Preparation of a Bead-Type Methylated Sericin Drug Delivery Carrier for the Treatment of *Helicobacter pylori* Infection

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In the present study, methylated silk sericin (MeSS) was prepared and a bead-type drug delivery carrier was formulated for the potent treatment of *Helicobacter pylori* infection. Sericin was reacted with methanol in the presence of hydrochloric acid as a catalyst under mild conditions to form an ester bond between the carboxyl group of sericin and the hydroxyl group of methanol. The methylation of silk sericin was confirmed by Fourier transform infrared spectroscopy (FTIR) and point of zero charge (pH\textsubscript{pzc}) studies. The formulated MeSS beads swelled more under acidic conditions, which is below the pH\textsubscript{pzc} of the MeSS. The release behavior of the model drug from the MeSS beads in the medium was extremely pH-sensitive. These results suggest that the formulated MeSS beads have the potential to be an effective antimicrobial agent carrier for the treatment of *H. pylori* infection.

**Key words**: Silk sericin, Esterification, Methylation, *Helicobacter pylori*, Drug delivery

INTRODUCTION

*Helicobacter pylori* is a gram-negative, spiral micro-aerophilic bacterium that is commonly found in the stomach (Hejazi and Amiji, 2002). It can penetrate the protective epithelial lining of the stomach and also attach to cells of the stomach (Kusters et al., 2006). Infection with *H. pylori* causes more than 90% of duodenal ulcers and up to 80% of gastric ulcers. It is estimated that, by the year 2020, *H. pylori* infection will be one of the top ten major causes of death worldwide (Sikora, 1999). Therapies for *H. pylori* infection have been developed, and there are many antibiotics that have activity against the organism in vitro. However, no single agent that completely inhibits *H. pylori* and prevents infection has been developed. To minimize the drug resistance of *H. pylori*, a combination of more than two antimicrobial agents along with a proton pump inhibitor is the recommended therapy for its effective eradication (McColl, 2010).

The incomplete efficacy of antimicrobial agents against *H. pylori* may be attributed to the short drug residence times and degradation by gastric acid; this prevents the maintenance of sufficient drug concentration in the gastric mucosa layer during constant time (Hwang et al., 1998). In order to release the drug at the desired concentration for a long time in a specific region, a targeted local drug delivery carrier is needed.

Sericin is derived from silkworm cocoons and comprises 25-30% of the cocoon. Sericin covers two strands of fibroin fibers and bonds them together like glue, to form a cocoon. Most of the sericin from the degumming process has been abandoned as wastewater, up until now. Many attempts have been made to find an application for this wasted resource in polymeric fields such as cosmetics, metal removal biosorbents, enzyme immobilization supports, tissue engineering scaffolds, and drug delivery carriers (Aramwit et al., 2010; Kundu et al., 2008; Kwak et al., 2014; Lee et al., 2005; Oh et al., 2011a). Li et al. (2008) investigated the therapeutic benefit of sericin against alcohol-induced gastric injury. Oh et al. (2007) prepared sericin beads and discovered its potential as an oral drug delivery carrier. The pH of the buffer solution significantly affects the drug-release profile because of its pH-dependent swelling behavior.

In the present study, we formulated methylated sericin beads that have different swelling behaviors compared to raw silk sericin, which has an isoelectric point close to acidic condition. Hydrochloric acid (HCl) catalyzes the reaction, which forms an ester bond between the hydroxyl group of methanol, and the carboxyl group of sericin, under mild reaction conditions. Confirmation of methylation was achieved with Fourier transform infrared spectroscopy (FTIR) and elemental analysis. Using 1 M lithium chloride (LiCl) and a dimethyl sulfoxide (DMSO) solvent system, methylated sericin beads were prepared. The swelling behavior was tested under various pH conditions. Finally, amoxicillin, one of antimicrobial agents for the treatment of *H. pylori*, was loaded into MeSS beads and the in vitro drug release profile was obtained.

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EXPERIMENTAL

Preparation of Silk Sericin (SS) and Methylated Silk Sericin (MeSS)

Silk sericin (SS) was extracted by boiling 20 g of Bombyx mori silkworm cocoons with 500 mL of distilled water in an autoclave for 1 h at 120°C. The extracted solution was filtered with a nonwoven filter in order to remove the undissolved cocoons. The SS solution was frozen at −70°C for 4 h and lyophilized. Methylated silk sericin (MeSS) was prepared according to the method of Fraenkel-Conrat and Olcott (Fraenkel-Conrat and Olcott, 1945). A total of 10 g of SS was suspended in 100 mL of methyl alcohol containing 0.1 mol of HCl as a catalyst for the esterification process, and the mixture was stirred at room temperature for 24 h. The MeSS was separated by centrifugation at 3300 rpm for 30 min. Then, it was repeatedly washed with distilled water to remove the remaining methyl alcohol and lyophilized.

Preparation of Silk Sericin (SS) and Methylated Silk Sericin (MeSS) beads

The beads were prepared by dissolving 2.5 g samples of SS and MeSS in 10 mL of 1 M LiCl and DMSO for 3 h at room temperature. After complete dissolution, the solutions were transferred to syringes and mounted on a syringe pump (KD Scientific, USA). The dope solutions were dropped into methanol, which was the coagulant. The drip rate was 20 mL/h, the size of the needle was 26 G, and the distance between the needle end and the surface of the coagulant was fixed at 3 cm. The beads that formed were kept in the coagulant for 1 h. Finally, the prepared beads were washed with distilled water.

Characterization

Elemental analysis was carried out using an elemental (C, N, S) analyzer (Flash EA 1112, Thermo Electron Corporation, USA). The analytical method was based on the flash dynamic combustion method, which produces complete combustion and elemental gases of the sample at 1800°C within a high-temperature reaction chamber. On the basis of the atom percentage of each element, the carbon:nitrogen (C:N) and hydrogen:nitrogen (H:N) ratio was calculated as follows:

\[
(1) \text{C:N ratio} = \frac{\text{Weight percentage of C}}{\text{Weight percentage of N}}
\]

\[
(2) \text{H:N ratio} = \frac{\text{Weight percentage of H}}{\text{Weight percentage of N}}
\]

The point of zero charge (pH_{pzc}) of the sericin sample was measured using the pH drift method. A total of 50 mL of 0.01 M sodium chloride (NaCl) solution was placed in a closed Erlenmeyer flask. The pH was adjusted over the range of 2-9 using 0.1 M HCl or 0.1 M sodium hydroxide (NaOH). Subsequently, 0.1 g of the sericin sample was added to the solution. The final pH was measured after 48 h of agitation and plotted against the initial pH. The pH at the point of intersection of the experimental curve and the line of the initial pH is the final pH_{pzc} of the sericin sample. The morphology of the SS and MeSS beads were analyzed using a field-emission scanning electron microscope (FESEM; Supra 55VP, Carl Zeiss, Germany).

Swelling Ratio Test

The prepared beads were dried in an oven at 50°C overnight. Twenty dried beads were immersed in buffer solutions at different pH values, including 2.2 (0.4 M glycine-HCl), 7.4 (0.2 M sodium carbonate, Na_2CO_3), and 9.4 (0.2 M Na_2CO_3), followed by agitation for 12 h at room temperature. The swollen beads were recovered from the buffer solution, and the liquid on the surface of the beads was gently removed using wipers. The swelling ratio of the beads was calculated as follows:

\[
\text{Swelling ratio} = \frac{W_s - W_d}{W_d}
\]

where \(W_d\) is the dry weight of the beads and \(W_s\) is the swollen weight of the beads.

In Vitro Amoxicillin Release Kinetics

Amoxicillin release behavior studies were carried out on the SS and MeSS beads by dissolving 100 mg of amoxicillin in 5 mL of 25% SS and MeSS dope solutions. Using the bead preparation method in section 2.2 above, 20 beads were collected and immersed in buffer solutions of varying pH including, pH 2.2 (0.4 M glycine-HCl), pH 7.4 (0.1 M sodium phosphate, Na_3PO_4), pH 9.4 (0.2 M Na_2CO_3). Aliquots were withdrawn at predetermined time intervals and an equal volume of buffer solution was replaced in each drug release solution to maintain a constant volume. Each withdrawn sample was analyzed using an ultraviolet (UV)/Vis spectrometer at 272 nm, and the cumulative drug release profiles were determined.

RESULTS AND DISCUSSION

Methylation of Silk Sericin (SS)

The aim of methylating the free carboxylic group is to introduce a methoxy group on the carboxylic acid of the silk protein. Fraenkel-Conrat and Olcott (1945) reported that many proteins are readily methylated at room temperature in alcohol containing mineral acids. The acid catalyzes the reaction involving specific carboxylic acids of protein, whereas the amino, thiol, and amide groups of amino acids are not affected (Seki et al., 2004). We pre-
pared methylated SS using methanol and HCl under mild reaction conditions. Fig. 1 shows the FTIR spectra of the SS and MeSS products. SS shows a band at 1400 cm$^{-1}$, which denotes OH-bending of the carboxylic acid. Conversely, MeSS did not exhibit this peak but showed new bands at 1739 cm$^{-1}$ and 1173 cm$^{-1}$, which denote the C=O and C-O stretching vibrations, respectively, of the methoxy group (Wheelwright et al., 2013). Moreover, the ratio of the band intensity at 2960 cm$^{-1}$ (CH$_3$ vibration) and 2932 cm$^{-1}$ (CH$_2$ vibration, [I$_{\nu \text{CH}_3}$/I$_{\nu \text{CH}_2}$]), which confirms the methylation process, increased from 1.010 with SS to 1.044 with MeSS, because the carboxyl group was methylated. An elemental analysis was performed to examine the methylation process further, and the results are shown in Table 1. The methylation process introduced the methoxy group, which was formed by the conversion of the -COOH to -the COOCH$_3$ group. This reaction increases the ratio of C and H compared to N which does not participate in the reaction. In the MeSS, the C:N ratio and H:N ratio were 3.4 and 6.31, respectively, while for the SS, the C:N ratio and H:N ratio were 3.13 and 5.19, respectively. This result indicates that the methylation of the carboxylic group was successfully accomplished with the methanol and HCl catalyst. From the results of the characterization experiment, it is clear that SS was successfully esterified to a methyl ester by the carboxylic acid specific reaction.

The effect of methylation on the charge balance MeSS was determined by estimating the pH$_{\text{pzc}}$ using the pH drift method. At pH < pH$_{\text{pzc}}$, the protein surface had a positive net charge, whereas at pH > pH$_{\text{pzc}}$, the surface had a negative net charge. Fig. 2 shows the result of the pH drift experiment; the data show that the pH$_{\text{pzc}}$ of the SS and MeSS are 5.0 and 8.6, respectively. The net charge of the protein depends on the quantitative relationship between the amine and carboxylic acid groups of the amino acid. The acid-catalyzed methylation reaction using methanol esterified the carboxylic acid of SS. Therefore, the surface of the MeSS might have acquired the highly positively charged group also.

**Preparation of the Silk Sericin (SS) and Methylated Silk Sericin (MeSS) Beads**

The 1 M LiCl and DMSO solution was an effective solvent system for the preparation of the high-concentration sericin dope solution. The bead-type sericin has been previously formulated using the dropping method, and the microparticles had been formulated by electrospraying (Kwak et al., 2013; Oh et al., 2011a). In this study, SS and MeSS beads were prepared using a 1 M LiCl and DMSO solvent system by the dropping method. Fig. 2 shows the FESEM images of the prepared SS and MeSS beads. Both SS and MeSS beads were spherical, with a

<table>
<thead>
<tr>
<th>Sample</th>
<th>C, %</th>
<th>N, %</th>
<th>H, %</th>
<th>C : N atom ratio</th>
<th>H : N atom ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>42.98</td>
<td>16.00</td>
<td>5.94</td>
<td>3.13</td>
<td>5.19</td>
</tr>
<tr>
<td>MeSS</td>
<td>42.35</td>
<td>14.50</td>
<td>6.54</td>
<td>3.40</td>
<td>6.30</td>
</tr>
</tbody>
</table>

![Fig. 1. Fourier Transform Infrared Spectroscopy (FTIR) Spectra of Silk Sericin (SS) and Methylated Silk Sericin (MeSS).](image1)

![Fig. 2. Point of zero charge (pH$_{\text{pzc}}$) of Silk Sericin and Methylated Silk Sericin (MeSS).](image2)
smooth surface morphology. Fig. 2 shows the average size of SS and MeSS beads in wet and dry condition. Comparing their sizes under wet condition, the SS bead was 2.01 ± 0.12 mm, while the MeSS was 2.27 ± 0.15 mm. This trend was similar under dry conditions as well, where the SS bead was 1.46 ± 0.07 mm and MeSS was 1.62 ± 0.09 mm. The difference in size might be due to differences in the viscosity of the resulting dope solutions used to produce the beads. Oh et al. (2011b) proposed the sericin refining method using ethanol precipitation. The low molecular weight sericin was removed by this fractionation process, and the hydrophobic ethanol-precipitated sericin fraction, had a higher viscosity than the SS. Similarly, in the methylation process of the SS, the methanol precipitated a certain sericin fraction, and it was assumed that there would also be an increase in viscosity.

**Swelling Behavior**

The degree of swelling is a critical parameter for drug delivery carrier which is closely related to drug loading and releasing properties. The swelling data of the SS and MeSS beads in different pH buffer solution is shown in Fig. 4. The SS and MeSS beads exhibited different swelling behaviors. The degree of swelling in the SS beads was greater at pH 9.4 compared with pH 2.2 and 7.4. Conversely, the MeSS beads had the greatest swelling capability at pH 2.2 compared with pH 7.4 and 9.4. The swelling behavior of protein beads is strongly affected by the ionic group composition and the pH of swelling the medium. The chemical composition of the protein and pH of the swelling medium are closely related to the net charge of the protein. At pH 2.2, SS beads had a positive charge related repulsive force, while at pH 7.4, they had a negative charge related repulsive force, which lead to the characteristic swelling behavior. Moreover, at pH 9.4 there was a stronger negative charge related repulsive force compared to pH 7.4. As a result, there was more repulsion and a greater swelling behavior. The MeSS beads, in contrast, showed a different swelling behavior because their pH_pzc was close to neutral at pH 7.63. Therefore, in the pH 7.4 buffer solution, it is difficult to drive the swelling...
behavior. However at pH 2.2, there was a strong positive charge related repulsive force and large swelling ratio. The resulting swelling behavior indicated that the SS beads have potential as an intestinal-specific oral drug delivery carrier. Furthermore, the MeSS beads show potential as a gastric-specific antimicrobial drug delivery carrier against *H. pylori*.

**In Vitro Release Behavior of Amoxicillin from Methylated Silk Sericin (MeSS) Beads**

The purpose of a drug delivery system is the control of the desired drug release behavior. This can be achieved with the carrier, ensuring that the drug is delivered to the infection site according to the desired time frame. The release profiles of unloaded and unencapsulated drugs have been shown to be rapid and complete (Wallace *et al.*, 2012). In our study, Figure 6 shows the cumulative release profiles of the amoxicillin from the SS and MeSS beads at 37°C for 10 h in buffer solutions with different pH values. The SS beads exhibited gastrointestinal targeted drug delivery, with much higher drug release at intestinal condition than gastric condition. This tendency is in agreement with the swelling behavior of the SS beads, which indicates that the main drug release mechanism is diffusion through the swollen beads.

As discussed earlier, the swelling ratio of the prepared MeSS beads under gastric conditions (pH 2.2) was greater than under intestinal conditions (pH 7.4). With the MeSS beads, approximately 80% of the loaded drug was released within 8 h at pH 2.2, whereas only 37% was released in a pH 7.4 buffer solution. The drug release profile also matched its characteristic swelling behavior.

**CONCLUSION**

Our study proves the possibility of enhancing the efficacy of antimicrobial agents against *H. pylori* by using modified silk sericin beads as a delivery carrier. Using methanol and HCl, the carboxyl groups of sericin were successfully methylated. The amoxicillin release profile depended on the swelling behaviors of beads. The MeSS beads have a greater gastric-specific drug release capability. Therefore, we expected that the modified MeSS bead is a potential drug delivery carrier system for the effective treatment of *H. pylori*.

**REFERENCES**


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