Synthesis , Characterization and Cosmetic Application of Self-Assembled Sericin-PEG Nanoparticle

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Summary

Silk Sericin(SS) is a natural protein extracted from cocoon of *bombix mori* and shows moisturizing effect to the skin due to a number of hydroxyl groups in the structure. But its application to cosmetics is limited due to its poor solubility in water. In order to solve this drawback and expand its application to cosmetics, polyethyleneglycol(PEG) was conjugated with sericin by reacting activated polyethyleneglycol(ActPEG). Reaction site of sericin is tyrosine residue, which was determined by using ^{1}H-NMR. Random coil structure of sericin was transformed to beta-sheet structure by conjugating polyethyleneglycol. It was confirmed that melting point of sericin-PEG conjugate was lowered compared to that of each sericin and PEG due to the interaction between sericin and PEG in crystalline structure. Self-assembled sericin-PEG nanoparticle was obtained by dialyzing with alcohol solution of sericin-PEG conjugate against water. The particle is spherical and has 200-400nm of size. The moisturizing ability of sericin-PEG nanoparticle was much higher than that of sericin itself. Incorporation of vitamin A into sericin-PEG nanoparticle was carried out by diafiltration method. The content of incorporated Vitamin A in sericin-PEG nanoparticle was 8.9 wt%. Releasing behaviour of

vitamin A incorporated into nanoparticle was tested in phosphate buffer, pH 7.4 at 37°C. and half-life of Vitamin A release was 43hrs. Sericin-PEG nanoparticle exhibited higher moisturing effect than sericin itself and distilled water, respectively. No toxicity and irritation were observed in animal tests. It can be expected that the self-assembled sericin-PEG nanoparticle can be developed for cosmetics.

Introduction

Nanoparticles, colloidal particles of sizes below 1 micrometer, have extensively been studied in various fields of life sciences such as separation technologies, histological studies, clinical diagnostic assays, drug delivery systems, and cosmetics [1-3]. Their applications have some advantages including easy purification and sterilization, drug targeting possibilities, and sustained release action [4]. Recently, several methods have been developed for the preparation of nanoparticles, among which, the diafiltration method based on the polymeric micelles have the following advantages; simple, no-aggregation, small particle size, high yield, and extremely spherical in shape [5].

In this study, we report on the preparation of self-assembled silk sericin(SS) nanoparticles and its cosmetical applications such as moisturizing effect, incorporation of Vitamin A into the particles and its release behavior, and safety test. SS is one of the silk protein constituents that gums together the fibroin filaments of cocoon silk and constitutes about 25% of the total cocoon weight. This natural protein, mostly removed through a degumming treatment of cocoon silk, is regarded as waste. The chemical structure of SS, very rich in serine (about 32%) having high content of hydroxyl group, influences the cosmetic activity of SS itself. Although sericin has a good water retention ability on cutaneous surfaces due to the presence of several hydroxyl groups [6], the use of SS in cosmetics is very difficult due to its instability in water and insolubility in organic solvents. To overcome these problems, SS nanoparticles were prepared after pegylation of SS. The introdution of poly(ethylene glycol) (PEG) into proteins through a chemical modification has been developed for the thiol-specific pegylation, N-terminal pegylation, non-terminal amine pegylation, controlled release, and hetero bifunctionals for targeting [7-10]. PEG possesses an amphiphatic behaviour – soluble both in water and various organic solvents. In addition, nontoxic and non-immunogenic PEG stabilizes the physiological function of proteins and bioactive substances [11]. In order to develop sericin-PEG nanoparticle as a cosmetic ingredient, we also tested its cosmetical functions such as moisturizing effect, incorporation ability of Vitamin A and its safety.

Materials and Methods

Materials

2-O-[Methoxy(polyethylene glycol)]-4,6-dichloro-s-triazine (actPEG, MW 5,000) and methoxy poly(ethylene glycol) (PEG-OMe, MW:5,000) were purchased from Sigma Chemical Co.(St.Louis, MO). All other chemicals were reagent-grade products obtained commercially.

Preparation of SS

SS was extracted by autoclaving at 120°C for 60 min using a high temperature and pressure degumming technique.

Preparation of Sericin-PEG conjugate

actPEG (135.37 mg) was added to 12 ml of 0.10 % (w/v) SS aqueous solution containing 0.1 M sodium borate (pH 9.4) at 4°C [8, 9]. The mixture was then reacted at 4°C overnight. Subsequently, the solution was dialysed against distilled water using dialysis membrane (M.W. CO: 12,000) for 2 days.

^{1}H-NMR Measurement

^{1}H-NMR spectra were measured at 25°C using AVANCE 600 spectrometer.

IR Measurement

Samples of sericin-PEG, SS, PEG, and sericin/PEG mixture prepared with KBr pellet were measured using an M series (MIDAC CORPERATION) FT-IR spectrometer.

Circular dichroism (CD) Measurement

CD spectra were measured using a Jasco J-715 spectropolarimeter equipped with a quartz cell having a pathlength of 10 mm at room temperature.

Differential scanning calorimeter (DSC) Measurement

DSC measurements were measured using DSC 2910 at a heating rate of 10°C min^{-1}.

Amino acid analysis

SS and sericin-PEG conjugate were hydrolyzed under vacuum in 6 N HCl at 110°C for 20 hrs. The hydrolysed samples were dried in a rotary evaporator at 40°C, and dissolved in 0.02 N HCl, and applied to an amino acid analyzer after filtering using a Pharmacia Biotech. System Biochrom 20 Plus type amino acid analyzer.

Preparation of SS nanoparticles

After dissolving 10.6 mg of sericin-PEG in 4 ml of ethanol, the solution was dialysed against distilled water using a dialysis membrane(M.W. CO: 12,000) for 2 days.

Particle Size Measurements

Particle sizes were measured using ELS-8000 (Otsuka Electronics).

Scanning electron microscope (SEM) Observation

Morphology of the nanoparticles was observed using JSM 5410 LV. One drop of the nanoparticle suspension was placed on a graphite surface. After air-drying, the sample was coated with gold using an Ion Sputter.

Transmission electron microscope(TEM) Observation

The nanoparticles were observed using TEM(JEM 1010, JEOL, Japan). One drop of the nanoparticles was placed on a copper grid and negatively stained with 1% uranyl acetate solution for 30 s. The grid was allowed to dry further for 10 min and examined with TEM.

Moisturizing effect of Sericin-PEG nanoparticle

Moisturizing effect of sericin-PEG nanoparticle was measured with Corneometer CM 825

(Courage+Khazaka, C+K, Germany)

Incorporation of Vitamine A

Incorporation of vitamin A was carried out by diafiltration method and the amount of incorporated Vitamin A was measured with UV-Vis spectrophotometer(HP G1103A). 30mg of Vitamin A and 100mg of sericin-PEG conjugate was dissolved in 30ml of ethanol and 10ml of distilled water was added to the solution. The mixed solution was dialyzed against water and freeze-dried to obtain 80mg of the product.

Releasing behavior of Vitamine A from Sericin-PEG nanoparticle

Releasing of Vitamin A from sericin-PEG nanoparticle was measured using UV-Vis spectrophotometer. After dissolving 80mg of Vitamin A loaded- sericin-PEG nanoparticle in 50ml of phosphate buffer, the solution was dialyzed in a cellulose membrane(Mw cut off = 12,000) at 37°C. The cumulative amount of released Vitamin A from the nanoparticles through dialysis membrane was determined by measuring the absorbance at 324nm.

Safety test of sericin-PEG nanoparticle

Safety of sericin-PEG nanoparticle was estimated according to *CTFA Safety Testing Guideline*[18].

Results and Discussion

Preparation of sericin-PEG conjugate

Five hundred and fifty mg of the product, sericin-PEG, was obtained from 50 mg of starting material, SS. Assuming that 100% of SS was recovered, the weight of the product was 8.4-fold higher than that of the starting material. Therefore, sericin-PEG is composed of PEG and SS at the weight ratio of 7.4:1 and contains 11.9 % SS. If the average molecular weights of the amino acid units constituting SS and actPEG are supposed to be 113 and 5000, respectively, the mole ratio of amino acid residue to PEG is calculated to be 6[=(1/113)/(7.4/5000)]. This result suggests that 16.7 mol% of amino acid residues in SS reacted with PEG.

Figure 1 shows ^{1}H-NMR spectrum of sericin-PEG conjugate. The results indicate that the

proton peaks at 6.68 and 6.96 ppm of the tyrosine residue in SS shifted downfield to 7.09 and 7.26 ppm, respectively, which suggests the change in the molecular environment of the tyrosine residue caused by the modification; that is, the shift is the results of the shielding effect of the triazine ring on the tyrosine residue [12]. Due to the electron-withdrawing effects of the PEG-triazine ring, the valence electron density around the protons attached to the carbon decreased. From the result of NMR, it can be said that the tyrosine residues of the sericin reacted with actPEG. Cyanuric chloride reacted with nucleophilic groups such as amino, imino, and hydroxyl groups [13, 14]. Therefore, the amino group of the lysine residue and the imidazole group of the histidine residue in sericin could react with actPEG [13, 14]. However, the peaks of these residues could not be detected by NMR measurement due to their very low contents in SS. Amino acid analysis revealed the contents of serine residues in SS and sericin-PEG were 40.5 and 27.9 %, respectively, an indication that serine residues in SS reacted with PEG, although other residues in SS did not change much after modification.

[Fig. 1]

Conformation of sericin in sericin-PEG

Figure 2 shows IR spectra of SS, sericin-PEG, PEG, and sericin/PEG mixture. In the spectrum of sericin-PEG, two new bands appeared at 2883 and 1114 cm^{-1} compared with the spectrum of SS. These new peaks were assigned to -CH_2- stretching [15] and C-O-C stretching [16], respectively, which indicate that PEG chains are introduced into SS. SS exhibited absorption bands at 1665 (amide I), 1535 (amideII), and 669 cm^{-1} (amideV), which are characteristics of random-coil conformation, whereas sericin-PEG exhibited absorption bands at 1646 (amide I), 1527 (amide II), and 669 (amideV), which are characteristics of β-sheet structure. The absorption bands of amides I, II, and V of SS appeared at 1646, 1530, and 669 cm^{-1}, respectively. The results suggest that the coexistence of PEG molecule in SS caused the conformational change from random coil to β-sheet.

[Fig. 2]

CD spectra of SS and sericin-PEG in aqueous solution are shown in Fig. 3. CD spectrum of SS showed a peak at 200 nm trough, an indication of a random coil conformation. On the contrary, the spectrum of sericin-PEG in aqueous solution exhibited a negative peak at 200 nm and a negative extreme at 220 nm, which were characteristic of β-sheet structure containing a random coil conformation (9, 17). The results suggest that conformational change of SS in sericin-PEG occurred

from random coil to β-structure after the introduction of PEG.

[Fig. 3]

Thermal properties of the sericin-PEG

Figure 4 shows DSC curves of PEG, SS, and sericin-PEG. Decomposition of SS began to occur at 200 - 220°C, whereas two endothermic peaks were observed at 52.4 and 196°C in sericin-PEG. The melting point of PEG was 56-59°C as already reported [9]. Thus, the endothermic peak of sericin-PEG at 52.4°C is considered to be due to the melting of PEG in sericin-PEG., probably caused by a decrease in the crystallinity PEG after the conjugation with sericin. Thermal decomposition temperature of SS (198°C) shifted to 196°C in sericin-PEG. In addition, the exothermic peak, which appeared at 162°C for sericin-PEG, can be attributed to the transition from random coil to β-structure of SS.

[Fig. 4]

Preparation of nanoparticles

Particle sizes of the sericin-PEG nanoparticles with a mean diameter of 204.3 nm prepared by the diafiltration method are shown in Table 1. The self-assembled polymeric nanoparticles were prepared from the sericin-PEG conjugate consisting of sericin and PEG as the hydrophobic and hydrophilic parts, respectively.

[Table 1]

Figures 5(a) and 5(b) show SEM and TEM photographs of sericin-PEG nanoparticles, respectively. Shapes of the nanoparticles were almost spherical, and the sizes ranged about 200-400 nm in diameter.

[Fig 5a and 5b]

Moisturizing effect of sericin-PEG nanoparticle

Figure 6 shows the moisturizing effect of sericin-PEG nanoparticle compared with sericin itself and distilled water. Sericin-PEG nanoparticle exhibited higher moisturing effect than sericin itself and distilled water due to the conjugation of hydrophilic PEG molecules with sericin.

[Fig 6]

Incorporation of Vitamine A and releasing behaviour from sericin-PEG nanoparticle

The content of Vitamin A incorporated into sericin-PEG nanoparticle was 8.9 wt%, which was measured by UV-Vis spectrophotometer. Figure 7 shows the releasing behaviour of Vitamin A from

sericin-PEG nanoparticle and the half-life of Vitamin A release was 43hrs.

[Fig 7]

Safety test of sericin-PEG nanoparticle

No skin and eye irritation were observed in the safety test of sericin-PEG nanoparticle. It does not also show any toxicity in acute dermal and acute oral tests. In the tests of the primary irritation potential and allergenic sensitization potential, no side effects, such as erythma or edema on the guinea-pig skin , were observed.

Conclusions

The sericin-PEG conjugate was prepared by reacting actPEG with sericin. Aliphatic and aromatic hydroxyl groups of the serine and tyrosine residues in SS as the reaction sites were clarified through amino acid analysis and ^{1}H-NMR measurement, respectively.

From IR and CD measurements, the introduction of PEG chains into SS was found to induce the conformational change from random coil to β-sheet. DSC thermogram of sericin-PEG suggests that SS chains in sericin-PEG affected the crystallization of PEG.

Release of Vitamin A incorporated into sericin-PEG nanoparticle was very slow. Sericin-PEG nanoparticle exhibited higher moisturizing effect than sericin itself and distilled water, respectively. No cytotoxicity and animal toxicity were observed in fibroblast cell culture and animal test, respectively. It can be expected that the self-assembled sericin-PEG nanoparticle can be developed as a ingredient for cosmetics.

Acknowledgement

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Table Ⅰ. Size distribution of Sericin-PEG nanoparticles

Sample	Particle size (nm)		
	Intensity	Number	Weight
Sericin-PEG conjugate	197.3±46	149.0±28.8	168.8±38.2

Figure captions

Fig. 1. ^{1}H-NMR spectra of SS (a), PEG (b), sericin/PEG mixtrue (c), and PEG-sericin conjugate (d).

Fig. 2. IR spectra of SS (a), PEG (b), sericin/PEG mixture (c), and sericin-PEG conjugate (d).

Fig. 3. CD spectra of SS (a) and PEG-sericin conjugate films (b).

Fig. 4. DSC thermograms of SS (a), PEG (b), and sericin-PEG conjugate (d).

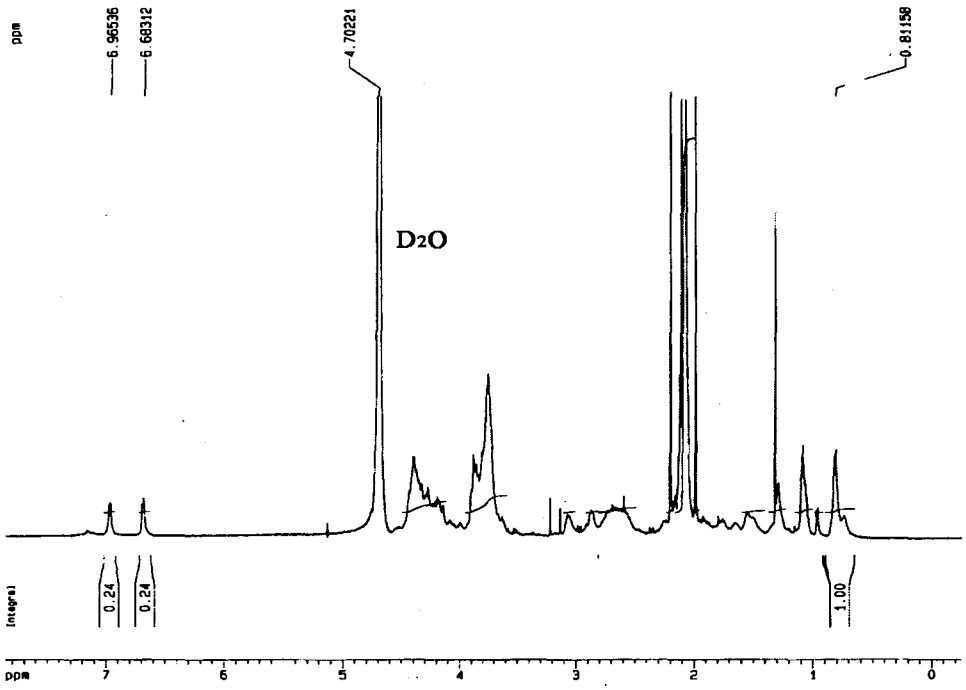
Fig. 5. SEM (a) and TEM (b) photographs of sericin-PEG nanoparticles.

Fig. 6. Comparison of moisturizing effect of sericin-PEG nanoparticle(a), Sericin(b), and distilled water(c)

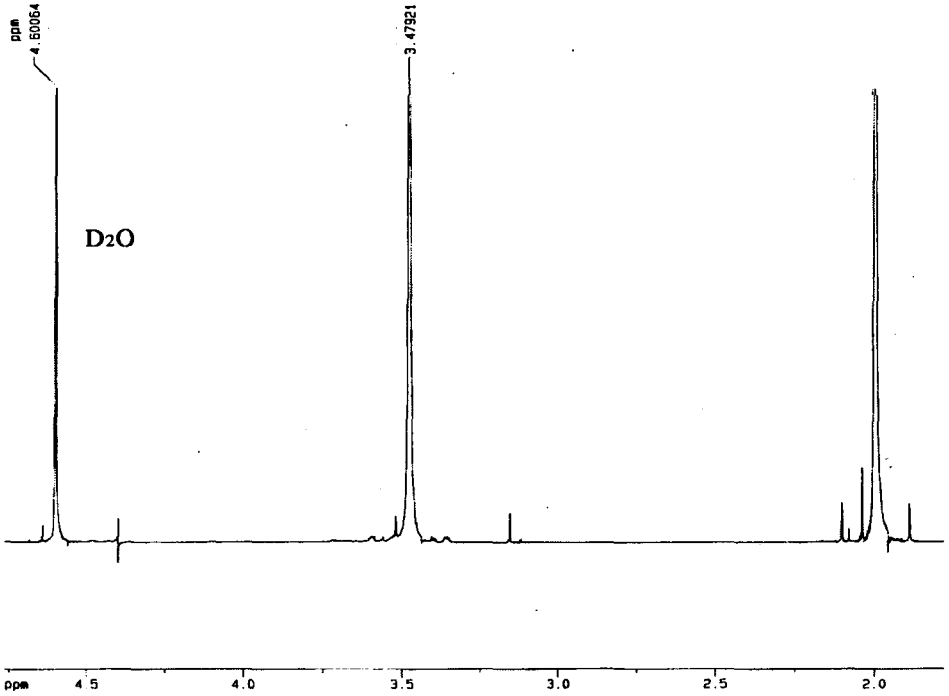
Fig. 7. Release behavior of Vitamin A loaded-sericin-PEG nanoparticle

Fig. 1

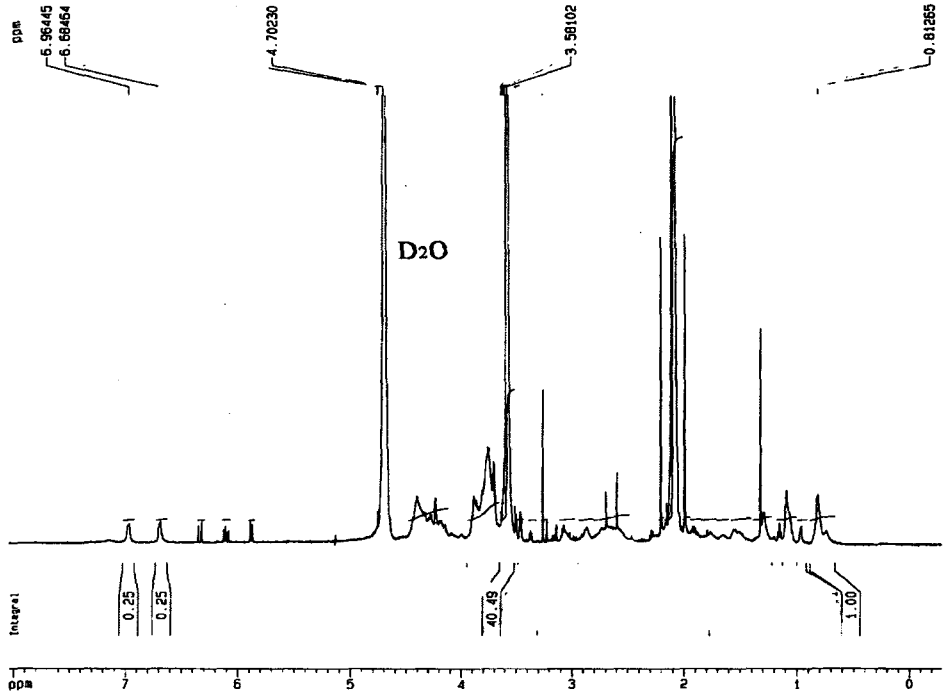
(a) Sericin in D_2O



(b) PEG in D_2O



(C) sericin/PEG mixture



(d) sericin-PEG conjugate

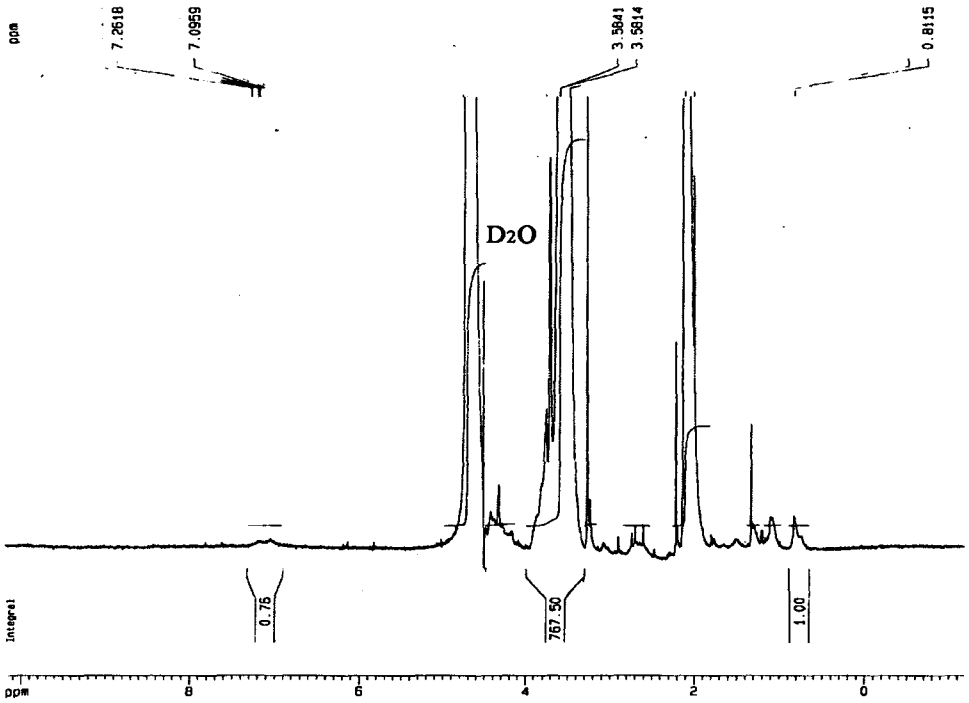


Fig. 2

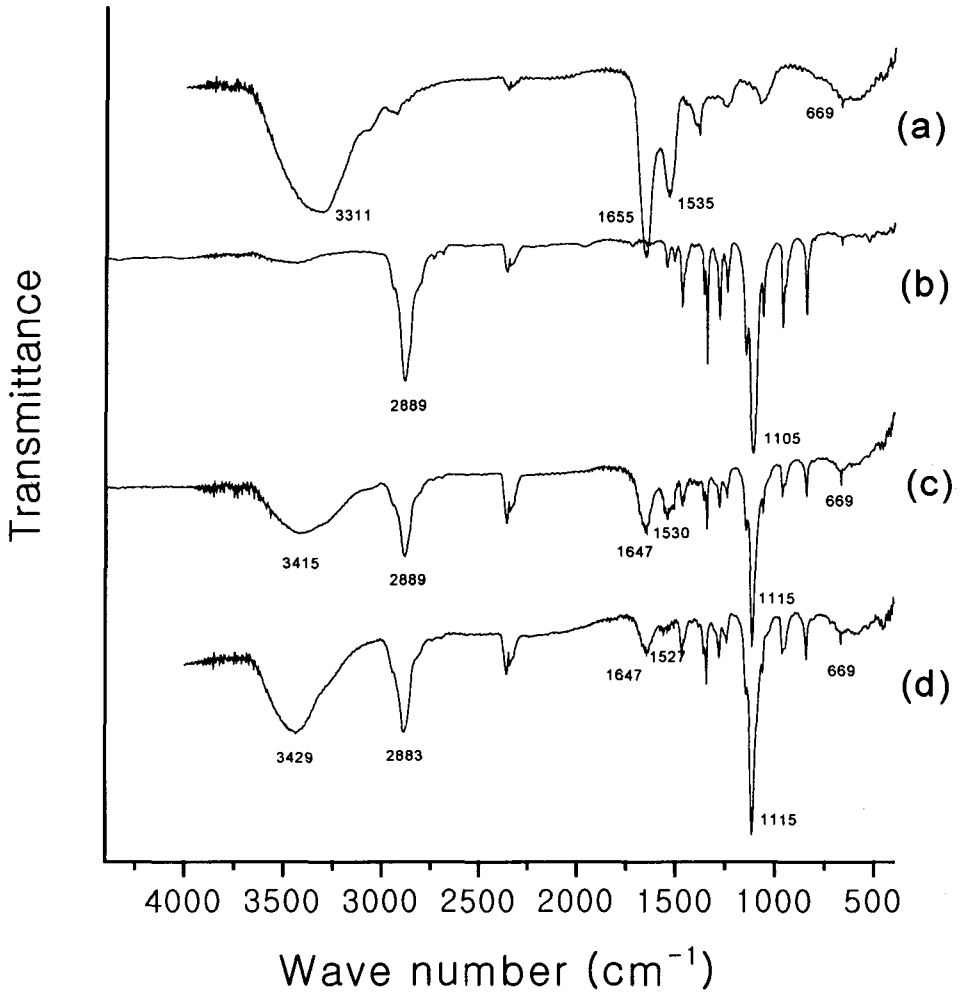
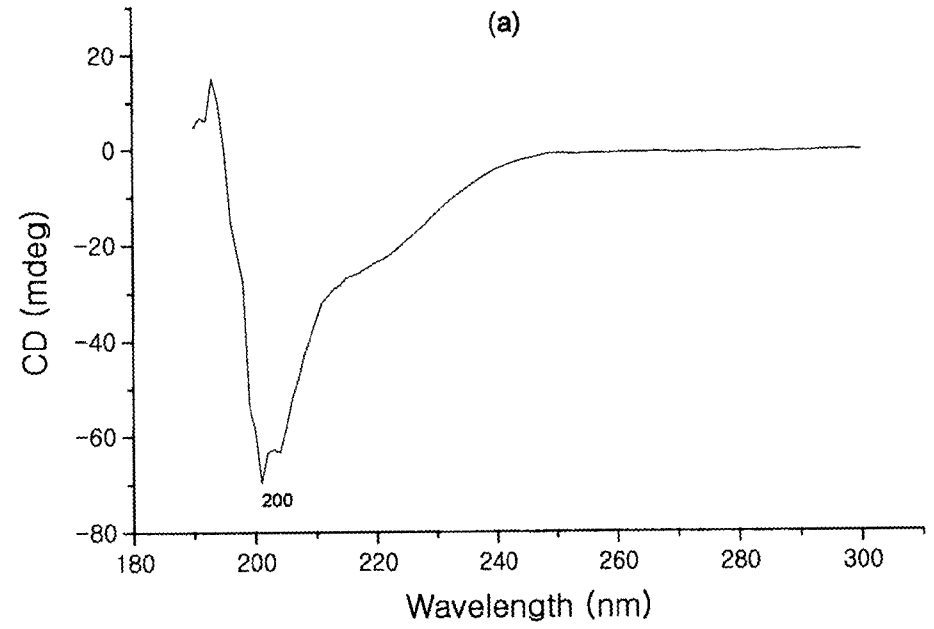

Transmittance
(a)
3311
1655
1535
669
(b)
2889
1105
(c)
3415
2889
1647
1530
669
1115
(d)
3429
2883
1647
1527
669
1115
4000 3500 3000 2500 2000 1500 1000 500
Wave number (cm^{-1})

Fig. 3


(a)
CD (mdeg)
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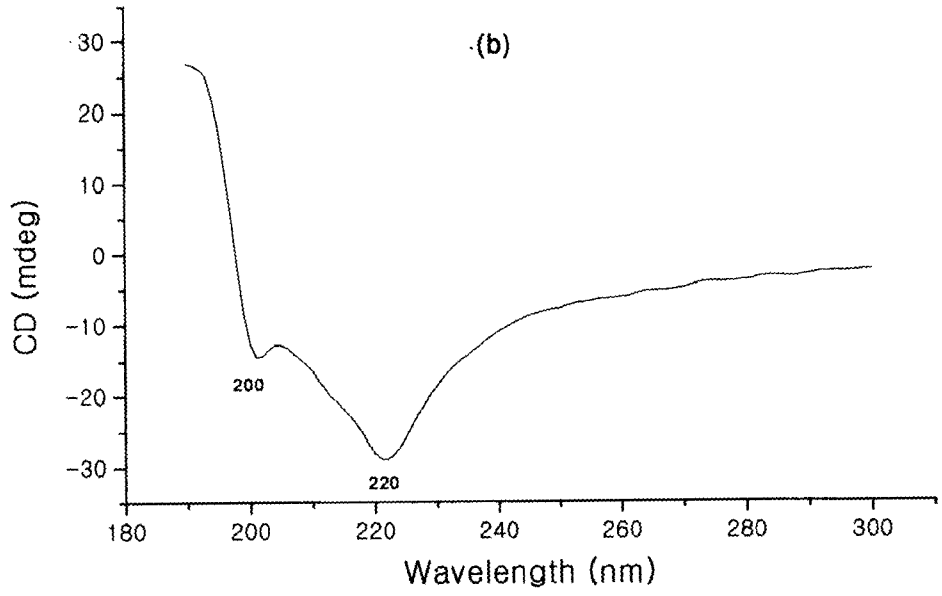
-20
-40
-60
-80
200
180
200
220
240
260
280
300
Wavelength (nm)


(b)
CD (mdeg)
30
20
10
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-10
-20
-30
200
220
180
200
220
240
260
280
300
Wavelength (nm)

Fig. 4

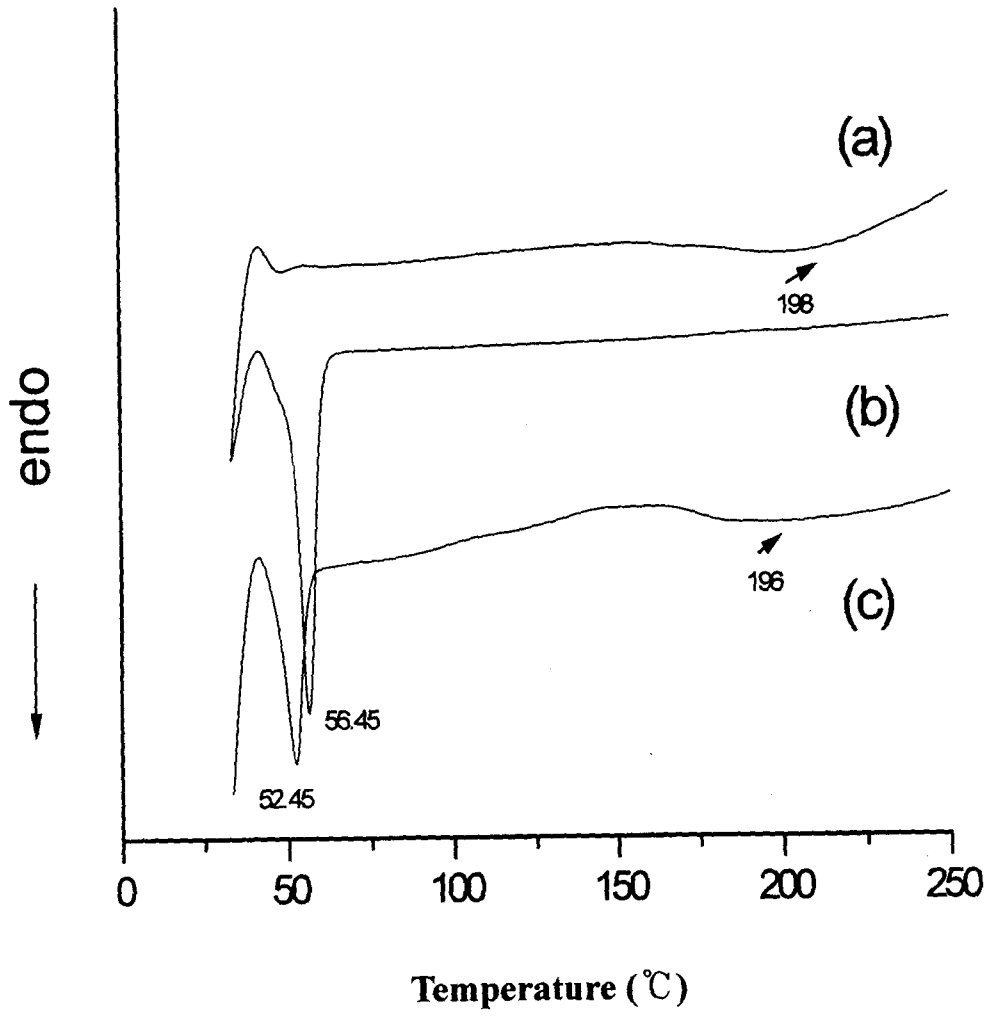
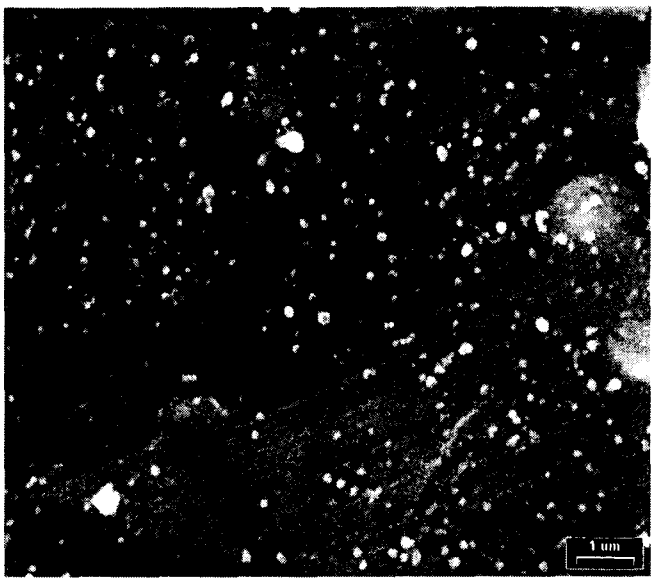


Fig. 5

(a)



(b)

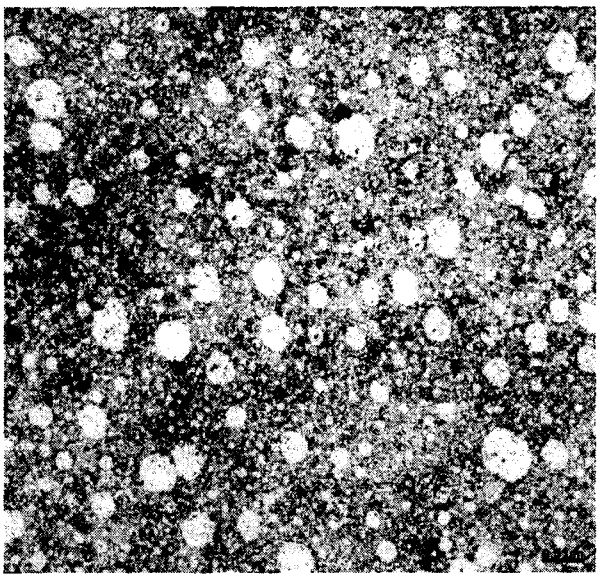


Fig. 6

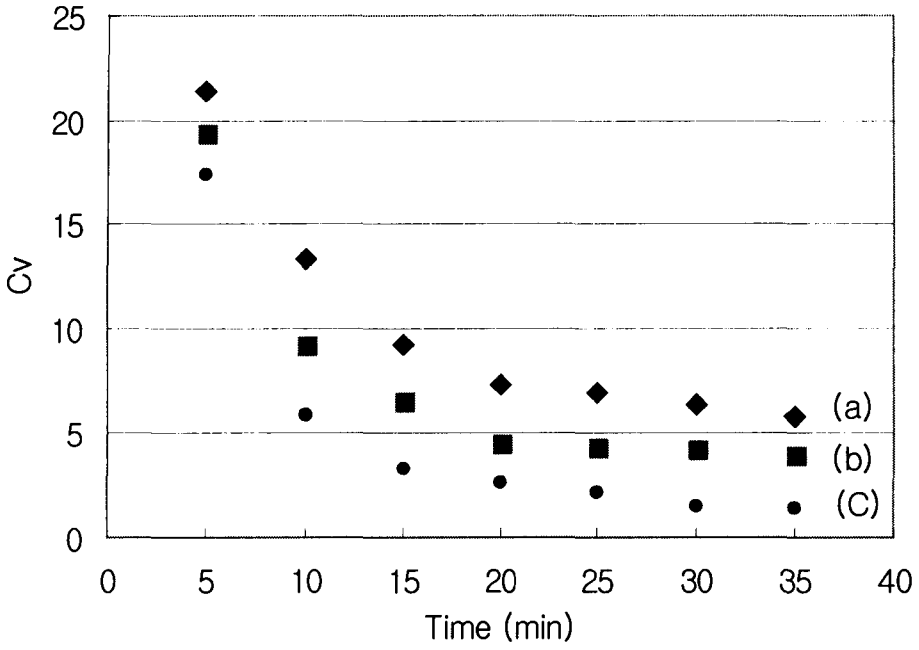
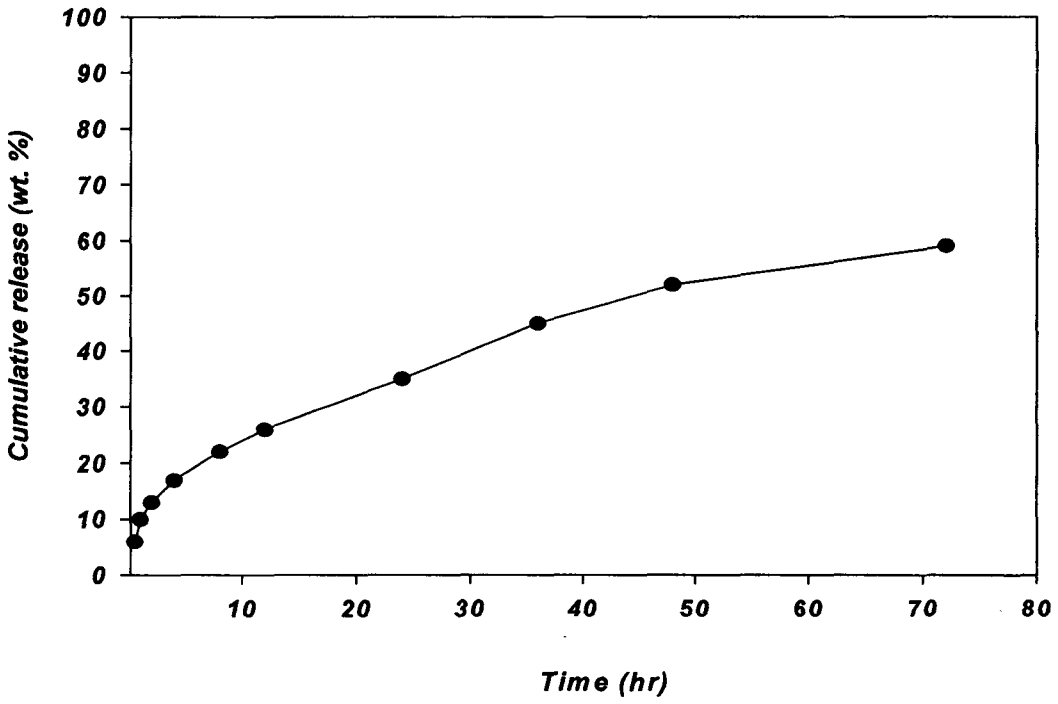


Fig. 7


Cumulative release (wt. %)
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Time (hr)