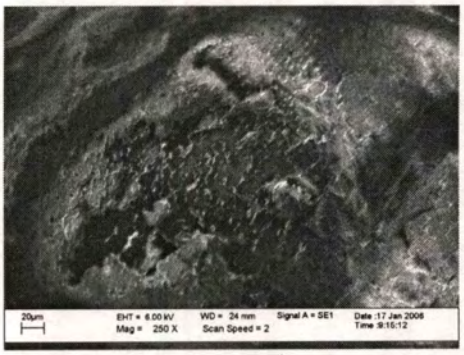
c) magnification 200x



d) magnification 200x



e) magnification 200x



f) magnification 250x

Figure 4.18 Surface morphology of microsphere particles at different positions a) particle 1 b), c), d) and e) particle 2 and f) particle 3

For all SEM pictures microspheres have non smooth surface and contain some pores. Pictures from particles 3 suggested that drugs might be contains inside microspheres.

Chapter 5
Conclusions

This research has focus on the preparation of microspheres from protein sericin of Thai silk. Chemical structures of sericin Nangnoi, Danchang, and Kaw suphan which characterized by FI-IR provided the similar pattern to amide functional groups. On the other hand, TGA thermograms suggested that each sericin composed of many differences fractions.

Under the suspension technique, the results from optical micrographs showed the microbubbles of protein sericin. However, the stability of these microbubbles were not enough for isolation due to low surface tension of bubble. Similarly results were found in the single emulsion system.

However, the stability of microspheres from sericin was obtained from double emulsion technique. Appropiate parameters for use in accordance with the present preparation include sericin from Nangnoi 1 g, vegetable oil 5 ml, drug 0.2 g. (Piroxicam), Span80 surfactant 0.1 ml. This can be accomplished through the temperature 150 ^{0}C. High temperature is more preferably to protein denaturization. Therefore, temperature of reaction is one of the most important parameters. Though glutaraldehyde has been reported as a protein crosslinking in many literatures, it was not succeed in this work. The results from Scanning Electron Microscope showed the average particles size of microspheres around 200-800 μm, non-smooth surface, various small pores and probably contain drugs inside. It was interesting to note that sericin from Danchang and Kaw suphan can not provided microspheres both in suspension and emulsion technique. This means that the molecular structure of sericin such as amino acid sequence and the repeating number of amino acid and conformation plays an important role.

Surface tension and viscosity of sericin solution, also, have influences on the microspheres formation because the high surface tension lead to the high viscosity.

In case of single emulsion , air can penetrated into sericin solution due to stirring. These air bubbles can struck inside sericin solution phase , as "a foaming liquid" due to viscosity of sericin solution.We can see these air bubbles through optical microscope but we can not isolated air bubbles from sericin solution phase.

In double emulsion, the drug (Piroxicam) can combined with molecule of protein by hydrogen bonding. Because their have the similar functional group called peptide bond (CONH). Hydrogen bond could be fulfilled as interior cohesion, resulting in droplets of microspheres formation. Then, this emulsion was pour into hot oil while stirring for denaturization of protein. Though the droplets of microsphere more dense than oil,they suspended in hot oil due to the continuous stirring.

Among three kinds of sericin only sericin Nangnoi give droplets of microsphere probably due to the suitable conformational structure.

Further suggestions

1. Sericin solution must be prepared before starting the experiment. Because the fresh solution is necessary.

2. Reaction time of double emulsion technique should be studied.

3. The other types of drug, sericin and protein crosslinking agent should be studied.

4. Drug contents in microsphere and drug diffusion property should be tested.

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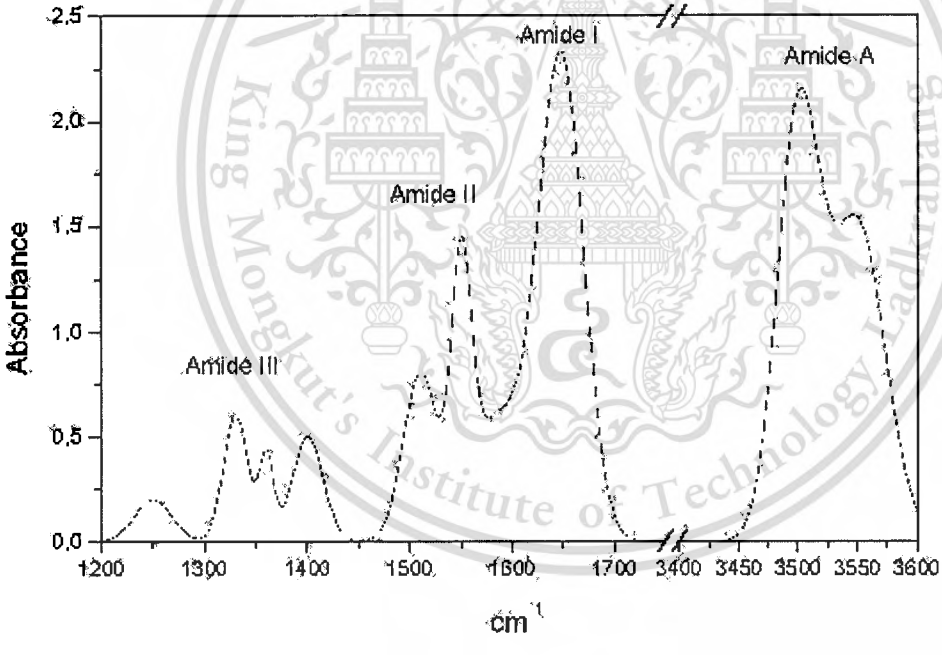
Appendix A

Amide vibrations [49]

The peptide group, the structural repeat unit of proteins, gives up to 9 characteristic bands named amide A, B, I, II ... VII. The amide A band (about 3500 cm^{-1}) and amide B (about 3100 cm^{-1}) originate from a Fermi resonance between the first overtone of amide II and and the N-H stretching vibration. Amide I and amide II bands are two major bands of the protein infrared spectrum. The amide I band (between 1600 and 1700 cm^{-1}) is mainly associated with the C=O stretching vibration (70-85%) and is directly related to the backbone conformation. Amide II results from the N-H bending vibration (40-60%) and from the C-N stretching vibration (18-40%). This band is conformationally sensitive. Amide III and IV are very complex bands resulting from a mixture of several coordinate displacements. The out-of-plane motions are found in amide V, VI and VIII.



Amide A is with more than 95% due to the the N-H stretching vibration. This mode of vibration does not depend on the backbone conformation but is very sensitive to the strength of a hydrogen bond. It has wavenumbers between 3225 and 3280 cm^{-1} for hydrogen bond lengths between 2.69 to 2.85 Å.

Amide I is the most intense absorption band in proteins. It is primilary goverend by the stretching vibrations of the C=O (70-85%) and C-N groups (10-20%). Its frequency is found in the range between 1600 and 1700 cm^{-1}. The exact band position is determined by the backbone conformation and the hydrogen bonding pattern.

Amide II is found in the 1510 and 1580 cm^{-1} region and it is more complex than amide I. Amide II derives mainly from in-plane N-H bending (40-60% of the potential energy). The rest of the potential energy arises from the C-N (18-40%) and the C-C (about 10%) stretching vibrations.

Amide III, V are very complex bands dependent on the details of the force field, the nature of side chains and hydrogen bonding. Therefore these bands are only of limited use for the extraction of structural information.

Amino acid side chain vibrations

The presence of bands arising from amino acid side chains must be recognized before attempting to extract structural information from the shapes of amide I and amide II bands. The contribution of the side chain vibrations in the region between 1800 and 1400 cm^{-1}. Among the 20 proteinogenous amino acids only 9 (Asp, Asn, Glu, Gln, Lys, Arg, Tyr, Phe, His) show a significant absorbance in the region discussed above. The contribution of the different amino acid side chains were fitted by a sum of Gaussian and Lorentziancomponents.

AS	vibration		cm^{-1}	A_0 (l/mol/cm)	FWHH (cm^{-1})	surface ($x10^{-4}$ l/mol/cm)
Asp	-COO st as	pH>pK (~4.5)	1574	380	44	5.5
	-COOH st	pH<pK (~4.5)	1716	280	50	4.1
Glu	-COO st as	pH>pK (~4.4)	1560	470	48	7.1
	-COOH st	pH<pK (~4.4)	1712	220	56	3.6
Arg	$-CN_3H_5^+$ st as		1673	420	40	4.3
	st s		1633	300	40	3.6
Lys	$-NH_3^+$ bd as		1629	130	46	1.8
	bd s		1526	100	48	1.3
Asn	-C=O st		1678	310	32	2.7
	$-NH_2$ bd		1622	160	44	2.5
Gln	-C=O st		1670	360	32	3.1
	$-NH_2$ bd		1610	220	44	3.5
Tyr	ring-OH	pH<pK (~10)	1518	430	8	1.0
	ring-O	pH>pK (~10)	1602	160	14	0.7
			1498	700	10	2.5
His	ring		1596	70	14	0.3

Phe	ring		1494	80	6	0.2
terminal						
	-COO st as		1598	240	47	3.5
	-COOH st		1740	170	50	2.1
	$-NH_3^+$ bd as		1631	210	54	3.8
	bd s		1515	200	60	4.3
	$-NH_2$ bd		1560	450	46	7.5

frequency, absorbance at the maximum (Ao), full width at half height (FWHH), surface of Gaussian band.

St = stretchingvibration
bd = bending
s = symetrical
as = asymmetrical.

Appendix B

Surface Tension [50]

Introduction

Water has many unusual properties as a result of its ability to hydrogen bond. For example, the density of ice is less than that of the liquid and the predicted boiling point is almost 200 degrees C higher than it would be without hydrogen bonding.

The water molecules at the surface of water are surrounded partially by air and partially by water. These surface molecules would be much more stable if they could be in the interior of the liquid where all their hydrogen bonds could be fulfilled (**cohesion**). Therefore, water normally tends to have the smallest surface possible, i.e. it has a high **surface tension**, in order to achieve the lowest possible energetic state.

If a solid material more dense than water is placed on the surface of water, what happens next depends on the nature of the material. If the material is **hydrophilic** ("water loving") it has a surface to which water is attracted. The **adhesion** of water to the surface of this material coats the surface of the object with water, reduces the surface tension, and causes the object to sink.

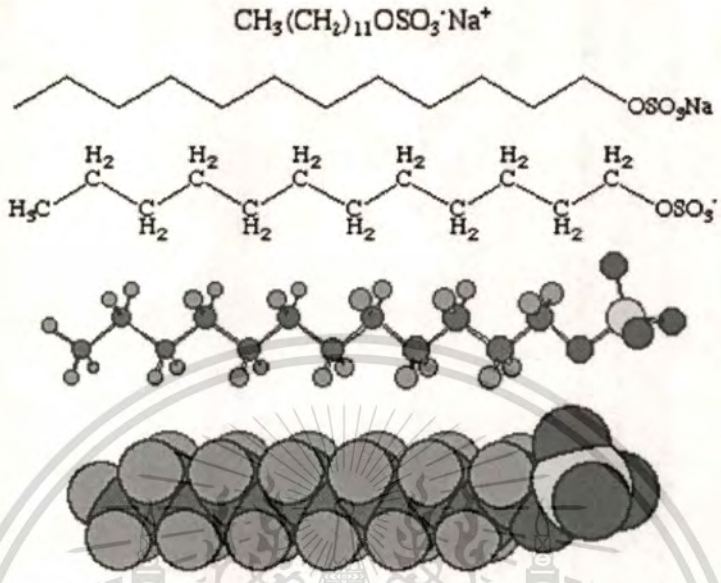
If the solid object is **hydrophobic** ("water fearing"),the unfavorable interactions between the water surface and the object make it difficult to wet the surface. Two forces now come into play -- the energy it would take to overcome this repulsion and the force of gravity. If the force of gravity is strong enough, it will prevail and the object will sink (assuming that the object has a density greater than water). If the gravitational force is less than the surface tension then the object will float on the surface of the water.

Surface tension is what permits water striders and other insects to walk across the surface of water and what enables a needle to float. Of course, the critical feature here is the amount of force per unit area -- put a needle into water end-on instead sideways and the needle will immediately sink.

How it works

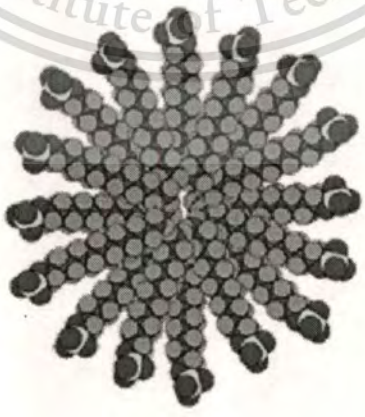
Detergents are a class of chemicals that contain hydrophobic (non-polar) hydrocarbon "tails" and a hydrophilic (polar) "head" group. This general class of molecules are called **surfactants**. Surfactants can interact with water in a variety of ways, each of which disrupts or modifies the hydrogen bonding network of water. Since this reduces the cohesive forces in water, this leads to reduction in the surface tension and our sulfur sinks.

A typical example of a detergent molecule is sodium lauryl sulfate (read that shampoo bottle of yours!). The structure can be represented in several different ways. Notice that in the models the Na ion has been left off because the anion and cation completely dissociate in water:



If you have the MDL Chime plug-in installed, you can play with this interactive 3-D model of a sodium lauryl sulfate molecule. You can rotate it, change the display features, enlarge/shrink, display solvent accessible surfaces and more...click and play:

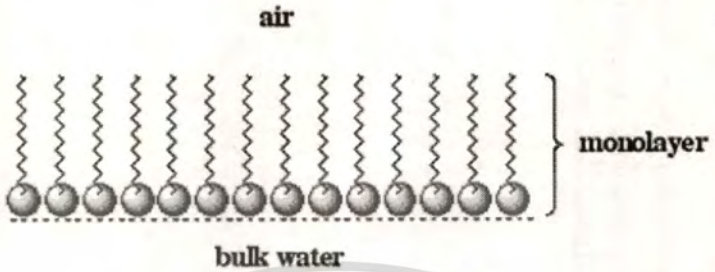
When a detergent is placed in water, the long non-polar hydrocarbon tails tend to aggregate because of favorable intermolecular interactions ("like dissolves like" in the interior and ion-dipole interactions at the exterior). The surfactant molecules thereby organize themselves into 3-dimensional spheres called **micelles** which have a hydrocarbon core and sulfate groups around the outer surface. Here's a 2-D representation:



Without detergent, we can not remove a greasy oily stain from clothing because grease and oil are large, non-polar, hydrophobic molecules. However, the interior core of a micelle is quite greasy just like an oily stain. When we

add detergent to our wash water, the oil or grease on our clothes can dissolve in the interior of the micelles and thereby go into solution.

Surfactants can also form other structures. Rather than form a sphere, some surfactants can coat the surface of the water to form a layer one molecule thick, a **molecular monolayer**. This is shown diagrammatically below:



A good example of a monolayer is oil on water. A small amount of oil can be spread over a large surface of water when the oil is only one monolayer thick! A variety of related structures are also known, particularly in cell walls (lipid bilayers etc.).

There are many, many other Real World examples and applications of surfactants! Here's just one: your body uses surfactants to reduce surface tension in the lungs. The human body does not start to produce lung surfactants until late in fetal development. Therefore, premature babies are often unable to breathe properly, a condition called Respiratory Distress Syndrome. Untreated, this is a serious illness and is often fatal, but administration of artificial surfactants virtually eliminates this health problem.

Appendix C

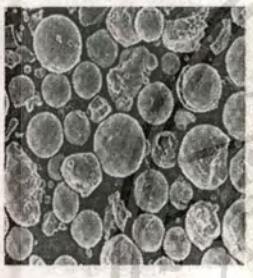
Powders and Granulates

Free-flowing powders and granulates are needed for a variety of industrial processes. These, however, do not always meet the exacting standards which modern manufacturing demands of them, due to their varying grain size distribution and odd shapes.

These properties are detrimental to efficient processing and lead to agglomeration, inexact dosage, abrading with loss of material, or low reproducibility of castings.

Pharmaceutical applications require highly reproducible dosage and the controlled release of active agents, which can not be achieved with conventional powders and granulates.

Powders manufactured by:

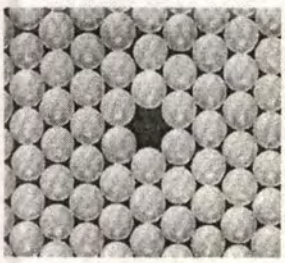


Spraying

Dispersion in liquids

The Answer to these Problems

The use of small and perfectly round Microspheres with exactly the same size circumvents all of the disadvantages that are encountered while using powders and granulates. These Microspheres are free-flowing and roll with practically no friction, that means there is no abrasion, guaranteeing a dust-free environment. Pharmaceuticals embedded in the Microsphere matrix are released continuously and at a constant rate.



Monodispersive alumina Microspheres

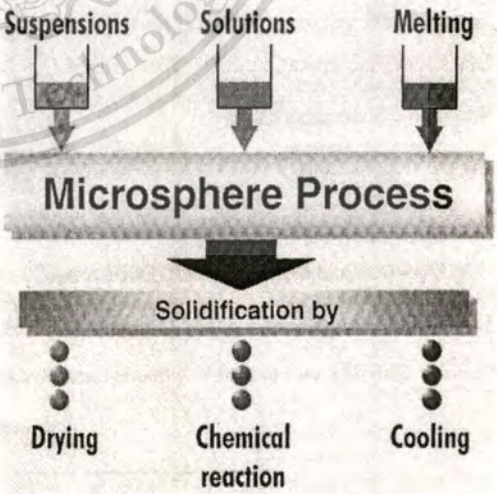
Ultra Spherical Microspheres

Microspheres with a monodisperse grain size distribution and the smallest divergence are manufactured by BRACE.

- perfectly spherical Microspheres
- monodisperse grain size, narrow size distribution with diameters between 50µm and 5000µm
- nonabrading, therefore dust-free
- free flowing, porous, large surface area, soft or rigid
- for embedding pharmaceuticals, biomass (e.g., yeast or enzymes) or other heterogeneous catalysts with or without coating.

The BRACE-Process

A liquid is gently pumped through a vibrating nozzle system whereupon exiting the fluid stream breaks up into uniform droplets. The surface tension of these droplets moulds them into perfect spheres in which gelation is induced during a short period of free fall. Solidification can be induced in a gaseous and/or liquid medium through cooling, drying, or chemical reaction. There are no constraints on the type of liquid—molten materials, solutions, dispersions, sols, or suspensions can be used to manufacture perfectly spherical Microspheres.



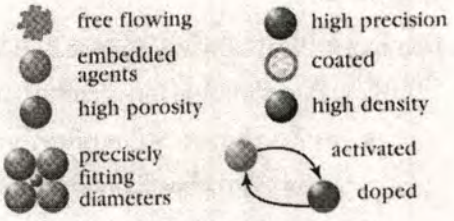
Types of Production Facilities

BRACE designs, constructs, and installs Microsphere production units according to customer needs. BRACE stocks a series of standard Microspheres and is able to produce Microspheres according to customer specifications. Additionally, BRACE offers the development of new encapsulation processes, enhancement of recipes or targeting new markets—from initial small scale up to full production and OEM manufacturing of spheres—is done by BRACE.

HARPER specializes in custom-designed process equipment incorporating state-of-the-art computer and PLC-based control instrumentation. System sizes range from pilot plant to fully automated production scale. Ancillary equipment, including fully automated material handling systems, can be provided as closed loop systems or interfaced to existing equipment.

Units are delivered with automated controls and can be delivered as remote controlled and enhanced solutions.

Types of Microspheres



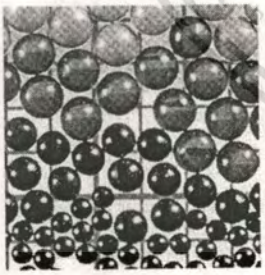
There are very few restrictions on the types of Microspheres than can be produced. With the right combination of liquid precursor, solidification process, and subsequent treatments, a wide range of Microspheres can be produced.

- Dry metal oxide Microspheres produced on the basis of a sol (Al_2O_3, ZrO_2, HfO_2, TiO_2, CeO_2, SiO_2, and mixed oxides) can be used as highly sinteractive press-feed for the production of high-tech ceramics. Through calcining, the pore size and surface area of the Microspheres can be tailored to exacting specifications.

These Microspheres make excellent catalyst carriers, homogeneous catalysts, or filtering materials. Unusually effective and abrasion resistant Microspheres for grinding other materials are made from sintered Al, Zr, and Hf-oxides.

Processing Characteristics

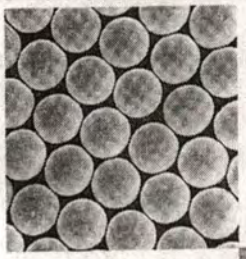
Microsphere production units have a minimal space requirement (15 to 40 ft²), the energy consumption is very low and they are noiseless during operation. These units operate at atmospheric pressure or slightly above and can be designed to be explosion-proof and/or according to the GLP/GMP guidelines. Microsphere production units from BRACE need practically no maintenance, therefore only a minimal staff is required.



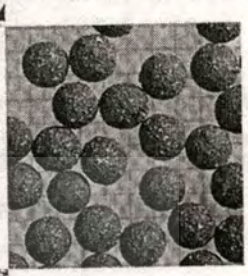
Metal oxide spheres as molded (yellow), dried (yellow transparent), calcined (black) and sintered (black, smallest). The shrinkage in diameter corresponds to their solid content during sintering.

- Microspheres produced from molten materials (inorganic, organic, alloys, and polymers) can be used for dosing, proportioning, compounding, coloring, and light stabilization. Microspheres with dissolved or embedded active agents, with or without coating, are used for numerous pharmaceutical and cosmetic products.
- Soluble chemical compounds can be incorporated into Microspheres by precipitation for use in the agricultural, food, pharmaceutical, and cosmetics industries.
- Suspensions are used to produce Microspheres with embedded enzymes or bacteria.
- With our special double nozzle systems, Microspheres with encapsulated materials can be obtained. Especially for the encapsulation of water, aqueous solutions or cells, a microsphere with a liquid core and a solidified shell can be produced. The shell and the core material can be chosen as appropriate: alginate, PVA, PEI, PEG, wax, metal oxides, gelatin, hydroxylcelluloses, etc.
- Mixing nozzle systems that allow mixing of agents are suitable to produce polymers by mixing the monomer with the catalyst "on time".

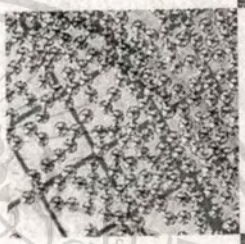
The finished Microspheres can be modified by subsequent washing, further chemical reactions, drying, calcining, sintering, impregnation, coating, coloring, sorting.



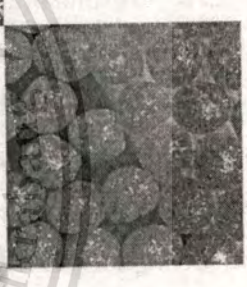
SEM image of SiO_2 Microspheres



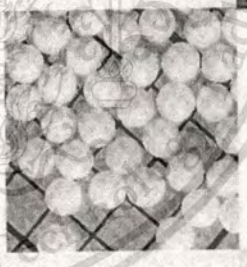
Filtering material out of Al_2O_3 with additives



Metal alloys for metallurgical and electronic applications

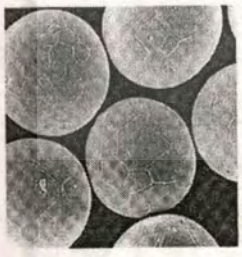


Pharmaceuticals embedded in Alginate for cosmetic use

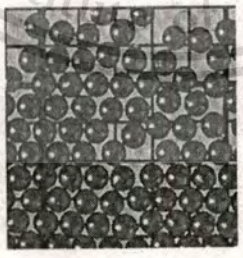


Pharmaceuticals embedded in wax

Colored polyethylene



SEM image of metal oxide Microspheres



Wax embedded liposomes