CHAPTER V
SILK SERICIN-ALGINATE NANOPARTICLES: PREPARATION AND ITS EFFICACY IN ANTI-INFLAMMATORY EFFECT

5.1 Abstract

In this study, silk sericin-alginate nanoparticles were prepared by emulsification method followed by internal crosslinking. The effects of silk sericin loading at various amounts on particle size, shape, thermal properties and release-characteristic were investigated. The initial silk sericin of 20, 40 and 80% w/w to alginate was incorporated into these alginate nanoparticles. The results from SEM images showed nanoparticles of about 71.30-89.50 nm with spherical shape. FTIR analysis suggested the interactions between alginate and sericin in the nanoparticles. TGA analysis showed no change in decomposition temperature with increasing initial silk sericin loadings. The release study was carried out in acetate buffer at the skin condition (pH 5.5; 32 °C). The release of silk sericin increased with increasing amount of silk sericin content. These silk sericin loaded-alginate nanoparticles were further incorporated into topical gel preparation and study their anti-inflammatory property using carrageenan-induced paw edema. The current study confirms the hypothesis that the application of silk sericin-alginate nanoparticles gel can inhibit the inflammation induced by carrageenan. The silk sericin-alginate nanoparticles gel's anti-inflammatory properties can allow for better wound healing.

Keywords: Silk sericin; Nanoparticles; Alginate; Carrageenan-induced paw edema; anti-inflammation

5.2 Introduction

Silk sericin (SS) is a globular protein, consisting of 18 amino acids, that is obtained from silk cocoons. SS acts as an adhesive and holds the two fibroin filaments together, leading to a cocoon formation. Because of tremendous studies on the advantages and biological properties of SS, this protein has become attractive in

many industries. SS provides a wide range of applications; antioxidant [1-5], anticoagulation [6], and anti-wrinkle [7]. This protein is also reported to suppress tumor growth [8, 9], and to reduce oxidative stress [9]. Panilaitis et al. (2003) concluded that SS itself does not significantly activate macrophage. In addition, the SS peptides have no immunogenicity in *in vivo* experiments and can be used in biomedical applications [11, 12]. SS accelerates cell proliferation and increases cell viability in various cell lines such as hybridoma [13], insulinoma [14] and human skin fibroblast [15]. Moreover, UVB-induced apoptosis in human skin keratinocytes can be inhibited by SS [4].

In wound application, the proliferation and attachment of human skin fibroblasts can be promoted when cultured in SS media [15] and SS also increases collagen production [16]. According to Aramwit et al. (2013), SS also plays a role in the suppression of carrageenan-induced inflammation which promotes wound healing.

Regardless of the myriad of benefits associated with SS, the SS solution is unstable and degrades due to pH and temperature. This instability makes the SS solution difficult to use. It is proposed in the current research that a SS gel preparation will be more beneficial and convenient for topical usage due to the nature of gels; easy application, simple formulation, and the ability to topically deliver a wide variety of active agents. Therefore, a SS gel and an encapsulated SS gel will be prepared in this study, to postulate that more stable and sustainable release of SS will be obtained.

In the pharmaceutical industry, encapsulation is the technique that is widely used to sustain or prolong drug release, to stabilize drugs from heat, moisture, light or vaporization, and reduce drug toxicity. Alginate microparticles have been used for the encapsulation of abundant biological and bioactive materials such as proteins [18-20], enzymes [21], antibodies [22], cells [23] and DNA [24]. Alginates are natural biodegradable polymers obtained from kelps (brown algae). The gelation of alginate can be simply induced by various divalent cations, especially Ca^{2+} which binds to two carboxyl groups on the neighboring alginate molecules resulting in the ionic inter-chain connections [25].

Entrapment of protein into alginate microparticles has several advantages; alginate matrix protects protein from environments, provides mucoadhesive property, possess low toxicity and low immunogenicity [26]. Hence, a mild protein encapsulation technique, emulsification followed by internal gelation, will be performed in the present work to fabricate silk sericin-alginate nanoparticles without the degradation of SS during the experiment.

The aim of the current study is to prepare SS-alginate nanoparticle and investigate their morphology, size, chemical structure, thermal properties and the release profile of SS from the SS-alginate nanoparticles. A final aim of this study is to formulate the SS-alginate nanoparticles topical gel and to further study the in vivo anti-inflammatory effect. It is hypothesized that the application of SS-alginate nanoparticles gel would inhibit the inflammation induced by carrageenan.

5.3 Materials and Methods

5.3.1 Materials

The fresh white cocoons from silkworms; Bombyx mori were provided by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand) which was produced in a well-controlled environment. Silk sericin was isolated from cocoons using a high temperature and pressure degumming technique [Lee, 2003]. Briefly, the silk cocoons were finely cut into small pieces and soaked in purified water (ratio of dry silk cocoon: purified water = 1 g: 30 mL). Subsequently, the mixture was degummed using autoclave (Tomy Seiko SS-320, Japan) at 120 °C for 60 min. Then, fibroin which water-insoluble was removed from the obtained solution by filtration and the SS solution was concentrated until the desired concentration is achieved, which determined by BCA Protein Assay Reagent (Pierce, Rockford, IL, USA).The obtained SS has molecular weight of ~ 25-150 kDa, as reported previously [27].

Sodium alginate was purchased from Aldrich (USA). Calcium carbonate ($CaCO_3$) and Span® 80 were obtained from Fluka (USA). Paraffin oil was supplied from Carlo Erba Reagents (Italy). Glacial acetic acid, isopropyl alcohol, and sodium acetate were purchased from Sigma-Aldrich (USA). All chemicals were used as received without further purification.

Male Albino Wistar rats (4 weeks old, weighing 150-200 g) were used for the anti-inflammatory study by carrageenan induced rat paw edema assay. Animals were housed in a standard laboratory condition of temperature (25 ± 2 °C), a 12 h/12 h light/dark cycle with free access to standard food (C.P. Company, Thailand) and water *ad libitum*. The animals were allowed to acclimate for 3-5 days before the experiments. At the end of experiment, the animals were sacrificed using carbon dioxide. The experimental procedures were carried out under sterile conditions in accordance with local guidelines for the care of laboratory animals of faculty of Pharmaceutical Sciences, Chulalongkorn University (Bangkok, Thailand).

5.3.2 Preparation of Alginate and SS-alginate Nanoparticles

Alginate nanoparticles were successfully prepared by using emulsification method and followed by internal crosslinking [28]. First, the certain amount of sodium alginate was completely dissolved in distilled water and then mixed with 5% (w/v) calcium carbonate aqueous solution by an ultra-turrax mixer for 5 min. The obtained solution was then poured into the mixture of paraffin oil and 1% (v/v) Span® 80 and mixed for another 15 min to allow the nanoparticles formation. Subsequently, paraffin oil and glacial acetic acid mixture was added to the oil phase to ionized calcium ion with continuous stirring for more 30 min, at this step the crosslinking of alginate nanoparticles occurred. Finally, the alginate nanoparticles were harvest using centrifugation and washed with isopropanol to get the desired alginate nanoparticles. For SS-alginate nanoparticles, the same procedure was carried out except in the first step SS was mixed with sodium alginate solution to achieve the content of SS ~ 20, 40 and 80 wt% bases on the weight of sodium alginate polymer.

5.3.3 Characterization and Testing

SS-alginate Nanoparticles Characterizations

5.3.3.1 Morphology Observations

The morphology of the prepared alginate and SS-alginate nanoparticles was examined using a scanning electron microscope (SEM, Hitachi S-4800, Japan). Prior to SEM operating, the specimens were dispersed onto a conductive tape and sputter-coated to achieve a thin layer of platinum under vacuum.

Then the specimens were imaged at magnification of 50,000 and 100,000 times with incident electron beam energy of 10 kV. The average size of alginate and SS-alginate nanoparticles were also determined by Semafore 5.21 software. At least 30 measurements on different nanoparticles were averaged to obtain a data point (n = 30).

5.3.3.2 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

To identify the interaction between alginate and SS in SS-alginate nanoparticles, FTIR analysis was assessed using an FTIR Spectrophotometer, (NEXUS-870, Thermo Nicolet Corporation) equipped with deuterated triglycine sulfate detector. Spectra were measured in the spectral region from 4000 cm^{-1} to 400 cm^{-1} in the transmission mode at a resolution of ± 4 cm^{-1} with scan frequency of 32 times. Optical grade potassium bromide was applied as the background measurement material to subtract from sample readings.

5.3.3.3 Thermal Analysis

Thermal behavior of alginate and SS-alginate nanoparticles were analyzed through Thermo-gravimetric analysis (Pyris Diamond TG-DTA, Perkin Elmer). Five milligram of each sample was heated in aluminum crucibles from ambient temperature to 650 °C with a constant heating rate of 10 °C/min in a nitrogen atmosphere and the percentage of weight loss was recorded over temperature.

5.3.3.4 In vitro Release Study

The release study of SS from SS-alginate nanoparticles was performed in acetate buffer (pH 5.5) at the skin condition of temperature 32 °C. To prepare acetate buffer, 150 g of sodium acetate was dissolved in 250 mL of distilled water. Subsequently, 15 mL of glacial acetic acid was slowly added into as-prepared sodium acetate solution. Eventually, the volume of solution was then adjusted to 1000 mL with distilled water.

For in vitro release characterization the experiments were carried out with the following procedures, approximately 300 mg of SS-alginate nanoparticles were incubated in 30 mL of citrate buffer which was maintained at 32 °C and 200 rpm during the assay. At specific time, between 0 min to 7 d, 100 µL of release medium was sampling to analyze the amount of SS released using a Pierce ®

bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, USA). Three parallel experiments were conducted and the results were presented as mean values.

Gel containing SS-alginate nanoparticles Characterization

5.3.3.5 Physical Appearance of Gel Formulation

Formulate gel preparation were visually inspected for their color, phase separation, and homogeneity. Also, the texture and skin feel when apply SS-alginate nanoparticles gel onto skin was investigated.

5.3.3.6 Accelerated Stability Study

The selected gel formulations were subjected to the accelerated stability test. Briefly, gel formulation was centrifuge at 3000 rpm or 10000 rpm for 5-30 min to investigate any changes in appearance which described the stability of the formulation.

5.3.3.7 Anti-inflammatory Evaluation

Anti-inflammatory property of selected SS-alginate nanoparticles gel and SS gel was evaluated using the carrageenan-induced edema model [29]. More specific, Forty-two Wistar rats were randomly distributed into seven groups of six rats for receiving topical gel treatment. The rats of control group were treated with gel base or alginate nanoparticles gel while the rats served as reference group were treated with diclofenac gel (Difelene Ltd). The four experimental groups received different formulations of 20 % SS- alginate nanoparticles gel, 80 % SS- alginate nanoparticles gel, 20 % SS gel and 80 % SS gel, respectively. Animals were fasted for 6 h before the experiment with free access to water.

Each rat was weighed and marked on right hind paw behind tibiatarsal junction, and then measured the paw volume as a baseline by water displacement using a plethysmometer (Model 7140, Ugo Basile, Comerio, Italy). 2.5 g of the gel formulations or the reference gel or gel base was applied topically onto the plantar surface of the right hind paw and rubbed for 15 sec until completely absorbed. 1h after the treatments, 0.1 ml of 1% w/v carrageenan solution was subcutaneous inject to the right hind paw. The paw volumes according to the inflammatory response were measured using a plethysmometer at 1 h time intervals

till 6 h after carrageenan injection. The percentage of edema inhibition was calculated in comparison to control group using following formula:

$$\text{Percentage of Inhibition} = \frac{(V_t - V_0)_{control} - (V_t - V_0)_{test}}{(V_t - V_0)_{control}} \times 100 \quad (1)$$

where Vt is paw volume at 1, 2, 3, 4, 5 and 6 h after carrageenan injection, V_0 is paw volume before the treatment

5.3.3.8 Histological Evaluation

After the carrageenan test, the animals were euthanized by carbon dioxide inhalation. The hind paws were removed and fixed by immersion in 10% neutral buffered formalin and decalcified. The tissues from paws were dissected and embedded in a paraffin block and thin sections (4 µm) were prepared. Subsequently, the tissue sections were mounted on glass slides and stained with Hematoxylin-Eosin (H&E) and captured digital images by Olympus BX50 optical microscope. At least ten fields from the inflammatory site were randomly observed in each section. The measurement of the inflammatory cell response, edema, hemorrhage, and necrosis were performed following International Organization for Standardization (ISO) 10993-1, 2003; Biological evaluation of medical devices-Part 1: Evaluation and testing was within a risk management process (Geneva, Switzerland).

5.4 Statistical analysis

Data are expressed as means ± standard errors of means (n = 3). Statistical analysis was performed based on the ANOVA followed by Tukey's test. The statistical significance for the entire test was accepted at a 0.05 confidence level.

5.5 Results and Discussion

SS-alginate nanoparticles Characterizations

5.5.1 Morphology Observations

The SEM images of the alginate nanoparticles and the SS- alginate nanoparticles are shown in Figure 5.1. All the particles were nearly spherical with some deflated surfaces and reasonably uniform in size in the range of 71.30-89.50 nm. The average size of the SS- alginate nanoparticles was found to be slightly smaller than alginate nanoparticles. As the alginate to SS mass ratio decreased from 5:1 to 1.25:1, the average size of SS-alginate nanoparticles was slightly decreased as shown in Table 5.1.

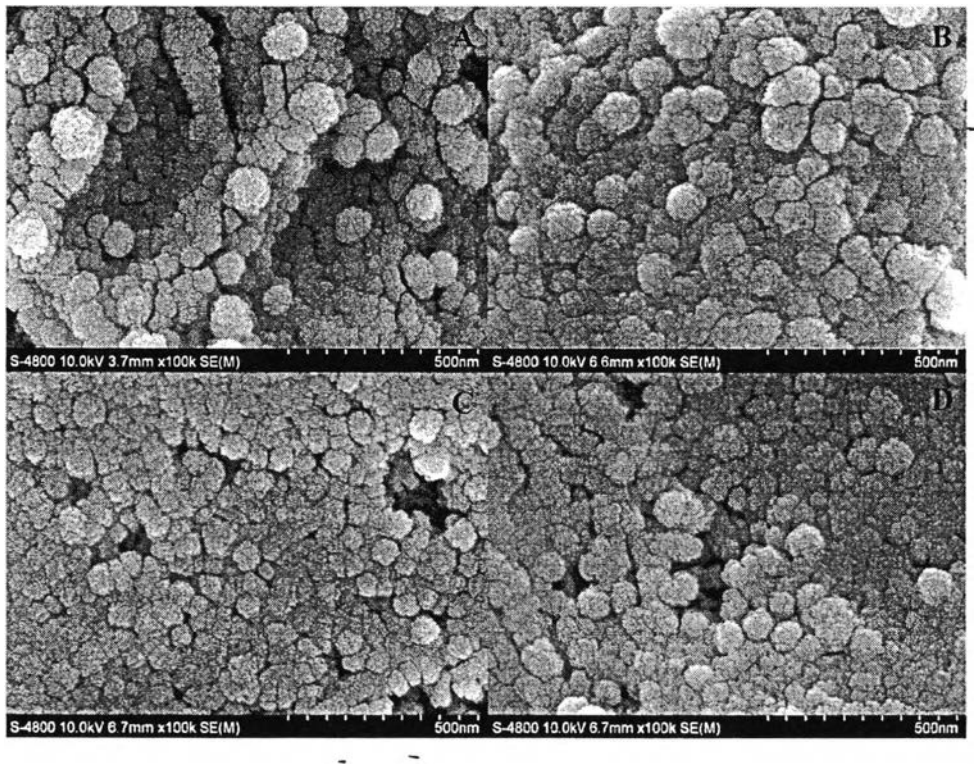

Figure 5.1 SEM micrographs of (a) alginate nanoparticles and SS-alginate nanoparticles at various SS contents: (b) 20 wt%, (c) 40 wt% and (d) 80 wt% relative alginate with a magnification of 100 000x.

Table 5.1 Average particles size of as-prepared nanoparticles

Sample	Alginate:SS mass ratio	Average particle size (nm)
Alginate nanoparticles	-	89.5
20 % SS-lginate nanoparticles	5:1	88.4
40 % SS-lginate nanoparticles	2.5:1	79.2
80 % SS-lginate nanoparticles	1.25:1	71.3

5.5.2 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectra were used to determine the chemical functional groups and molecular interactions between alginate and SS as shown in Figure 5.2. The slightly difference can be observed between alginate nanoparticles and the SS-alginate nanoparticles at various SS content. The strong and broad peaks in the 3500-3300 cm^{-1} ranged corresponding to O-H stretching and intermolecular hydrogen bonding. Around the wave numbers of 1700-1400 cm^{-1}, the observed peaks belonged to the C=O stretching (amide). The FTIR spectrum of the alginate nanoparticles shows two distinctive absorption bands at around 1609 cm−1 and 1419 cm−1 relative to both asymmetric and symmetric C=O stretching absorptions of the carboxylic acid salts. As would be expected, the band intensities of the alginate's distinctive absorptions (C=O symmetric stretching and saccharide bands) slightly shifted to a higher wave number and decreased as the SS content increased as shown in Figures 2, suggesting interactions between alginate and SS in the nanoparticles. These interactions may be the hydrogen bonds between the carbonyl groups of SS and hydroxyl groups of alginate.

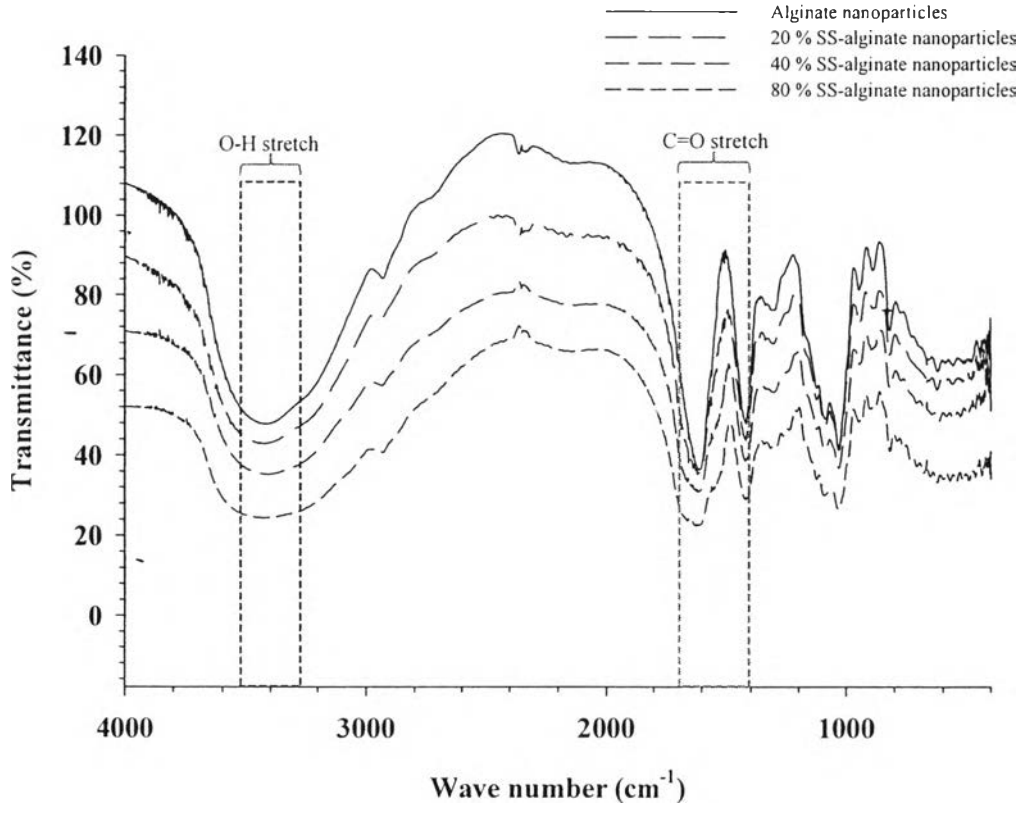

Figure 5.2 FTIR spectra of the alginate nanoparticles and SS-alginate nanoparticles.

5.5.3 Thermal Analysis

The TGA curves of alginate nanoparticles and the SS- alginate nanoparticles are shown in Figure 5.3. There was no change in decomposition temperature when increasing SS content. Two regions of degradation were observed, first begins from 240 °C to 270 °C and second from 280 °C to 480 °C.

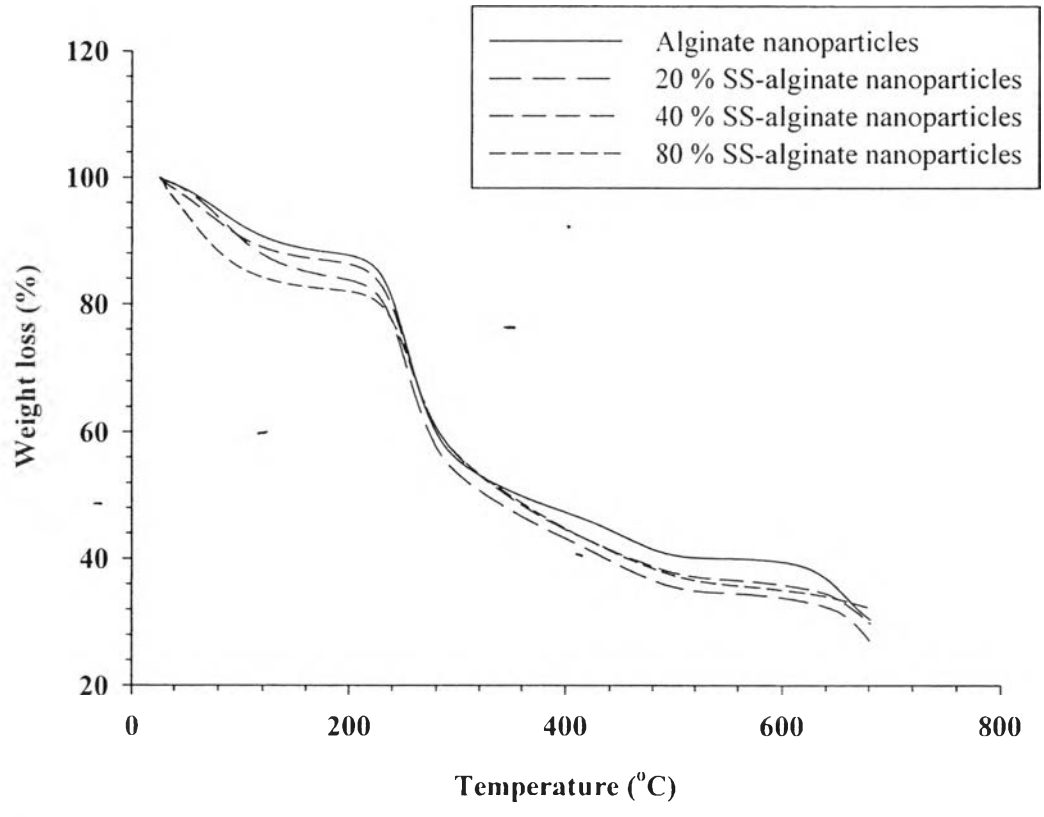

Figure 5.3 TGA curves of alginate nanoparticles and the SS- alginate nanoparticles.

5.5.4 *In Vitro* Release Study

The release characteristic of SS from SS-alginate nanoparticles was investigated by the total immersion method in acetate buffer at the skin condition. The cumulative amount of SS released from these materials is reported in Figure 5.4. Evidently, the cumulative amount of SS released from these samples in the acetate buffer occurred rather rapidly during the first 8 h after submersion in the releasing medium and it increased gradually afterwards. As expected, the maximum amount of SS released from these materials increased with increasing the amount of SS loaded in alginate nanoparticles.

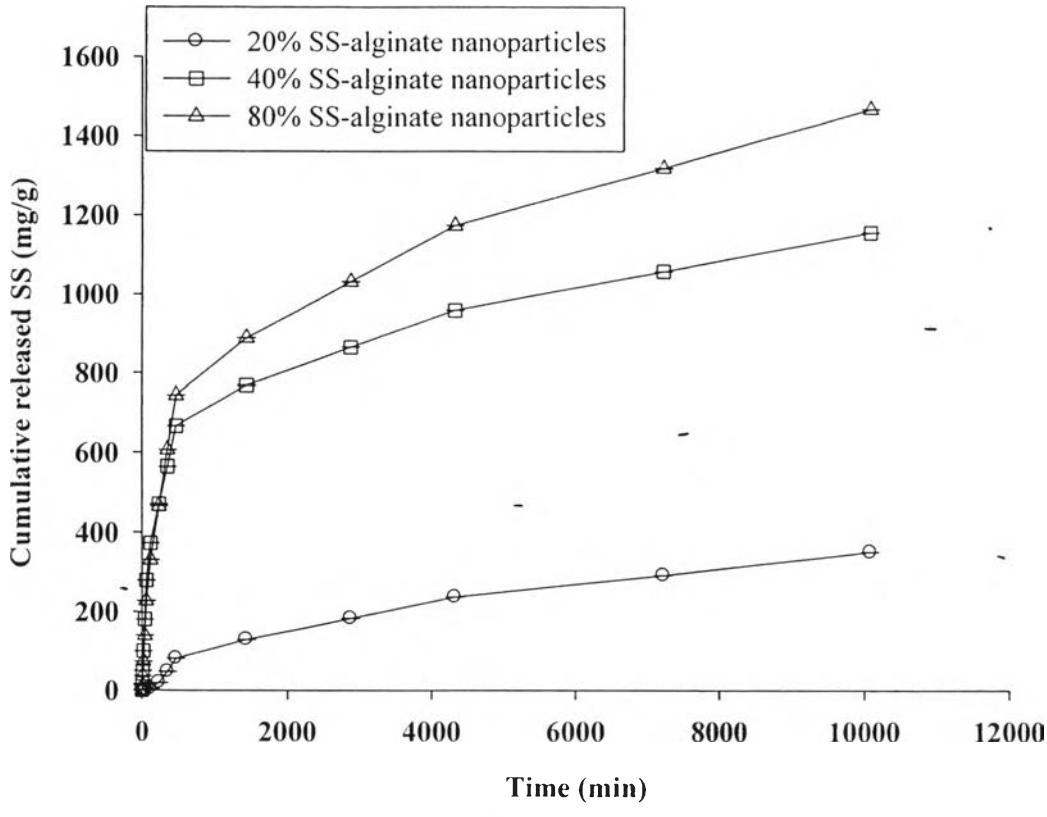

Figure 5.4 Cumulative release profiles of SS from SS-alginate nanoparticles. The error bars represent the standard deviation based on three replicates analysis (*$p<0.05$).

Gel containing SS-alginate nanoparticles Characterization

5.5.5 Physical Appearance of Gel Formulation

Figure 5.5 illustrates the as-prepared gel formulations. All formulation was rubbed on the back of the hand and no noticeable solid components found which can be implied that all samples had a uniform consistency. Also, any irritation was not observed upon application of gel samples to the skin.

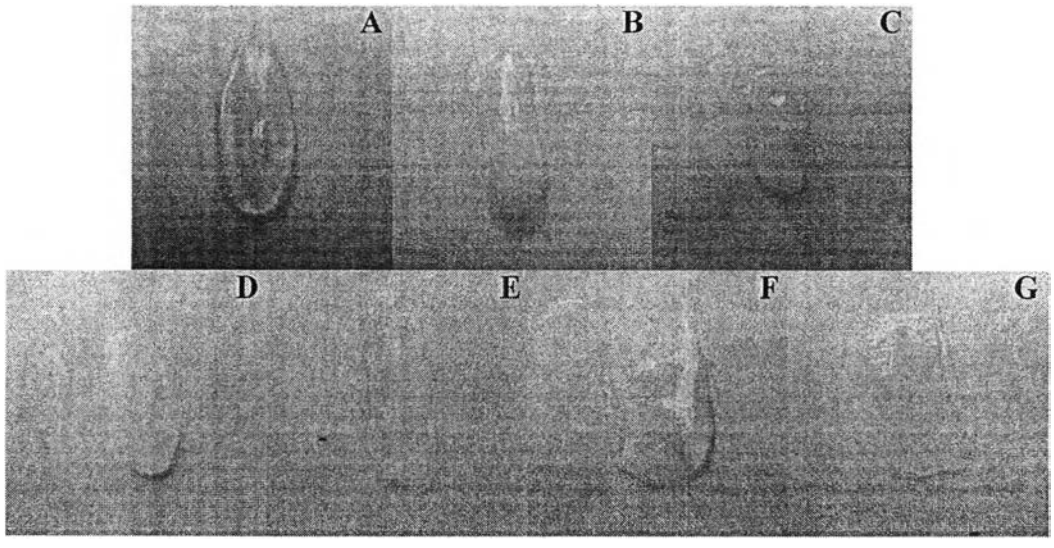

Figure 5.5 Gel formulations a) gel base, b) diclofenac gel, c) alginate nanoparticles gel, d) 20 % SS-alginate nanoparticles gel, e) 80 % SS-alginate nanoparticles gel, f) 20 % SS gel, and g) 80 % SS gel.

5.5.6 Accelerated Stability Study

The gel formulation was performed stability studies by centrifuge test. The results of the accelerated stability studies revealed that the formulation was stable under accelerate conditions, no significant alteration in the physical appearance of the gels after subjecting the formulations to 3000 rpm and 10000 rpm for 30 min.

5.5.7 Anti-inflammatory Evaluation

The pharmacological screening was performed by the carrageenan-induced edema model to evaluate the possible anti-inflammatory activity of the as-prepared gel formulation. Subcutaneous administration of carrageenan into the right hind paw produced significant edema, with a maximum response being observed 3-4 h after carrageenan injection, as shown in Table 5.2.

Table 5.2 Average rats' paw edema volume of the treatments in carrageenan-induced paw edema

Treatments	Paw edema (mL) ± S.E.M.					
	1 h	2 h	3 h	4 h	5 h	6 h
Gel base	0.56±0.08	0.61±0.11	0.810.08	1.17±0.12	0.94±0.07	0.85±0.11
Diclofenac gel	0.52±0.11	0.50±0.09	0.60±0.08	0.46±0.09	0.65±0.09	0.65±0.07
20 % SS gel	0.69±0.05	0.96±0.06	1.21±0.10	1.36±0.12	1.14±0.11	1.16±0.12
80 % SS gel	0.64±0.07	0.90±0.10	0.99±0.13	1.01±0.09	1.31±0.10	0.98±0.09
Alginate nanoparticle gel	0.78±0.10	0.95±0.16	1.25±0.16	1.03±0.14	1.08±0.14	0.79±0.12
20 % SS-alginate nanoparticle gel	0.80±0.10	0.90±0.14	1.11±0.12	0.91±0.16	0.74±0.08	0.72±0.13
80 % SS-alginate nanoparticle gel	0.73±0.14	0.73±0.11	0.95±0.16	0.68±0.15	0.68±0.09	0.45±0.07

The percentage of edema inhibition is shown in Figure 5.6. The anti-inflammatory effect of diclofenac gel (commercial) achieved the highest percentage of edema inhibition about 60.46% at 4 h after carrageenan injection. While, all SS formulations exhibited less anti-inflammatory effect when compare to diclofenac gel. In addition, edema inhibition of 80% SS-alginate nanoparticles gel and 20 % SS-alginate nanoparticles were comparative better and longer period compare to 80 % SS gel and 20 % SS gel. Hence, the SS-alginate nanoparticles gel provided the sustain release of SS as hypothesized.

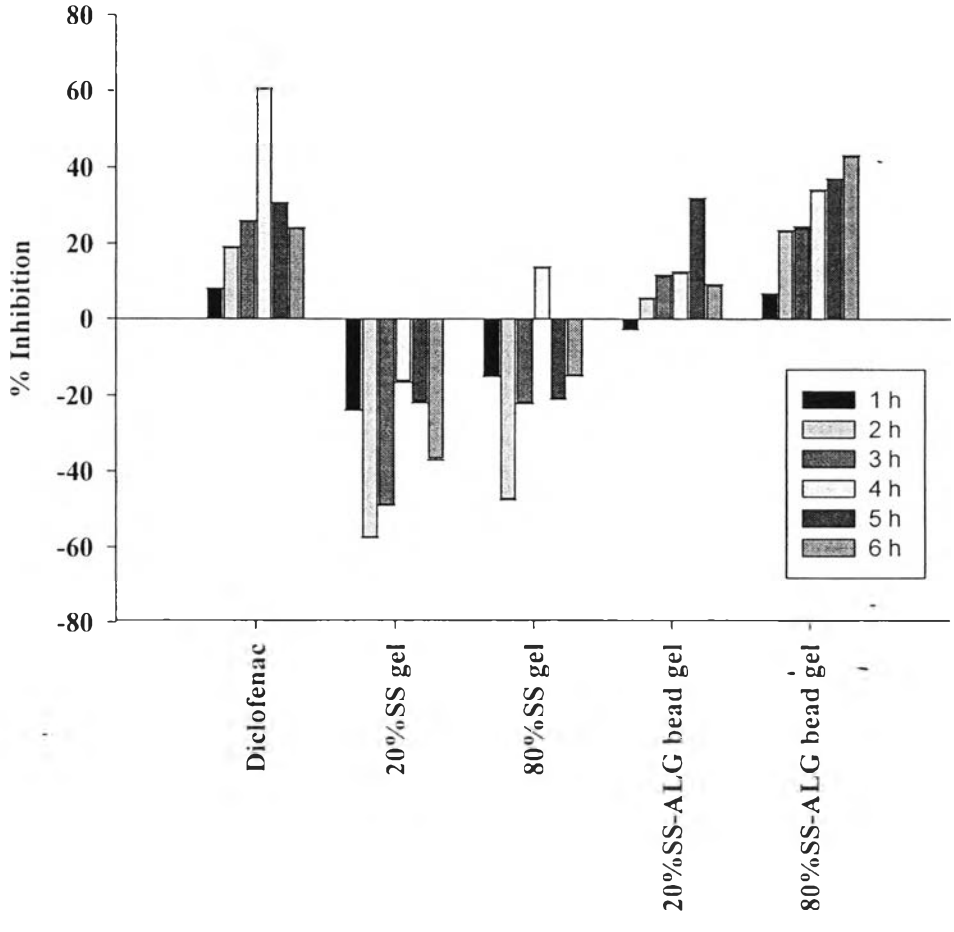

Figure 5.6 The percentage of edema inhibition from sericin of different formulations at various time points.

5.5.7 Histological Evaluation

According to the histological examination, paw biopsies revealed pathologic changes after carrageenan injection, including cellular infiltration and edema as shown in Figure 5.7. In rats treated with commercial diclofenac gel, no sign of tissue damage or cellular infiltration was observed when compared to untreated rats. In the control groups treated with gel base and alginate nanoparticles gel, polymorphonuclear cells (PMN) which play a central role in inflammation were found in a high number. Also, edema and hemorrhage were observed which indicate highly inflammation. There were less cellular infiltration and less edema in rats treated with 80% and 20% SS-alginate nanoparticles gel. While 80 % SS gel and 20 % SS gel treated tissues prior to carrageenan injection showed a massive cellular infiltration in the dermal layer.

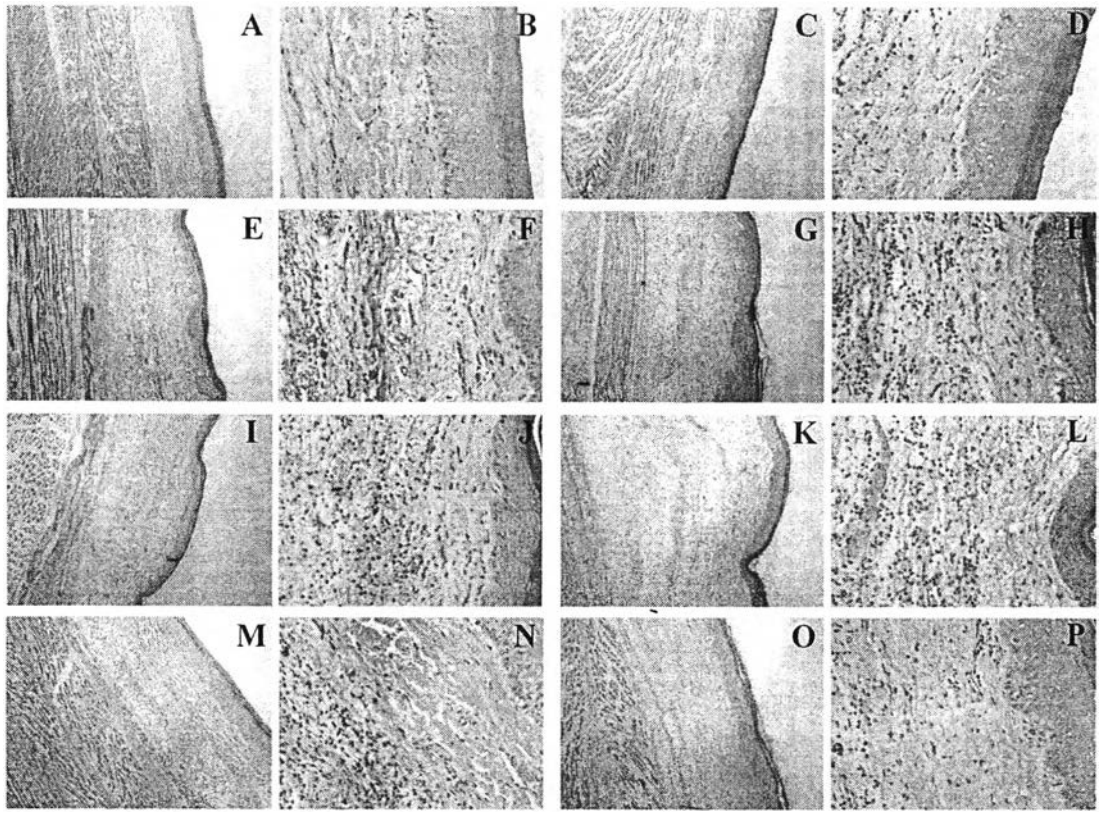

Figure 5.7 Histopathological examinations in rats' tissue after 6 h at 1% carrageenan injection (100X, 400X). (A), (B) Normal histological structure of the epidermal and dermal layers with no obvious cellular infiltration. (C), (D) diclofenac gel-treated tissues. (E), (F) gel base-treated tissues and (K), (L) alginate NPs gel show massive cellular infiltration, edema and hemorrhage. (G), (H) 20% SS gel-treated tissues and (I), (J) 80% SS gel-treated tissues. (M), (N) 20% SS-alginate NPs gel-treated tissues and (O), (P) 80% SS-alginate NPs gel-treated tissues showed less cellular infiltration in the dermal layer compared with the control.

5.6 Conclusions

The current study has shown that SS can be incorporated into alginate nanoparticles by simple method. SS loading at various amounts slightly affected particle size and amount of SS released. These as-prepared SS-alginate nanoparticles were further formulated to topical gel. The gel preparation was stable under

accelerated condition. Eventually, the application of SS-alginate nanoparticles gel can sustainably inhibit the inflammation induced by carrageenan.

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