CHAPTER III

RESEARCH METHODOLOGY

Materials and Methods

Chemicals

1. HPLC method validation

   Acetonitrile HPLC grade (Labscan Asia, Thailand)

   Distilled water

   Potassium orthophosphate (Sigma, Germany)

   Dipotassium phosphate (Sigma, Germany)

   80% phosphoric acid (Labscan Asia, Thailand)

2. Niacinamide loaded sericin nanoparticles Preparation

   Dowcorning™ 345

   Dowcorning™ 5225C

   Sericin powder (Thailand institute of nuclear technology)

   Niacinamide (Cosmetic grade, Namsiang)

   Calcium chloride (Merck, Germany)

   Ethanol 95% (Sigma, Germany)

   Acetone (Labscan Asia, Thailand)

Apparatuse

HPLC (SIL -20ACHT, Shimatzu)

Particle size analyzer (ZetaPAL® Brookhaven instruments cooperation, USA)

Scanning electron microscopy (Model 1455vP, LEO Electron Microscopy Ltd., UK)

Ultracentrifuge (Model Mikro 120, Hettich, Germany)

pH meter (Model delta 320, Mettle Toledo, China)

Platinum EPS C18 100A Rocket Column 3 μm (53mm x 7 mm)

Silverson®LM Homogenizer

Hot plate stirrer (Model RCT M, Ika

Ultracentrifuge (Model Mikro 120, Hettich, Germany)

HPLC chromatographic conditions

The analysis of niacinamide was quantified using HPLC (SIL -20ACHT, Shimatzu). The samples were detected using reverse-phase column (Platinum™ C18-EPS (53x7mm, 3 μm)). The injection volume was 20 μl and the temperature of column was maintained at 35^0C. The mobile phase was consisted of 50 mM potassium phosphate buffer (pH 7.0) and acetonitrile (99:1). The flow rate was 1.0 ml/min and time of analysis was 10 minutes. The absorbance was measured at 220 nm.

Method for validation

As the HPLC column was changed during the study, method validations were conducted.

Method 1

Calibration curve

Ten milligrams of standard niacinamide were placed in a 10 ml volumetric flask and dissolved in distilled water (stock solution). Six additional calibration levels were prepared by diluting this solution 1:1 with distilled water. Within the range of concentrations injected (200 – 3.725 μg/ml), the combined calibration data (the limit of detection will be determined by serial dilution and calibration curves were generated by linear regression based on peak area)

Limit of detection (LOD)

The lowest concentration that can be detected but not quantified accurately is defined as the detection limit. An estimation of the detection limit can be achieved by the determination of the signal/noise ratios of 3:1.

Precision (intra and inter-day) of assay was verified by analyzing sample 3 dilutions on 3 consecutive days.

Accuracy

Accuracy was determined by a comparison between the theoretical concentrations of standard added to sample solution and those obtained within the chromatographic analysis. Each sample solution was injected in triplicate.

Method 2

Method 2 was used for skin permeation study.

Calibration curve

Ten milligrams of standard niacinamide were placed in a 10 ml volumetric flask and dissolved in distilled water (stock solution). Seven additional calibration levels were prepared by diluting this solution 1:1 with distilled water. Within the range of concentrations injected (100 – 0.0977 μg/ml), the combined calibration data (the limit of detection and quantification will be determined by serial dilution and calibration curves were generated by linear regression based on peak area)

Limit of detection (LOD)

An estimation of the limit can be achieved by the determination of the signal/noise ratios of 3:1

Limit of quantification (LOQ)

The lowest concentration that can be quantified with acceptable accuracy and precision is defined as LOQ.

Precision and reproducibility

Precision (intra and inter-day) of assay was verified by analyzing dilutions of samples on 3 consecutive days.

Accuracy

Accuracy was determined by a comparison between the theoretical concentrations of standard added to sample solution and those obtained within the chromatographic analysis. Each sample solution was injected in triplicate.

Optimization of sericin nanoparticle preparation

The niacinamide-loaded sericin nanoparticles were prepared by water in silicone emulsion technique as follow: Sericin solution was prepared by dissolving sericin powder in distilled water at room temperature. Then, the niacinamide was dissolved directly into the sericin solution to a final concentration of 20% (w/w of polymer). The sericin-niacinamide solution was then emulsified into Dow Corning 345 containing 5% w/w Dowcorning™ 5225C under mechanical stirring (6500 rpm). The system was maintained under mechanical stirring for 15 minutes and then the ionic gelation of the sericin nanospheres was achieved by adding calcium chloride ($CaCl_2$) solution with equal volume of sericin polymer solution. After a prefixed cross-linking time of 24 hours, the nanospheres were isolated by centrifugation with 12,000 rpm for 10 minutes. The silicone residue was removed by washing with 95% of ethanol for three times. The dried particles were stored at room temperature. Some

parameters that influenced the particles formation were also studied such as calcium chloride concentration (2%, 4%, 6% and 8%), sericin concentration (0.5%, 1% and 2%), niacinamide concentration (0.5%, 1%, 5% and 20%), homogenizing time (15, 30 and 60 min) and speeds of homogenizer (6500, 9500 and 13500 rpm).

Characterization of sericin nanoparticles

Particle size and polydispersity index (PI)

The sizes of sericin particles were evaluated by ZetaPALS® (Zeta Potential and Particle Size Analyzer) using distilled water as a liquid medium. The mean particle size and PI values of the aqueous sericin nanoparticles dispersions were obtained by the auto-measuring mode at a fixed angle of 90 °C, the wavelength of 659 nm in 10 mm diameter cell. All samples were diluted with distilled water and each sample was run for 5 times of the measurement.

Morphology determination

The morphology of the sericin nanoparticles was examined by scanning electron microscopy (SEM). The particle suspension was diluted with distilled water and spread on a cover slip. The samples were dried for 3 hours by incubating in oven at 50°C. Next the sample was coated with gold-palladium using sputter coater prior to the SEM examination.

Niacinamide entrapment efficiency and niacinamide loading efficiency studies

Twenty milligrams of niacinamide-loaded sericin nanoparticles were suspended in 1 ml of phosphate buffer (pH 7) for 24 hours at room temperature. Then, the suspension was centrifuged at 12,000 rpm for 10 minutes. The supernatant was then filtered (0.2 μm Nylon syringe filters, Whatman, UK). The filtrate was analyzed using HPLC. All samples were analyzed in triplicate. The entrapment efficiency and loading capacity were calculated according to the following expression

Entrapment efficiency

$$\text{Entrapment efficiency (\%)} = \frac{(\text{amount of niacinamide in particles/amount of niacinamide loaded})}{} \times 100$$

Loading efficiency

$$\text{Loading efficiency (\%)} = \frac{(\text{total amount of niacinamide in particles / amount of particles})}{} \times 100$$

Stability evaluation of niacinamide-loded sericin nanoparticles

Twenty milligrams of niacinamide-loaded sericin nanoparticles were placed into centrifugate tubes containing 1 ml of phosphate buffer pH 5.5 and 7.4. The mixtures were kept at 25°C and 50 °C for 1 month. Then, the mixtures were centrifuged at 12,000 rpm for 10 min and the supernatants were analyzed by HPLC.

Evaluation of the *in vitro* release

Twenty milligrams of niacinamide-loaded sericin nanoparticles were placed into each 8 centrifuge tubes containing 1 ml of dissolution media and incubated at 37°C. The pH of dissolution buffer was 5.5 and 7.4. After centrifugation of the samples (12,000 rpm, 10 minutes) at predetermined time intervals, 1 ml of supernatant was withdrawn and the amount of niacinamide released from the nanoparticles was analyzed using HPLC.

Skin permeation study

The permeation study of niacinamide-loaded sericin nanoparticles was performed using Franz diffusion cells according to OECD No 428 (OECD, 2004) guideline. Franz diffusion cells had 0.5 cm of diameter of donor compartment and 13 ml of receiver compartment. Abdominal skin was used as a permeation membrane (the research for this research received the approval of the Naresuan university institutional review board for ethical research on humans: reference number 291/56). Fat and connective tissue were removed by forceps and scissors. The skin was stretched over receiver compartment and then donor compartment was joined together using a clamp. The cell was connected to water jacket providing temperature at 37 ± 1 C^{o}. A 30 minutes period of equilibration was allowed before the start of the experiment. The receiver compartment containing HEPES pH 7.4 was stirred by magnetic stirrer at 400 rpm. The sample was applied on epidermal side of the skin. Receiver solution (1 ml) was collected at 0, 30 minutes, 1, 2, 4, 6 and 8 hours respectively and then fresh medium was replaced with the same volume. The quantity of niacinamide in the sample was quantified by HPLC.

Lotion formula	
Ingredient	%w/w
Mineral oil	4
Isopropyl myristate	1
Cetyl alcohol	1
Glyceryl monostearate	4
Aracel 165	5
Tween 80	2.5
Glycerine	3.5
Deionized water	79

Statistical analysis

Student's *t*-test was used for comparison between two independent groups. The one-way ANOVA was also used to compare the means of three or more independent groups. The *p*-value of less than 0.05 was considered as significance.

CHAPTER IV

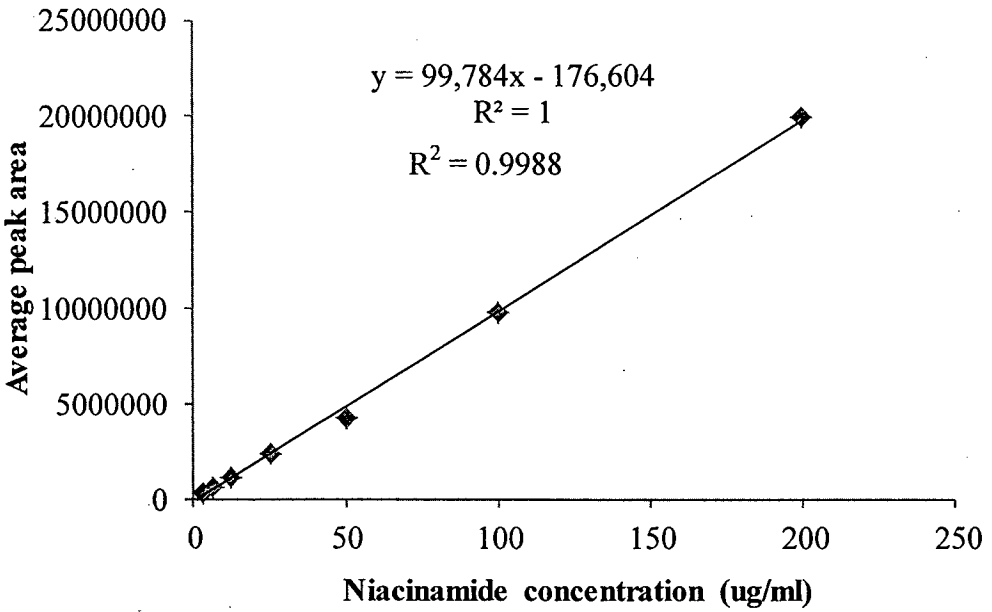
RESULTS AND DISCUSSIONS

HPLC method validation: Method 1

The HPLC analysis method 1 was developed and used for entrapment efficiency, *in vitro* release and stability studies. The calibration data of standard niacinamide are shown in Table 4. Linearity of all response was obtained in the range of 3.125 - 200 µg/ml. The detection limit determined at signal to noise ratio was 0.015 µg/ml.

Table 4 Calibration data of the standard niacinamide

Linearity range (µg/ml)	3.725 - 200
Regression equation	y = 99784x-17660
Correlation coefficient	0.9988
Limit of detection (µg/ml)	0.015



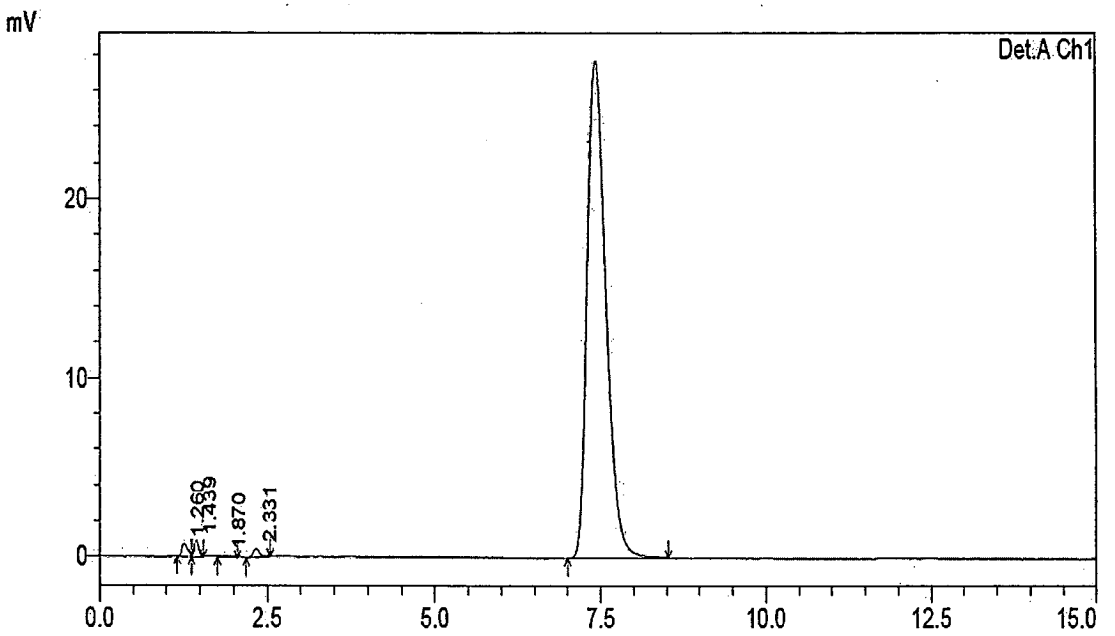


Figure 14 Chromatogram of the standard niacinamide

The accuracy of the method was determined using the standard addition method. Three different concentrations of standard niacinamide were added to a sample and analysed. Each set of additions was repeated three times. The results were shown in Table 5. The accuracy values were in range of 96 – 102%

The inter-day precision of the method was obtained from 3 dilutions of standard niacinamide for 3 consecutive days. The inter- and intra-day precision can be determined from %RSD. In this study, percentages of all RSD were less than 5 indicating that the method had high precision.

Table 5 Accuracy data of the HPLC analyses of the standard niacinamide

Concentration (μg/ml)	% Accuracy (±SD)
6.25	100.09 ± 3.19
12.5	98.99 ± 0.13
25	101.10 ± 0.34

Table 6 Intra and inter-day precision of the HPLC analyses of the standard niacinamide

Concentration (μg/ml)	Intra-day (%RSD)			Inter-day (n=3)
	Day 1	Day 2	Day 3	
6.25	5.87 (3.38)	6.13 (2.18)	5.81 (2.30)	5.94 (3.00)
50	43.71 (2.44)	46.26 (2.22)	43.71 (1.83)	44.56 (3.32)
100	89.58 (1.73)	94.69 (1.96)	89.59 (1.66)	91.29 (3.24)

HPLC validation: Method 2

The HPLC analysis method 2 was similar to that method 1 except that the different C-18 column was used. This method was used in permeation study. The calibration data of standard niacinamide are shown in Table 7. Linearity of all response was obtained in the range of 0.0977 - 100 μg/ml. The detection limit was determined at signal to noise ratio was 0.020 μg/ml. The qualification limit was 0.0977 μg/ml.

Table 7 Calibration data of the standard niacinamide

Linearity range (μg/ml)	0.0977 – 100
Regression equation	y = 68,882x+18653
Correlation coefficient	0.9998
Limit of detection (μg/ml)	0.020
Limit of qualification (μg/ml)	0.0977

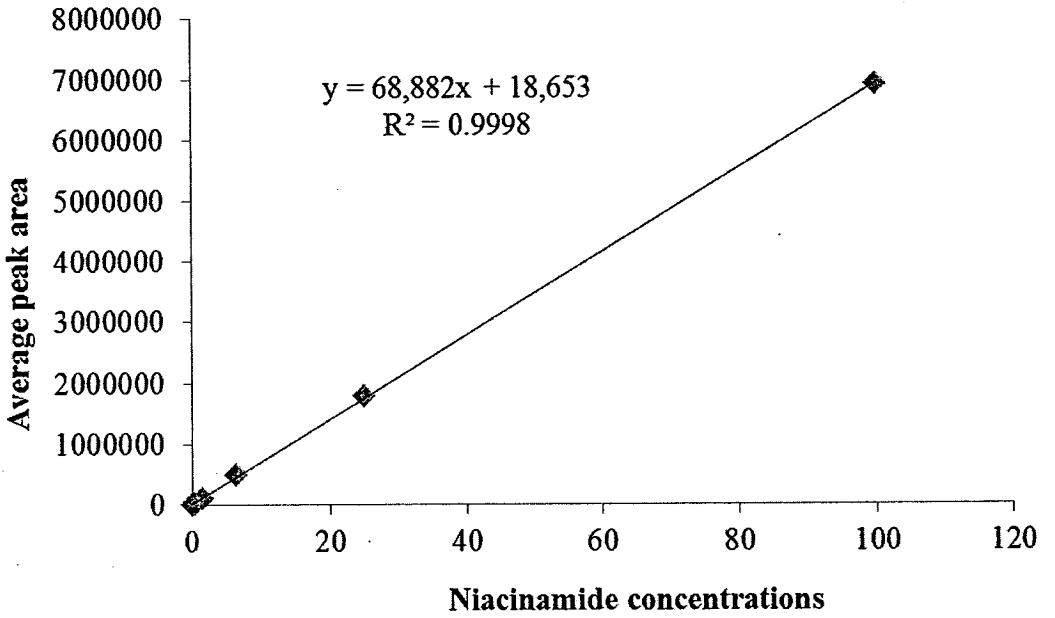


Figure 15 Calibration curve of the standard niacinamide

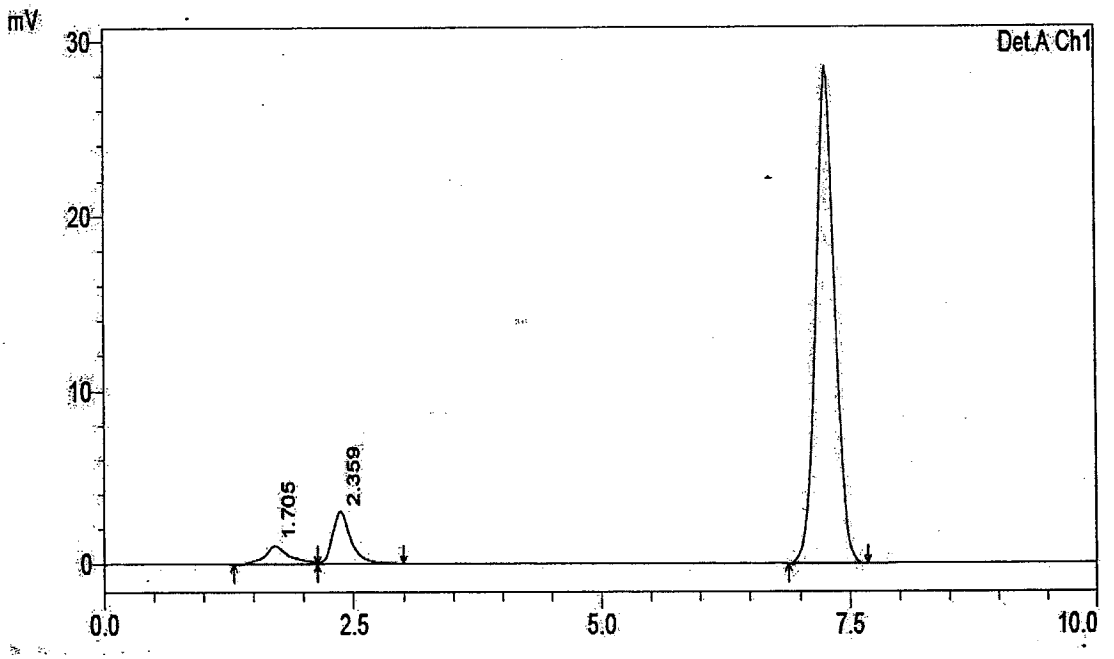


Figure 16 Chromatogram of the standard niacinamide

The accuracy and precision of the developed method was evaluated and results are expressed as percent recoveries of the components in the samples. As shown in

Table 8, the accuracy of the method was assessed by comparing the amount of analyte determined versus the known amount spiked at three different concentration levels. The accuracy values were approximately in range of 94 – 101%.

The precision study was evaluated on the basis of the % RSD value. The relative standard deviation was less than 5%. Results of precision study are shown in Table 9.

Table 8 Accuracy data of the HPLC analyses of the standard niacinamide

Concentration (μg/ml)	% Accuracy (±SD)
0.781	94.47 ± 3.34
12.5	99.16 ± 2.18
100	100.79± 2.41

Table 9 Intra and inter-day precision of the HPLC analyses of the standard niacinamide

Concentration (μg/ml)	Intra-day (%RSD)			Inter-day (n=3)
	Day 1	Day 2	Day 3	
0.781	0.86 (4.67)	0.88 (4.01)	0.86 (1.41)	0.87 (2.37)
12.5	12.22 (0.35)	12.34 (0.44)	12.23 (1.07)	12.26 (0.56)
50	51.92 (4.47)	49.66 (2.13)	50.14 (2.80)	51.17 (1.33)

Sericin nanoparticles preparation

Effect of Sericin and Cacium chloride concentrations

The sericin nanoparticles were prepared using water in silicone emulsion technique. Sericin solution containing 20% niacinamide was emulsified into silicone oil (DC 345) containing 5% silicone emulsifier (DC5225C) while stirring at 6500 rpm

for 60 minutes. Thereafter, the ionic gelation of sericin nanoparticles was achieved by adding calcium chloride solution with equal volume of sericin solution. After 24 hours, the sericin nanoparticles were isolated by centrifugation. The parameters that influenced the particles formation were varied and the results were presented in Table 10 and Figure 17.

Table 10 Average mean diameter and polydispersity index of sericin nanoparticles produced under various condition (n=3)

Sericin (%)	$CaCl_2$ (%)	Mean diameter (nm±SD)	Polydispersity index (±SD)
0.5	2	251.75 ± 3.42	0.18 ±0.02
0.5	4	244.47 ± 3.46	0.18 ±0.02
0.5	6	250.09 ± 2.16	0.21 ±0.03
0.5	8	259.85 ± 3.08	0.21 ±0.02
1	2	232.43 ±4.08	0.15 ±0.01
1	4	239.56 ± 3.74	0.16 ±0.01
1	6	246.87 ± 10.21	0.20 ±0.02
1	8	253.07 ± 4.78	0.19 ±0.02
2	2	226.27± 3.38	0.15 ±0.001
2	4	254.06± 2.57	0.20 ±0.01
2	6	262.09± 1.25	0.22 ±0.03
2	8	244.94± 8.64	0.22 ±0.04

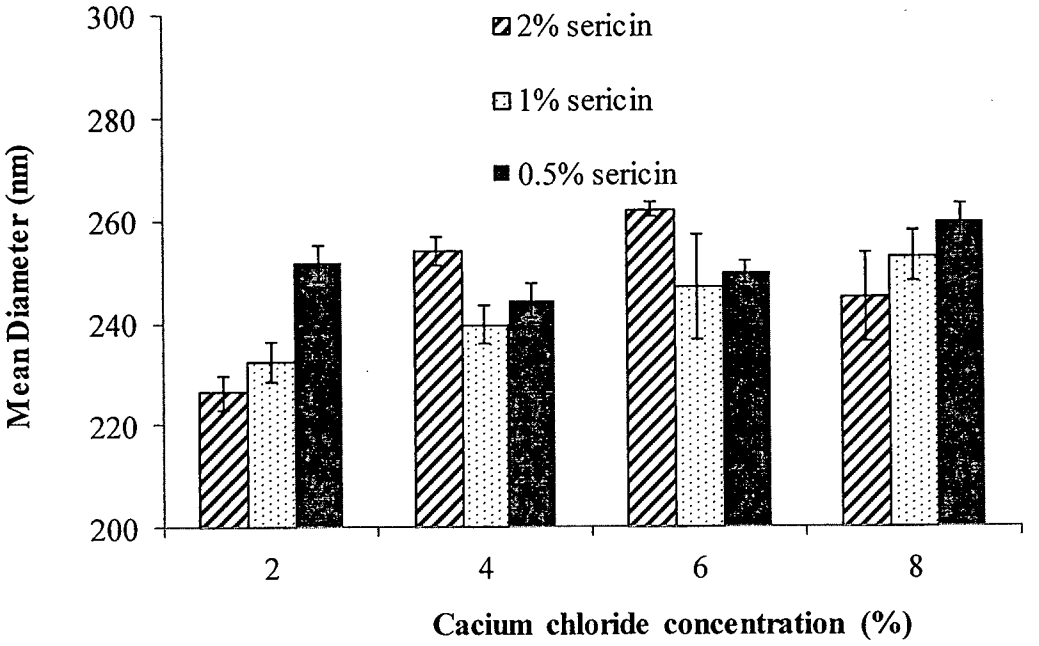


Figure 17 Comparision of sericin nanoparticle size obtained from various sericin and niacinamide concentrations (n=3)

Particles produced from different sericin and calcium chloride concentrations varied in size. The smallest particles were observed with 2% sericin and 2% calcium chloride. With increasing the concentrations of sericin and calcium chloride, mean diameter of particles tended to increase. For polydispersity index, only calcium chloride concentration affected the polydisersity value. With increasing calcium chloride concentration, the larger value was observed due to the broader size distribution of particles.

Effect of homogenizing time and speed

The homogenizing time was varied at 15, 30 and 60 minutes while sericin and calcium chloride concentrations were fixed at 2%. The speed of homogenizer was also fixed at 6500 rpm. The homogenizing time affected the particle size. The particle size did significantly change with 60 minutes of homogenizing time. Extending the homogenizing time allowed the formation of smaller particles with narrow polydispersity value. The speed of homogenizer was varied at 6500, 9500 and 13500

rpm while other parameters were fixed. The sizes of particles produced at several of speed homogenizers were not significantly different.

Table 11 Average mean diameter of sericin nanoparticles produced by various homogenizing time and speed

Preparing conditions		Mean diameter (nm±SD)	Polydisersity index (±SD)
Speed of homogenizer (rpm)	Homogenizing time (min)		
6500	15	245.26 ± 8.53	0.16 ± 0.01
6500	30	248.96 ± 8.74	0.21 ± 0.03
6500	60	226.27± 3.38	0.15 ± 0.001
9500	60	234.87± 3.95	0.16 ± 0.04
13500	60	224.19 ± 8.42	0.19 ± 0.01

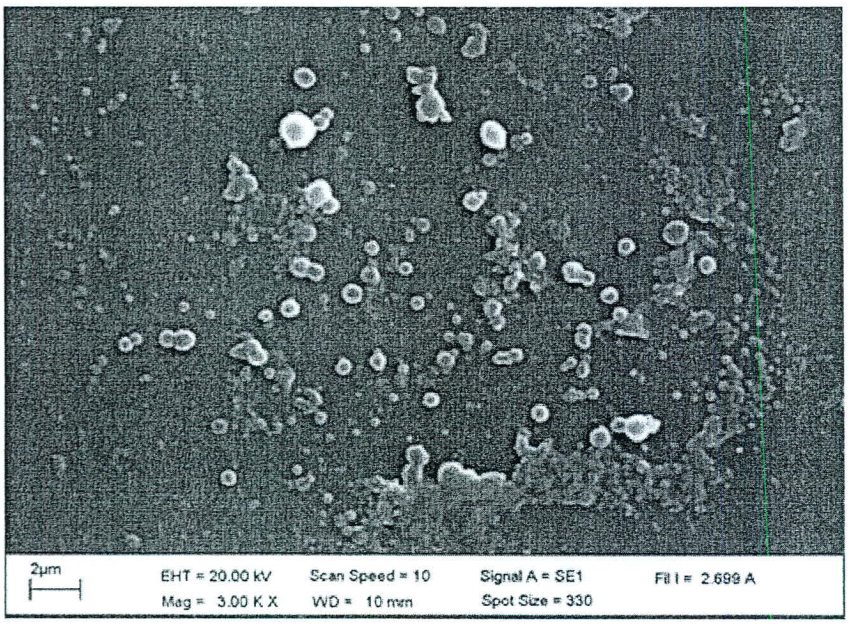


Figure 18 Electron micrograph of sericin nanoparticles

Entrapment efficiency

The percentage of niacinamide entrapped in sericin nanoparticles was prepared with various conditions were showed in Table 12. The entrapment efficiency was

found to be more in high calcium chloride concentration. The highest entrapment was produced from 8% calcium chloride.

Table 12 Percents of niacinamide entrapment at various calcium chloride concentrations while sericin concentration, homogenizing time and speed were fixed at 2%, 60 minutes and 6500 rpm, respectively

$\%CaCl_2$	% entrapment (±SD)
2	0.0088 ± 0.0045
4	0.0493 ± 0.0082
6	0.1056 ± 0.0350
8	0.2556 ± 0.0271

In an attempt to reduce an energy using in the process, the speed of homogenizer was decreased to 1000 rpm, sericin concentration was fixed at 2% while calcium chloride concentration was 8%. The homogenizing time was varied.

Table 13 Average mean diameter of sericin nanoparticles produced under various homogenizing time and speed

Preparing Conditions		Mean diameter (nm) ± SD	Polydisersity index (±SD)
Speed of homogenizer (rpm)	Homogenizing time (min)		
1000	15	264.66 ±9.83	0.12 ±0.01
1000	30	266.27 ±5.21	0.19 ±0.01
1000	60	250.62 ± 9.35	0.19 ±0.04
6500	60	226.33 ± 3.38	0.15 ±0.001

The result showed that the size of sericin nanoparticles obtained from 1000 rpm of homogenizing speed was a slightly different to particle obtained from 6500

rpm. Therefore, the optimum conditions were 1000 rpm and 15 minutes of homogenizing time because the particle can be produced gentlely.

Sericin nanoparticles produced with various concentrations of calcium chloride showed very low entrapment efficiency. The experiment for improving the entrapment efficiency was concerned. Concentration of sericin, concentration of calcium chloride, time for homogenization and homogenizing were fixed at 2%, 8%, 15 minutes and 1000 rpm respectively. In preparation process, 15% acetone was added into sericin solution before emulsified to DC345. The addition of organic solvent may allow the ease of solidification process. After 24 hours of cross-linking time, the system was heated. The entrapment efficiency was determined and the results are shown in Table 14.

Table 14 Percents of niacinamide entrapment and loading at various conditions

Preparing condition				Entrapment (%)	Loading (%)
Niacinamide (%)	DC5225C (%)	Heating temperature (°C)	Heating time (hr)		
20	5	50	1	0.42 ± 0.28	0.13 ± 0.02
20	5	50	5	1.55 ± 0.20	0.19 ± 0.01
5	5	50	5	2.08 ± 0.43	0.34 ± 0.05
5	5	80	5	4.09 ± 0.05	0.49 ± 0.03
1	5	80	5	29.22 ± 8.44	0.49 ± 0.06
0.5	5	80	5	58.69 ± 6.99	0.47 ± 0.03
0.5	1	80	5	59.41 ± 8.25	0.50 ± 0.04

The percent of niacinamide entrapped in sericin nanoparticles was increased when decreased niacinamide loading amount. On the other hand, the percent of entrapment was increased when increased the heating temperature and heating time. Possible explanation for these finding is that there is water remaining after stop the cross-linking process. Therefore, niacinamide might be still solubilized in the water.

Thus, heating the system and adding organic solvent could speed up the water removal time. Thus, particle's entrapment efficiency can be improved. Moreover, the entrapment efficiency obtained with 1% and 5% of DC5225C were not significantly different. So, the optimum concentration of DC5225C was 1%.

The drug loading capacity of the different procedures for loading niacinamide also shown in Table 14. The percentage of niacinamide loading was improved by increased the heating temperature and time, whereas the percent of loading was increased when decreasing the niacinamide concentration. The loading capacity of niacinamide was limited at niacinamide concentration lower than 5% and 80°C of heating temperature. The percent niacinamide loading at optimum condition was 0.504.

Stability of niacinamide-loded sericin nanoparticles

In this study, the stability of niacinamide in sericin nanoparticles was determined for 1 month under various temperatures and pH conditions. The niacinamide solution was used as a control.

Generally, niacinamide was very stable to oxygen, heat and light [42]. From the figures 19 and 20, after test condition, niacinamide remaining in sericin nanoparticles was more than 85%. One month of storage under accelerated temperature (50 °C), the percentage of niacinamide from nanoparticles and niacinamide solution were not different. These results suggested that temperature did not affected chemical stability of niacinamide.

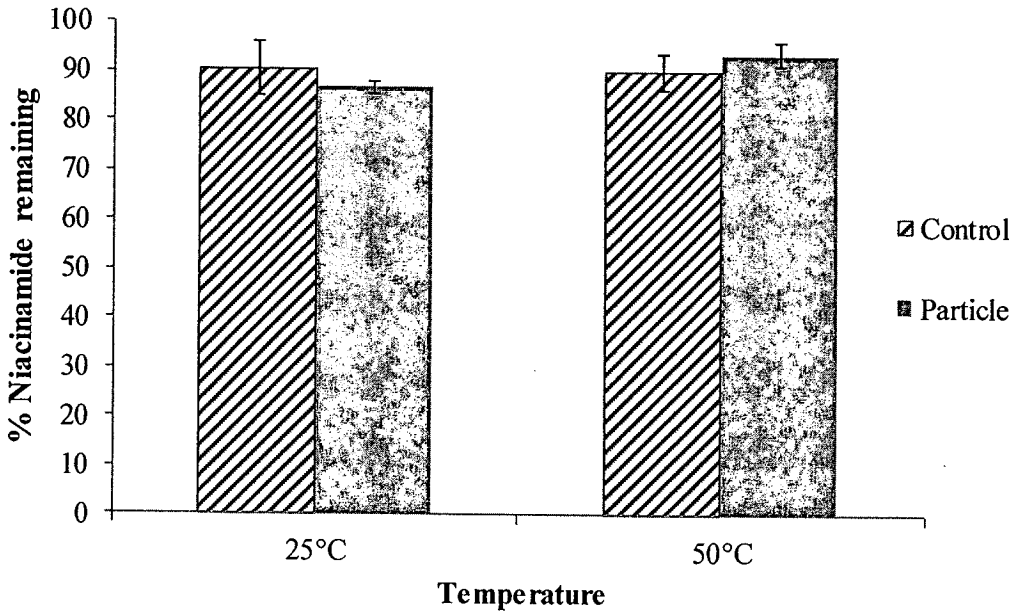


Figure 19 Comparison on the stability of free and entrapped niacinamide in sericin nanoparticles at various temperatures at pH 5.5

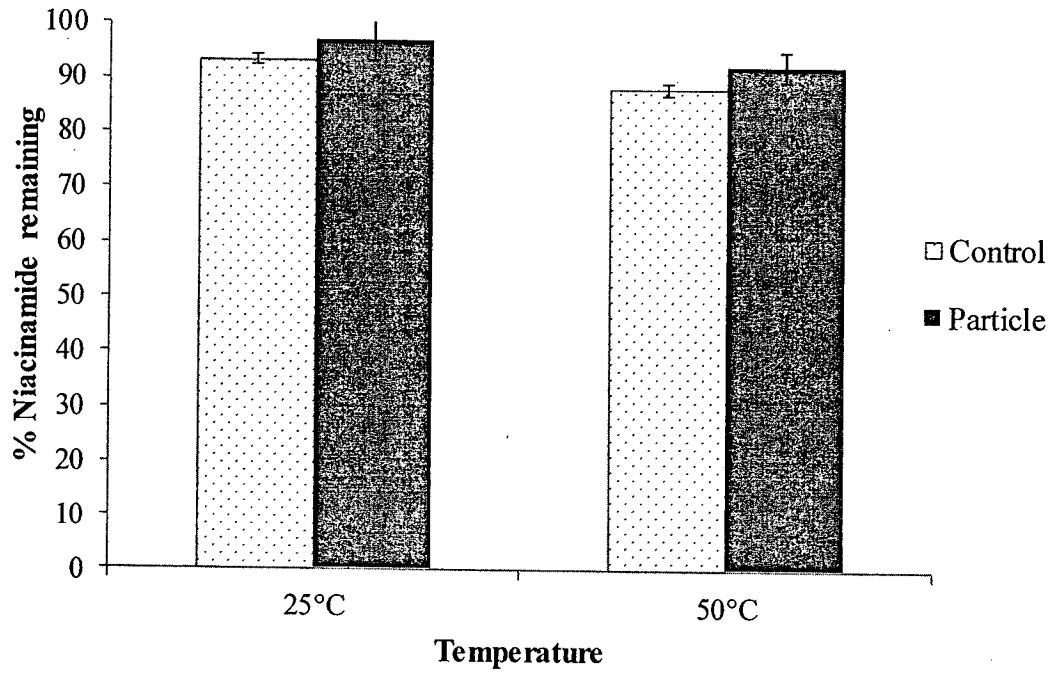


Figure 20 Comparison on the stability of free and entrapped niacinamide in sericin nanoparticles at various temperatures at pH 7.4

The niacinamide remaining in sericin nanoparticles at all pH and temperatures were stable more than 85 %. These results suggested that niacinamide-loaded sericin nanoparticles can be incorporated into cosmetic formulation presenting wide range of temperatures and pH values.

In vitro release

In this study, membrane-free model was used. Drug releasing behavior of niacinamide-loaded sericin nanoparticles was investigated using the in vitro releasing tests in phosphate buffers pH 5.5 and 7.4. Niacinamide released from sericin nanoparticles was investigated and reported as the plots of percentages of released niacinamide against times.

Figures 21 and 22 shows the release profile of niacinamide from sericin nanoparticles against time after suspending in buffers pH 5.5 and 7.4. They clearly showed that 50% of niacinamide were rapidly released from nanoparticles within 30 minutes and completely released within 2 hours. At pH 7.4, the percentage of niacinamide released at 30 minutes (65.78%) was higher as compared to that (49.43%) at pH 5.5. The complete released of niaciamide at pH 7.4 was achieved within 1 hour.

These results indicated the swelling of the sericin nanoparticles. The swelling rate of sericin nanoparticles depends on pH. It can be explained that the swelling behavior of the protein matrix depends on its net charge. The isoelectric point of sericin is about pH 4, therefor the net charge of sericin at pH 5.5 is nearly zero. Since, there were equal numbers of negatively charged and positively charged amino acids. Thus, the attractive force between negative and positive charges prevented the swelling of sericin molecules. With increasing the pH, negatively charged amino acids were increased, the net charge of sericin became negatively so a repulsive force made it easier to swell [7]. At pH 7.4, sericin molecules had more repulsive force compared to pH 5.5. Therefore, the release rate of niacinamide from sericin nanoparticles at pH 7.4 was more rapid than that at pH 5.5

Moreover, interaction between polymers and cross-linking agent also affected the release profile. The formation of sericin nanoparticles was achieved by interaction between negatively charged sericin molecules and calcium cation. The hydronium cations were attracted to the anions, while the other hydroxide anions were attracted to

the cations, so the ionic bonds eventually break easily. From this result, the release rate of niacinamide in sericin nanoparticles was fast and complete within 2 hours.

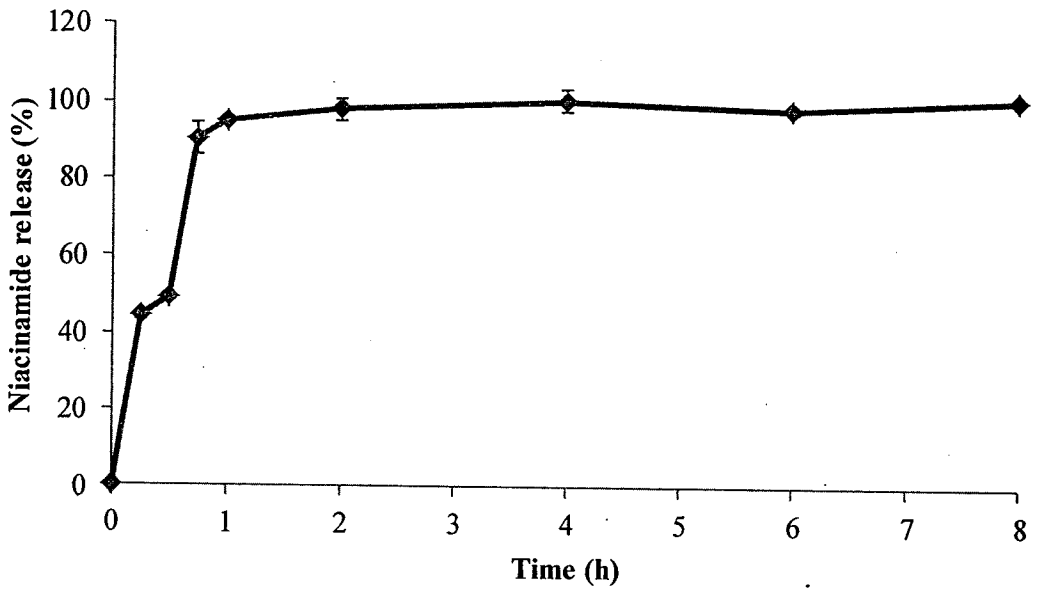


Figure 21 Release of niacinamide from sericin nanoparticles against time after suspending in buffer pH 5.5

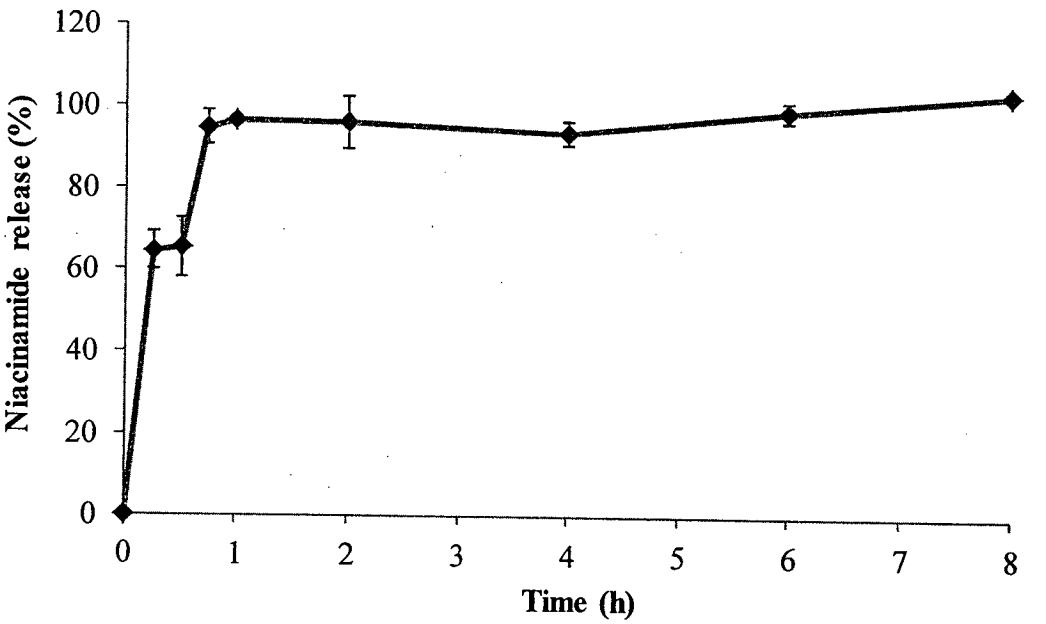


Figure 22 Release of niacinamide from sericin nanoparticles against time after suspending in buffer pH 7.4

Skin permeation study

In vitro skin permeation was performed using Franz diffusion cell model. The human abdominal skin obtained from Yanhee hospital was used as membranes. The procedure followed OECD NO 428 protocol. Niacinamide permeation from niacinamide-loaded sericin nanoparticles lotion was compared with niacinamide lotion. The amount of niacinamide in various parts of skin and franz cell compartments were determined. The percentages of niaicnamide in donor, receiver, total strips, and living skin strata were shown in Table 15.

Table 15 Amount of niacinamide contents from niacinamide-loaded sericin nanoparticles and niacinamide lotion in each skin layer and fractions of Franz cell compartment (n=5)

Formulation	%Niacinamide in skin layer and Franz cell parts (±SE)				Recovery (%)
	donor	total strips	living skin strata	receiver	
niacinamide lotion	59.80± 2.86	12.95 ± 0.49	14.89± 1.88	Lower than LOQ	87.65± 8.47
niacinamide-loaded sericin nanoparticles lotion	50.96±1.64	22.60±1.66	19.20±1.82	3.39 ±0.48	96.15 ± 6.10

The total amount of niacinamide permeated from niacinamide-loaded sericin nanoparticles was 3.39±0.47 % while niacinamide lotion was lower than LOQ. The cumulative amount of niacinamide permeated from nanoparticle in total strips was higher about 2 times compared with niacinamide lotion. The remaining niacinamide from niacinamide-loaded sericin nanoparticles in living skin strata was higher than that of niacinamaide lotion.

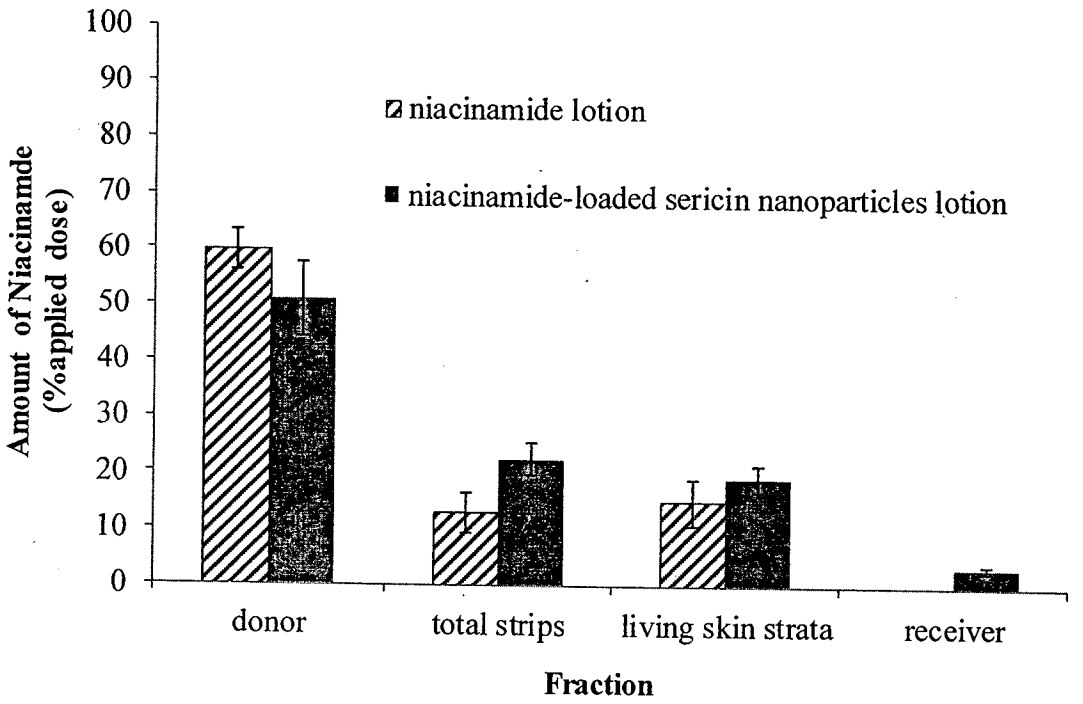


Figure 23 Comparision of amount of niacinamide in various fractions

Due to the data of permeation study, the results supported that niacinamide-loaded sericin nanoparticles enhanced niacinamide skin permeation.