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Combined Silk Fibroin Microneedles for Insulin Delivery

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Abstract

To reduce the pain caused by subcutaneous injections, the microneedle patches as the new transdermal drug delivery method are gaining increased attention. In this study, we fabricated a composite insulin-loaded microneedle patch. Silk fibroin, a natural polymer material, was used as the raw material. The tip of the microneedle had good dissolving property and was able to dissolve rapidly to promote the release of insulin. The pedestal had the property of swelling without dissolving and was carrying insulin as a drug store. The insulin carried by the pedestal could release continuously through the micropore channels created by the microneedles. This kind of microneedle could achieve a sustained release effect. It was observed that the Insulin had a good storage stability in this kind of microneedles, and it maintained more than 90% of its biological activity after 30 days. The results of transdermal delivery of diabetic rats showed that the microneedle patches displayed apparent hypoglycaemic effect and indicated a sustained release effect. These drug-loaded silk microneedle patches may act as potential delivery systems for the treatment of diabetes.

Keywords: silk fibroin, microneedles, insulin, transdermal delivery

1. Introduction
Diabetes is a common metabolic disease, which is characterized by high blood sugar. Long-term diabetes can cause other disorders, such as retinopathy, renal failure, neuropathy, and cardiovascular disease. In recent years, the number of people with diabetes has increased, particularly in developing countries. Currently, insulin is the most used drug for the treatment of diabetes. It can be delivered by subcutaneous injection in various ways, such as using syringes, insulin pens, and insulin pumps. However, the repeated injections cause the severe pain and increase risk of infection.

With the advancement in micro-manufacturing technology, the microneedles are being developed. This painless and convenient method of drug delivery is receiving increased attention from the researchers. This system has many applications in disease treatment, immunobiological administration, disease diagnosis and cosmetic field. The lengths of the microneedles are usually between 50 and 900 μm, which are of sufficient lengths to penetrate the stratum corneum of the skin. Therefore, the drug can enter the dermis through a transdermal route and is delivered to the whole body through microcirculation. The early microneedles were mostly silicon, glass, and metal microneedles. Nowadays, the polymer microneedles with high toughness and good biocompatibility exhibit better prospects. Usually the biodegradable polymers are widely used in scientific research. These kinds of microneedles can be degraded in the body, which can reduce the possibility of infection and the generation of medical waste.

Silk protein fibroin is a common natural polymer. Compared to other natural polymers, fibroin has better mechanical properties. Fibroin with excellent toughness and ductility is often used as a tissue engineering and biomedical materials. At the same time, silk fibroin has good biocompatibility, and good tolerance and biodegradability in vivo. This material hardly causes immune rejection. The versatility of silk fibroin makes it widely used for drug delivery. Fibroin is easy dissolve to get aqueous solution and is used to prepare soluble microneedles. However, microneedles made from unmixed fibroin are prone to rupture and rapid dissolution may lead to sudden drug release. Thus, some modifications are required to improve the toughness and dissolution time and finally to extend the drug release time from the microneedles.
In this study, unmixed silk fibroin is used as the dissolvable microneedle tip, and the pedestal is provided to improve the swellability of fibroin, which will act as the drug store. This type of microneedle may easily penetrate the skin, and the needle tips can open the channels of the skin surface and dissolve rapidly to release insulin. The pedestal is slowly swelled to provide power for subsequent insulin release. This structure can avoid the rapid release of the drug while the soluble ingredients dissolve.

2. Materials and Methods

2.1 Experimental materials

Fresh mulberry silkworm cocoons of *Bombyx mori* from Xiancan Silk Biotechnology Co. Ltd. (Suzhou, China), dialysis bags from Puyi Biotechnology Co. Ltd. (Shanghai, China), proline from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), insulin from Meilun Biotechnology Co. Ltd. (Dalian, China), the insulin ELISA kit from Kejing Biotechnology Co. Ltd. (Jiangsu, China), and streptozotocin (STZ) from Macklin (Shanghai, China) were purchased for this experiment. Male SD rats and New Zealand rabbits were provided by the Experimental Animal Center of Suzhou University.

2.2 Preparation of silk protein fibroin solution

The fibroin solution was prepared as described previously. The silkworm cocoons were treated in a 0.3% (w/v) Na$_2$CO$_3$ /0.1% (w/v) NaHCO$_3$ solution at 100 °C for 30 min. This was repeated three times to fully remove sericin (glue protein). After washing and drying at 60 °C, the dried fibroin fibres were dissolved in a 9.3M LiBr solution for 1 h. The dissolved fibroin solution was dialyzed with deionized water at 4 °C for 3 days to remove small molecules. The fibroin fibres that were not completely dissolved were filtered off, and the obtained fibroin solution was stored at 4 °C.

2.3 Preparation of microneedles

A mixed solution of insulin and fibroin was prepared as described below. Insulin was dissolved in Tris-HCl buffer solution (pH = 7.0), and mixed solutions were prepared according to the insulin/fibroin mass ratios of 1:100, 1:50, and 1:20. The mixed solution was poured into a polydimethylsiloxane (PDMS) mould, and was evacuated.
3 times to remove the bubbles from the solution on the mould. Then, the excess solution on the surface of the mould was removed. They were dried at 25°C for 8h to obtain the microneedle tip. A fibroin/proline/insulin mixed solution with a mass ratio of 50:10:1 was prepared. This was used as the pedestal for the composite microneedles. The solution was put onto the mould and dried to obtain composite drug-loaded microneedles as shown in Figure 1.

Figure 1. Preparation of composite insulin-loaded silk fibroin microneedles

In this experiment, the microneedles with needle tips loaded with insulin/silk protein at the ratio of 1:50 were selected. The drug load was 2%. The experimental animals were the diabetic rats. Diabetes cannot be treated with a low loading capacity. Pre-experimental displayed that: The microneedles with a low loading ratio of insulin/silk fibroin 1:100 were ineffective due to too low concentration of insulin released. Some rats still kept high blood glucose and couldn’t achieve normal blood glucose in time after administration of such microneedles. The microneedles with a high drug-loading ratio of 1:20 can achieve the expected effect due to high concentration of insulin released. However, some rats died due to the too low blood glucose. So we selected appropriate microneedles with an insulin/fibroin ratio of 1:50.

2.4 Mechanical properties

To study the force required for the microneedle to penetrate the skin, the mechanical properties of the microneedle were tested with a texture analyser. A single piece of microneedle (15 * 15) was taken and affixed to the compression sensor. Simultaneously, the rabbit skin was fixed below the test bench and compressed at a constant test speed of 10mm/min (n= 3). The mechanical properties of pure fibroin microneedles, methanol-tREATED insoluble fibroin microneedles (methanol was sprayed on the surface of pure fibroin microneedles followed by drying), composite fibroin microneedles, and composite fibroin microneedles with different loading capacities
were tested. The rabbit skin that was punctured by the microneedles was removed. The pinhole marks on the skin surface were observed.

2.5 Dissolution and swelling performance

The dissolution and swelling properties of pure fibroin microneedles, methanol-treated insoluble fibroin microneedles, and composite fibroin microneedles were tested. A sample of 0.1g (5 parallel samples of each group) was acquired by adding PBS buffer solution (pH = 7.4) at a bath ratio of 1:100, and was shaken in a constant temperature water bath at 37 °C for 24h. After removing the microneedles, they were rinsed with deionized water. A filter paper was used to absorb surface moisture. Then, the weights were measured. After centrifugation at 3500 rpm for 15 minutes, the supernatant was collected, and the absorbance was measured at 278 nm using an ultraviolet spectrophotometer. The solubility of the microneedles in the buffer solution was estimated from a standard curve. The dissolvability and swelling rates of the microneedles were calculated according to the following formula:

\[
\text{swelling ratio}(\%) = \frac{m_1-m}{m \times N \times [1/(1+S)]} \times 100\% \quad (1)
\]

\[
\text{dissolve ratio}(\%) = \frac{100C}{m \times N \times [1/(1+S)]} \times 100\% \quad (2)
\]

where \(m\) is the initial mass of the microneedles, \(m_1\) is the mass after swelling, \(N\) is the solid content of the microneedles (the ratio of the mass to the initial mass after drying in an oven at 100 °C for 4 h), and \(S\) is the mass ratio of proline to fibroin, \(C\) is the solubility of the microneedles.

2.6 Stability of insulin in microneedles

To understand the storage stability of insulin in microneedles, both the newly prepared insulin/fibroin microneedle tips and the insulin/fibroin microneedle tips that were left at room temperature for 30 days were dissolved in phosphate buffer solution. Insulin ELISA kit was used to measure the insulin activities of the following four groups of solutions: a newly prepared insulin/fibroin solution, insulin/fibroin solution placed for 30 days, newly prepared insulin microneedle tips dissolved in PBS (pH = 7.4), and insulin microneedle tips dissolved in PBS and kept for 30 days.

2.7 In vitro release of insulin
PBS buffer solution was used to simulate the release of insulin in body fluids. The microneedles were inserted into the cleaned rabbit skin and the needle tips were fixed downwards in the middle of the diffusion cell. Then 15 mL PBS solution was added into the diffusion cell to ensure the rabbit skin was in contact with the buffer solution. The solution was magnetically stirred at 300 rpm. Samples of 1 mL were taken at regular intervals, and at the same time, 1 mL of fresh PBS buffer solution was added into the diffusion cell. The insulin release was measured by an insulin ELISA kit.

2.8 Transdermal delivery of insulin into the rats

Male SD rats (150-250g) were used for this experiment. Streptozotocin (STZ) was dissolved in citric acid-sodium citrate buffer solution to prepare a 2% mass concentration solution for modelling type 1 diabetes. All rats were given a single intraperitoneal injection with 60 mg/kg of STZ solution after 12 h of fasting. After 3 days, the blood glucose was stable and was above 16.7 mmol/L. This indicated that the modelling was successful.

The rats were shaved and cleaned before the experiment. The diabetic rats were divided into the following groups: (1) blank group, no administration to diabetic rats; (2) injection group, intraperitoneal injection of insulin solution (5IU) to diabetic rats; (3) microneedle group, insulin microneedles were administered on the back of rats (5IU); (4) unloaded microneedle group, the microneedles without drugs were attached to the backs of the rats. The blood samples were taken from the tail vein every hour before and within 8 hours after administration, and the blood glucose levels were measured with a blood glucose meter. Then, the blood samples were centrifuged at 3000 rpm for 10 minutes to separate the serum, and the serum insulin concentration was measured by an insulin ELISA kit.

2.9 Analysis of pharmacodynamics and pharmacokinetics of insulin

The minimum blood glucose level ($C_{min}$) and the time point of minimum glucose level ($T_{min}$) were determined by the percentage change curve of blood glucose over time relative to the initial level. The relative pharmacological availability (RPA) was calculated by the following formula:

$$RPA(\%) = \frac{(AAC_{MN} \times Dose_{IP})}{(AAC_{IP} \times Dose_{MN})} \times 100$$  (3)
Here, $A_{AC \text{MN}}$ represents the area above the curve after administration of insulin-loaded microneedles, and $A_{AC \text{IP}}$ represents the area above the curve after insulin injection.

The maximum serum insulin concentration ($C_{\text{max}}$) and the time point of maximum serum insulin concentration ($T_{\text{max}}$) were obtained from the curve of serum insulin concentration over time. The relative bioavailability (RBA) was calculated using the following formula:

$$\text{RBA} (\%) = \frac{A_{\text{UC MN}} \times \text{Dose}_{\text{IP}}}{A_{\text{UC IP}} \times \text{Dose}_{\text{MN}}} \times 100$$  \hspace{1cm} (4)

where $A_{\text{UC MN}}$ represents the area under the blood concentration-time curve after administration of insulin-loaded microneedles, and $A_{\text{UC IP}}$ represents the area under the blood concentration-time curve after insulin injection.

2.10 Statistical analysis

The results of this study were all expressed as mean ± SD. A difference of $p<0.05$ was considered to be statistically significant.

3. Results

3.1 Characterization of microneedles

The microneedles prepared by the mould are slender and tapered, and 600 μm long and about 330 μm wide. A microneedle patch consists of 225 (15 * 15) microneedles, which are evenly distributed. In a transdermal drug delivery system, the microneedles only need to pierce the epidermal layer of the skin. Then, the drug diffuses into the dermal layer and is absorbed by capillaries into the body’s circulation. The thickness of the human epidermal layer is 70-120 μm. The microneedles are sharp enough to penetrate the epidermis.
Figure 2. Characterization of microneedles under a stereomicroscope. (A) Single microneedle under the microscope, with a height of about 600 μm. (B) The observed microneedle patch array under the microscope, which has loading capacity about 5 IU/piece.

The composite structure provides the microneedles with their high loading capacity. Only insulin is added to the silk fibroin solution in soluble needle tips, which greatly increase the upper limit of insulin loading capacity. The maximum loading capacity can reach up to 7%, which can achieve the effect of quickly stopping the blood sugar level from rising after meals. The swelling pedestal serves as a slow-release drug pouch with a maximum drug load of 5%. This structure has a higher loading capacity than the ordinary microneedles. A small piece of microneedle is enough to achieve the desired treatment effect. It is painless and minimally invasive.

3.2 Mechanical properties of microneedles

The microneedles were pierced slowly and uniformly into the rabbit skin. The test process is shown in Figure 3(A). Figure 3(B) is a diagram of the surface of the skin after the microneedles had been inserted. The uniform pinholes left by the microneedles were clearly visible on the skin, which indicated that each needle was able to penetrate the skin evenly. Figure 3(C) shows the displacement-force curves of silk fibroin microneedles (SF), methanol-treated intolerable microneedles (METH), and composite silk fibroin microneedles (SF + pro). All three types of microneedles can penetrate the skin with small force, which is convenient to use. Figure 3(D) shows the test results of the mechanical properties of the microneedles with different loading capacities, prepared as described in section 2.3. As the loading capacity increased, the microneedles were softened and the force was increased. Therefore, to ensure the therapeutic effect of the administered amount, it was easier to pierce the skin by adding an appropriate amount of the drug to the microneedle.
Figure 3. Mechanical performance test of the microneedles. (A) Schematic diagram of the testing process; (B) The skin surface after microneedle penetration; (C) Mechanical properties of the microneedles prepared from different materials; (D) Mechanical properties of microdermal microneedles with different loading capacities.

3.3 Microneedle dissolution and swelling

The microneedles prepared by unmixed silk fibroin are almost completely dissolved and deformed in PBS buffer solution. Therefore, it is difficult to test the swelling rate. The dissolve rate is as high as 60%. When treated with methanol, the swelling and dissolve rates were greatly reduced. They were almost insoluble in the solution. The composite microneedles showed an improvement of swelling and dissolvability rates. The swelling rate of the composite microneedles reached to 180%, and the dissolution rate in the solution was about 25%. These partially dissolved microneedles with high swelling rates could control the drug release easily.
3.4 Insulin stability in the microneedles

To determine the storage stability of insulin in silk fibroin solution and of insulin-loaded silk fibroin microneedles at room temperature, the prepared microneedles were dissolved in PBS solution. The insulin content was measured by an insulin ELISA kit. The results showed the difficulties in maintaining biological activity of insulin for a long period at room temperature in silk fibroin solution (Figure 5). After 30 days, the activity decreased by more than a half. When the insulin and fibroin solutions were used to make the microneedles, a good biological activity could be maintained. When left at room temperature for 30 days, the biological activity could still be maintained at about 90%. This provides a good basis for the use of insulin and silk fibroin-loaded microneedles.
3.5 In vitro release of insulin from composite microneedles

To study the process of insulin release from the microneedles, the microneedles were inserted into the rabbit skin to check the cumulative drug release rate in PBS buffer solution. From Figure 6 (A), it is observed that both the soluble microneedles and the composite microneedles could extend a very high cumulative release rate. With the extension of time, the cumulative release rate could reach more than 90%, while the insulin in methanol-treated insoluble microneedles were released slowly within 9 h. The cumulative release rate was very low. As compared to the rapid release of insulin from the soluble microneedles, the cumulative release rate of the composite microneedles reached more than 50% in the first 2 h. This indicated that insulin was released rapidly with the dissolution of the needle tips. It was then observed that the release of insulin from the microneedles gradually became slower. Nine hours later, the cumulative release rate gradually reached to more than 90%.
3.6 Hypoglycaemic effect in rats

To study the hypoglycaemic effect of insulin-loaded silk fibroin microneedles in vivo, the back of SD rats was shaved and cleaned with alcohol, and the rats were administered according to the groups. As shown in Figure 7, the microneedle tips are almost completely dissolved after administration. The dissolution of the needle tips left the rat skin surface with tiny channels, which facilitated the contact of body fluids with the swelling pedestal. The swollen structure made the pedestal absorbed the body fluids and expanded. The body fluids formed a dynamic cycle to promote the release of insulin in the pedestal.
Figure 7. *In vivo* experiments with insulin-loaded microneedles in rats. (A) and (B) Photographs of rats treated with insulin-loaded microneedles; (C) Photographs of the skin surface of rats after microneedles were removed; (D) Microscopic morphology of the microneedles before administration; (E) Microscopic morphology of the microneedles after 4 h of administration in rats.

The blood glucose level curve of rats in each group over time is shown in Figure 8 (A). Both the microneedle group and the injection group showed significant hypoglycaemic effect; however, the injection group had a more significant hypoglycaemic effect. The blank group and the unloaded microneedles had no hypoglycaemic effect. The injectable administration made the blood glucose of rats to reach at the lowest level in the second hour. It returned to the initial levels after 4 h. After administering the rats with the same insulin content through microneedles, the blood glucose reached the lowest levels in 3 h, and returned to the original levels after 6 h. The unloaded microneedles had no effect on the blood glucose levels in the rats.

The drug-loaded microneedles released faster *in vivo* because the blood vessels in the dermis could absorb most of the polymer in the epidermis to promote percutaneous absorption. This permitted the drugs into the tissues through the systemic circulation. The blood flow accelerated the drug release. As compared to the static environment of drug release *in vitro*, the release of insulin in the body was faster. This decreased after reaching the highest point because insulin is metabolized in the body.
Figure 8. Drug release curve in diabetic rats. (A) Blood glucose levels over time; (B) Serum insulin concentrations over time.

Figure 8 (B) shows the differences in serum insulin levels in each group of rats. In the injection group, serum insulin rapidly increased to the maximum in 1 h after administration. The microneedle administration caused the serum insulin levels to reach the maximum in the second hour. The maximum was lower than the injection administration group. After 2 h, the serum insulin content of the microneedle group was always higher than that of the injection group. These results show that microneedles can release insulin slowly and smoothly, and the insulin may last longer in the blood. Thus, the use of such prepared microneedles is a stable and efficient way for insulin administration.

The pharmacodynamic parameters of blood glucose levels and the pharmacokinetic parameters of serum insulin concentrations are shown in Tables 1 and 2. Table 1 shows that the relative pharmacological availability (RPA) of insulin in the microneedles is 92.7 ± 28.2%, which can achieve a therapeutic effect close to the injection. The pharmacokinetic parameters of serum insulin are shown in Table 2. The relative bioavailability (RBA) of insulin from the microneedles reached 98.8 ± 12.6%. This indicates that insulin is almost completely released with the dissolution of the microneedles. Thus, the silk fibroin drug loading system can maintain the high level of insulin activity.
Table 1. Pharmacodynamic parameters of blood glucose levels in diabetic rats after administration of insulin-containing microneedles and insulin injection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$C_{\text{min}}$ (%)</th>
<th>$T_{\text{min}}$ (h)</th>
<th>AAC$_{0→8}$ (%)h</th>
<th>RPA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNs</td>
<td>57.7±4.0</td>
<td>3</td>
<td>99.9±30.5</td>
<td>92.7±28.2</td>
</tr>
<tr>
<td>Injection</td>
<td>46.1±5.2</td>
<td>2</td>
<td>107.8±25.8</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Pharmacokinetic parameters of serum insulin concentration in diabetic rats after administration of insulin-containing microneedle, and insulin injection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$C_{\text{max}}$ (μIU /mL)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>AUC$_{0→8}$ (μIUh/mL)</th>
<th>RBA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNs</td>
<td>133.3±10.4</td>
<td>2</td>
<td>363.2±46.3</td>
<td>98.8±12.6</td>
</tr>
<tr>
<td>Injection</td>
<td>199.5±17.5</td>
<td>1</td>
<td>367.5±47.2</td>
<td>100</td>
</tr>
</tbody>
</table>

4. Discussion

Figure 9. Mechanism of insulin release in microneedles.

The release mechanism of microneedles in the skin is shown in the figure 9. After the microneedles penetrate the skin, the needle tips first come in contact with the skin. Body fluids in the skin quickly dissolve the needle tip and insulin is released into the skin. The micropores form on the surface of the skin by the dissolution of the needle tip, and body fluid enters the micropores to contact the pedestal, which promotes the swelling of the pedestal and insulin release simultaneously and continuously. This structure can meet the needs of patients to quickly lower blood sugar after a meal. At the same time, it will not cause hypoglycaemia due to excessive insulin release and achieve a sustained release effect.
The needle tip made of pure silk material guarantees the sufficient mechanical properties of the microneedle. At the same time, the excellent biocompatibility of silk fibroin enables insulin to be stably stored in microneedles. In vitro release process, it can be seen that insulin is rapidly released within the first 2 hours. Then the release rate is slowed down, and the release is completed within 9 hours. In rats, the release rate is faster due to the biological circulation. Compared with injection, compound microneedle administration has a longer duration of action and a more moderate effect. The micropores formed by the dissolution of the needle tip promote the release of insulin. This makes the availability of insulin in the microneedle close to the effect of injection, and reduce drug waste.

5. Conclusions

In this study, composite silk fibroin microneedles were developed for transdermal delivery of insulin. The good solubility and biodegradability of the silk fibroin support the stable release of insulin in the rats without rejection. At the same time, insulin could maintain its biological activity in silk fibroin microneedles and achieve a hypoglycaemic effect. The microneedle administration could achieve a sustained release effect as compared to the regular injections. This system effectively avoids hypoglycaemia and pain caused by the injections. The delivery of insulin using silk fibroin microneedles shows great application prospects in the treatment of diabetes.

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