pH-Responsive Cellulose-Based Microspheres Designed as an Effective Oral Delivery System for Insulin

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ABSTRACT: Functional modified cellulose microsphere (CMs) materials exhibit great application potential in drug various fields. Here, we designed pH-responsive carboxylated cellulose microspheres (CCMs) by the citric/hydrochloric acid hydrolysis method to enhance oral bioavailability of insulin by a green route. The CMs were high purity cellulose that dissolved and regenerated from a green solvent by the green sol–gel method. The prepared microspheres were characterized by spectroscopic techniques, such as field emission scanning electron microscopy (FE-SEM), Fourier transform infrared spectrum (FT-IR), X-ray diffraction (XPS), etc. The spherical porous structure and carboxylation of cellulose were confirmed by FESEM and FT-IR, respectively. Insulin was loaded into the CCMs by electrostatic interactions, and the insulin release was controlled through ionization of carboxyl groups and proton balance. In vitro insulin release profiles demonstrated the suppression of insulin release in artificial gastric fluid (AGF), while a significant increase at artificial intestinal fluid (AIF) was observed. The insulin release profile was fitted in Korsmeyer–Peppas kinetic model, and insulin release was governed by the Fickian diffusion mechanism. The stability of the secondary structure of insulin was studied by dichroism circular. Excellent biocompatibility and no cytotoxicity of designed CCMs cast them as a potential oral insulin carrier.

1. INTRODUCTION

Oral administration of drugs is desirable due to the convenience and increased compliance to patients, especially for chronic diseases, such as diabetes, that require frequent administration. Although, enzyme degradation and hydrolysis in the gastrointestinal tract could limit the effectiveness of insulin.15 Insulin is a hormone, which regulates blood sugar, advised to diabetic patients, and it also undergoes degradation in the presence of gastric enzymes and acids due to destruction of disulfide bonds.3−5 pH-responsive carriers offer excellent potential as oral therapeutic systems by enhancing the stability of insulin delivery in stomach and achieving controlled release in intestines. This motivates the development of an oral insulin transport carrier to improve its bioavailability, and various studies have been reported.6−12 Considering the advantages of natural materials, researchers are looking for nontoxic, biocompatible, biorenewable, and low-cost natural materials to replace synthetics.

Several naturally derived polymers have been investigated to design pH-responsive insulin carriers owing to their renewable and biocompatible nature.13,14 Some researchers designed natural polymer-based insulin delivery systems, but most of them utilized toxic reagents in complex preparation methods.13,15 Cellulose microspheres (CMs), has been widely explored for its high porosity, hydrophilicity, biocompatibility, and great modification potential.16 Therefore, cellulose-based microspheres can reasonably be used as a drug carrier. Although the microsphere system is not as convenient as the nanometer system, still they have the advantages of controlling the amount of insulin released in the stomach to protect insulin from the inhospitable environment of the stomach.17 Microspheres can reduce the dosing frequency and thereby improve patient compliance. CMs are easy to prepare by the sol–gel method from the green solvent (sodium hydroxide/urea aqueous solution). The high porosity, appropriate specific surface area, and hydrophilicity of cellulose-based microspheres play key roles in insulin loading and controlled release ability.18 In addition, in accordance to the structure and physicochemical properties of the insulin, the interfacial microstructure (such as pore size and particle size) of cellulose-based microspheres can be controlled to design high-performance oral insulin carriers to improve the bioavailability of insulin.18 Since cellulose is nondigestible and nondegradable in the human body, cellulose-based materials are in general considered as noncytotoxic and biocompatible.
microsphere-loaded insulin can be released continuously in the body without affecting the carrier’s structure.

Insulin is a proteinaceous drug with amino and carboxyl groups in its structure with an isoelectric point (pI) of 5.5–6.4. The pI of cellulose was 3.0. The carboxylation of CMs introduces anionic functional groups that reduced the pI of carboxylated cellulose microspheres (CCMs), which helps to bind with the insulin drug via electrostatic interactions. The citric/hydrochloric acid hydrolysis method is a green chemistry approach to fabricate CCMs, where hydronium ions (H₃O⁺) from HCl dissociation hydrolyze the amorphous domains of CMs and catalyze the esterification of hydroxyl groups on the exposed CMs simultaneously. Optimal pH environments promote insulin loading and release via electrostatic interactions between insulin and CCMs.

In order to design a new oral insulin carrier by a simple green route, pH-responsive CCMs were prepared though the citric/hydrochloric acid hydrolysis method to control oral insulin delivery. CCMs’ morphology, insulin loading and controlled release profiles, and the mechanism involving the interactions between insulin and CCMs were investigated. The circular dichroism (CD) analysis of native insulin and released insulin in vitro cytotoxicity of CMs and CCMs were also evaluated to establish the effectiveness of designed materials in drug delivery systems.

2. RESULTS AND DISCUSSION

2.1. Morphology and Structure Characterization. FESEM images of CMs, CCMs, and insulin−CCMs are shown in Figure 1. All the samples exhibited good spherical shape and a highly crowded porous three-dimensional (3D) structure. The porous structure was regulated by the gelation and regeneration processes of cellulose solution when treated with a large volume of nonsolvent and a relatively high temperature; the phase separation happened during the process, and the solvent-rich regions in cellulose solution contributed to the pore formation. The porous structure of CMs is conducive to the chemical reaction and drug loading, which made cellulose-based microspheres ideal candidates as insulin carriers. Generally, the presence of pores facilitates the penetration of water/insulin solution into CCMs, consequently benefits the diffusion and absorbency of insulin. Compared with CMs (Figure 1b), the pores of CCMs (Figure 1e) increased slightly. It may be due to the electrostatic repulsion between carboxyl groups of CCMs resulted in a larger pore of CCMs than that of CMs. Insulin−CCMs exhibited smaller pores than CMs and CCMs (Figure 1h). The reason could be that positively charged insulin was loaded into negatively charged CCMs by electrostatic interaction to occupy the space of pore of CCMs at pH = 4 PBS. The element content of the surfaces of CMs, CCMs, and insulin−CCMs were measured by energy dispersive X-ray spectroscopy (EDS) patterns (Figure 1c,f,i). Compared to CMs and CCMs, the presence of sulfur element on the surface of insulin−CCMs indicated that insulin was loaded into the CCMs.

Correlation between the chemical structures of CMs, CCMs, and insulin−CCMs was studied with FT-IR spectra (Figure 2a). There was a broad peak between 3000–3300 cm⁻¹, which could be attributed to −OH groups of cellulose. Compared with CMs, a new carbonyl peak was observed at 1730 cm⁻¹, indicating the presence of carboxyl groups (−COOH) on CCMs. This could be caused by the hydronium ions (H₃O⁺) from HCl catalyzed during the esterification of hydroxyl groups on the exposed CM chains with carboxyl groups of C₆H₈O₇. The graft yield and grafting efficiency of CCMs were 12.2 and 4.2%, respectively. The results indicated that the carboxyl groups of citric acid were successfully grafted into CCMs via the esterification reaction.

Generally, drug carriers require high-temperature treatment before using, so it was necessary to evaluate the thermal stability of CMs and CCMs. The thermogravimetric analysis (TGA) and differential thermogravimetric analysis (DTGA) for CMs and CCMs are displayed in Figure 2b.

Interestingly, CCMs exhibited a slightly lower onset degradation temperature (111.28 °C) than that of CMs (124.2 °C). This might be a result of the insertion of carboxyl group that disrupted the hydrogen bonding of CMs. Moreover, the small weight loss at the low-temperature range was due to the evaporation of absorbed water, whereas the two other weight losses were due to pyrolysis of hydrocarbon chains. Plus, the main degradations of CMs and CCMs did not start at the same point; the thermographs showed different degradation behaviors for CMs and CCMs, which indicated that acid hydrolysis could have increased the degree of crystallinity and thus led to the different degradation behaviors. With the increase of temperature, CMs and CCMs showed the degradation at the temperature range of 150–600 °C. The peak of DTGA represented the temperature of the maximum weight reduction. Based on the DTGA analysis, it can be observed that small degradation at 250 °C existed in CMs and then followed by a relatively small peak. Meanwhile, CCMs started the main decomposition at about 250 °C, and the degradation increased until the peak appeared, which suggested that CCMs possessed higher thermal stability than that of CMs. This result revealed the successful insertion of the carboxyl group on CMs. CCMs can withstand the high temperature of autoclaving and sterilization before insulin loading, and the structure was not destroyed by the high temperature, so CCMs were suitable as insulin carriers.

To explore insulin loading and release mechanisms, the zeta potential of CCMs was measured from pH 1.0 to 13.0. The results are shown in Figure 2c. When pH > 2.3, the net negative zeta potential indicated that the ionization of carboxylic acid (COOH → COO⁻) increased the absolute value of the zeta potential, resulting in more negative
The original pI of insulin fell in the range of 5.5−6.4,19 and insulin showed a positive zeta potential at pH = 4 PBS. It indicated that insulin was loaded into CCMs by electrostatic action. When pH < 2.3, CCM dispersions showed a positive zeta potential, indicating the protonation of carboxylic acid. The hydrogen bonding interactions between the protonated carboxylic groups of CCMs might preserve the structure of CCMs in a compact collapsed state, preventing the release of insulin in AGF. However, insulin is negatively charged and CCMs are negatively charged in AIF. The electrostatic repulsion between COO\(^{-}\) of CCM chains enlarged the pore size of CCMs and promoted water molecules into CCMs. At the same time, the electrostatic repulsion between insulin and CCMs also promoted the insulin diffusion and release.

The esterification reaction among the CMs and citric acid can be verified by using the XPS technique too. Figure 3 shows the peak spectra of C1s. The peaks at 284.8 and 286.3 eV belong to C−C and C−O−C, respectively.28 Compared with Figure 3b, a new peak appeared at 288.6 eV in Figure 3c, which was attributed to the ester (O−C==O) peak.29 In addition, the change of the peak (centered at 284.8 and 286.3 eV) area and the appearance of a new peak (O−C==O) (centered at 288.6 eV) in Figure 3c indicated the successful esterification reaction of hydroxyl groups on CMs with carboxylic groups of citric acid. This further showed the hydrolysis mechanism of citric acid/hydrochloric acid, which is also in accordance with the FT-IR results.28,29

2.2. Swelling Properties of CCMs. To examine the effect of solution pH on water uptake, the swelling ratio (SR) was determined using CCMs gravimetric ratios in AGF and AIF, respectively. The swelling property of CCMs was calculated with eq 3. CCMs showed higher swelling in AIF (598.3%) than in AGF (455.7%). This observation can be explained by the ionization and protonation equilibrium of the carboxyl groups (pK\(_a\) = 4.3) in CCMs. In AGF, the carboxyl groups were protonated to promote the formation of intramolecular hydrogen bonds. Thereby, CCMs exhibited lower swelling ratios. While in AIF, the swelling ratios were higher than those in AGF. The reason was that the hydrogen bonds were broken in AIF and generated the electrostatic repulsion forces among the ionized carboxylic acids,30,31 benefiting the diffusion of water molecules into CCMs, which therefore led to quick swelling. CCMs were responsive to changes in pH values, they can protect insulin against the gastric fluid in AGF while quickly swelling in AIF to facilitate the diffusion and absorption of insulin.

2.3. Insulin Loading and Controlled Release Performance of CCMs. To study the loading and releasing properties of insulin, a constant flow pump was used to simulate gastrointestinal absorption. CCMs were added in a glass column (internal diameter = 1.1 cm). The pH = 4 insulin solution was used as eluent solution, which was collected after passing through the column with CCMs at predetermined time intervals to calculate insulin loading efficiency (ILE) and insulin loading quality (LC). The values of ILE and LC depended on the insulin concentration, insulin/CCMs ratio, solvent pH, etc.30 The ILE and LC were observed as 2.6% and 335 mg g\(^{-1}\) via eqs 4 and 5, respectively. This may be due to the opposite charges between insulin and CCMs at pH = 4. Insulin is positively charged at pH = 419 and CCMs are negatively charged at pH = 4 under room temperature as is shown by zeta potential of CCMs (Figure 2c). This promoted insulin loading via electrostatic interactions.
To determine the release properties of insulin from insulin-CMs and insulin−CCMs, the constant flow pump was used to simulate gastrointestinal absorption. AGF and AIF were used as eluent solutions, which were collected after passing through the column with CMs and CCMs at predetermined time intervals to obtain the breakthrough curves, respectively. The release behavior of insulin in AGF and AIF were studied, respectively (Figure 4). As shown in Figure 4a, 68.44% of insulin were released in AGF, and 65.05% of insulin were released in AIF, respectively. The results showed that oral insulin delivery could not be controlled by CMs. Figure 4b shows the insulin release curves versus time from insulin−CCMs in AGF and AIF, respectively. In AGF, 32.75% of insulin was released fast, followed by 17.05% of release. In AIF, the release rate was fast, with 44.5% of the total insulin release after 10 min, and then an equilibrium plateau was gradually established at about 85.3%. The burst release phenomenon was due to the fact that insulin was attached to the surface of CCMs.32,33 Controlled release of insulin from insulin−CCMs can be regulated by the proton balance of carboxyl groups and the charge of insulin in AGF and AIF. In AGF, the hydrogen bonding interactions between the protonated carboxylic groups of CCMs might preserve the structure of insulin in compact collapsed state, preventing the release of insulin.13 In AIF, the carboxyl groups of CCMs existed as COO−. The electrostatic repulsion between COO− enlarged the pore size of CCMs and promoted water molecules into CCMs. Insulin, with a molecular weight of about 6000, is a hydrophilic water-soluble protein,34 and it is also negatively charged in AIF. The electrostatic repulsion between insulin and CCMs also promoted the diffusion of insulin into water molecules. The insulin molecule contains six positively charged and 10 negatively charged amino acid residues. Due to a handful of positive charge in the insulin molecule and the formation of the hydrogel barrier after the CCM swelling, insulin was trapped in CCMs and could not be released completely. This indicated that the carboxyl groups of CCMs played an important role in the oral delivery of insulin.

For the stomach and intestine environments, the calculated parameters of the Korsmeyer model are summed up in Table 1. The n values were less than 0.45; the correlation coefficients for the model ranged from 0.9397 to 0.9981, indicating that insulin was released according to the Fickian diffusion.

### Conformation of Insulin Release

The folding and conformation of insulin correlated with its activity and can change during the formulation process. Therefore, it is crucial to prepare controlled release systems that can release insulin in its active form. The qualitative and quantitative information about protein conformations were provided by CD spectra.35 From Figure 5, the released insulin from insulin−CCMs displayed similar characteristic peaks at about 208 and 223 nm on CD spectra compared with native insulin, suggesting that the secondary structure stability of released insulin was preserved during the loading and release processes. The CD results confirmed that there were no obvious conformational changes for the released insulin from insulin−CCMs.

### In Vitro Cell Viability of Samples

Considering the safety of oral preparations, it is necessary to evaluate the toxicity of CMs and CCMs.36 The cytotoxicities of CMs and CCMs were tested by the MTT method. The biocompatibility of CMs and CCMs was assessed by A549 cells (Figure 6). It can be seen from Figure 6 that the viability of the cells was above 90%, indicating that both CMs and CCMs were biocompatible.
noncytotoxic and had good biocompatibility. When the concentrations of CMs and CCMs reached 200 μg mL⁻¹, the cell viability exceeded 100%. This suggested that high concentrations of cellulose-based microspheres promoted cell growth, which was indicative of low toxicity of cellulose microspheres. Results showed that the CMs and the reagents used in the preparation of CCM carriers were nontoxic. CCMs have a potential application to safe oral insulin administration.

2.6. Design Strategy and Insulin-Loaded and Controlled Release Mechanisms of CCMs. In this work, CCMs were used as the carrier to improve the oral bioavailability of insulin. The design mechanism of CCMs is depicted in Scheme 1. Based on the design principle of CCMs, the carboxyl group of citric acid was combined with the hydroxyl group of CMs through the esterification reaction so that CCMs is pH sensitive. The pI of insulin is 5.5–6.4, and the pI of the CCMs is 2.3. Insulin is positively charged and CCMs are negatively charged at pH = 4 PBS. Insulin was loaded into CCMs by electrostatic interactions. Scheme 1 shows the controlled release mechanism of oral insulin from CCMs. The controlled release of insulin can be explained by the ionization and proton equilibrium of the carboxyl groups (pKₐ = 4.3) of CCMs. In AGF, pH = 1.2 PBS was lower than the pKₐ of the carboxyl groups, which made them positively charged. The carboxyl groups of CCM existed in the protonated form (–COOH). Hydrogen bond interactions between –COOH caused CCMs to shrink. CCMs were in a tight collapsed state, inhibiting the entry of water molecules and the diffusion of insulin. In AIF, pH = 7.4 PBS was higher than the pKₐ of the carboxyl groups, making them negatively charged. –COOH existed in the form of –COO⁻, and the electrostatic repulsion between –COO⁻ caused the CCMs to swell. It was beneficial to the diffusion of water molecules into the CCMs and promoted the release of insulin. At the same time, the electrostatic repulsion between insulin and CCMs also promoted insulin diffusion and release.

3. CONCLUSIONS

In this work, CCMs were prepared by a green route via the citric/hydrochloric acid hydrolysis method from CMs, which were prepared through the sol–gel transition method from green cellulose solvent. CCMs were chosen to be used as a candidate material of an oral insulin carrier. The oral bioavailability of insulin was insured by the pH sensitivity of CCMs. In vitro release studies demonstrated that release of insulin was 48.87 and 85.12% in AGF and AIF, respectively. The release curve of insulin from CCMs conformed to Fickian diffusion by the Korsmeyer–Peppas model fitting. The CD results that the secondary structure stabulity of released insulin was preserved during the loading and release process. Cell viability revealed that the CCMs were noncytotoxicity. These results indicated that the encapsulation of insulin into CCMs was a key factor in the improvement of its oral absorption and oral bioactivity; the designed CCMs had the potential for oral insulin delivery.

4. MATERIALS AND METHODS

4.1. Materials. Cellulose (cotton linter pulp; α-cellulose > 95%) was purchased from Hubei Chemical Fiber Group Ltd. (Xiangfan, China). Its viscosity average molecular weight (Mᵥ) was 12.5 × 10⁴ Da. Citrate was purchased from Aladdin. Insulin (porcine pancreatic) was provided by Xuzhou WanBang Co. Phosphate buffer saline (PBS), AIF, (pH = 7.4 PBS), and AGF (pH = 1.2 PBS) were prepared to simulate the pH environment of the human stomach and intestines. The pH = 1.2 PBS was prepared with 7 mL of hydrochloric acid (HCl) and 93 mL of dipotassium hydrogen phosphate (KH₂PO₄).
acid (37 wt %) and 2.0 g of sodium chloride dissolved in 1 L of deionized water (DI water). Potassium dihydrogen phosphate (6.8 g) was dissolved in DI water, and the pH value was adjusted to 7.4, followed by diluting the solution to 500 mL to prepare pH = 7.4 PBS.

4.2. Preparation of CCMs. CMs were prepared through the sol−gel transition method according to our previous work.40 CCMs were fabricated according to the citric/hydrochloric acid hydrolysis method.28 Briefly, 8 g of CMs and the acid mixture (90% citric acid/10% hydrochloric acid (v/v)) were added to a 500 mL three-necked flask and stirred constantly (300 r min−1) at 80 °C for 4 h. After the reaction was completed, the suspension was quickly cooled to room temperature. Then, CCMs and mixed acids were separated by filtration processes, and CCMs were further washed three times with DI water to remove unreacted acid mixtures. Finally, CCMs were dried in a vacuum oven at 60 °C for 48 h.

4.3. Characterizations. The morphology and structure of CMs, CCMs, and insulin−CCMs were investigated by FE-SEM (GeminiSEM 300), and the surface elements were analyzed by EDS. Samples were crushed into powder for other characterizations, and pellets were pressed with potassium bromide for FT-IR analysis. FTIR spectra were recorded on an FT-IR spectrometer (170-SX, Thermo Nicolet Ltd., U.S.A.). The CMs and CCMs chemical states of the elements were determined by XPS (Kratos, U.K.). Thermal properties of the elements were determined by the Malvern nanoparticle size potentiometer (Zetasizer Nano S90). A secondary structure of insulin was evaluated by Chirascan Plus (Applied Photophysics, United Kingdom).

4.4. Graft Yield and Graft Efficiency of CCMs. The graft yield and graft efficiency of CCMs were calculated by the gravimetric method using the following relationships:

\[
graft \text{ yield (GY\%) } = \left( \frac{W_g - W_0}{W_0} \right) \times 100\%
\]

\[
graft \text{ efficiency (GE\%) } = \left( \frac{W_g - W_0}{W_m} \right) \times 100\%
\]

where \(W_0\) is the CMs before reacting, \(W_g\) represents the weight of the CCMs after reacting, and \(W_m\) denotes the weight of the citric acid used for reacting.41

4.5. pH-Responsive Swelling. CCMs were immersed in AGF and AIF until the swelling equilibrium is reached to study the pH swelling properties of CCMs by the gravimetric method. Dry CCMs (\(G_i\) (10 mg)) were added to 50 mL of AGF and AIF soaking for 24 h, respectively. The immersing fluid was removed by centrifugation, and the weight of the wet CCMs (\(G_s\)) was recorded. The SR was calculated using the following equation:

\[
SR \text{ (\%) } = \left( \frac{G_s}{G_i} \right) \times 100\%
\]

where \(G_s\) is the weight of wet CCMs, and \(G_i\) is the weight of dried CCMs.26

4.6. In Vitro Insulin Loading and Release Properties. Insulin was loaded into CCMs by electrostatic action. CCMs (0.4 g) were added in glass column (internal diameter = 1.1 cm). The column was pretreated with PBS eluent water of pH = 4. pH = 4 insulin solution was then used as the eluent solution. The eluent solution was collected after passing through the column with CCMs at predetermined time intervals to calculate the insulin loading efficiency and insulin loading quality. The concentration of insulin was detected by UV/vis spectrophotometer at wavelength of 276 nm and quantified by comparing with a standard curve.42 When the concentrations (C) of insulin at pH = 4 PBS were 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg mL−1, the absorbance (A) at the wavelength of 276 nm were 0.000, 0.212, 0.395, 0.620, 0.820, and 1.046, respectively. Then the standard curve equation \(A = -0.004 + 1.040C \quad (R^2 = 0.999)\) was obtained by linear fitting. Finally, the concentration of insulin was calculated according to the standard curve equation. ILE (%) and LC(mg g−1) were calculated using the following equations:

\[
\text{ILE (\%)} = \left( \frac{C_0 - C_1}{C_0} \right) \times 100\%
\]

\[
\text{LC} = \left( \frac{C_0 - C_1}{V/m} \right)
\]

where \(C_0\) and \(C_1\) are the initial insulin concentration (mg mL−1) and the final or equilibrium insulin concentration (mg mL−1), respectively. \(V\) represents the volume of insulin solution (mL), and \(m\) denotes the dry weight of CCMs (g).

In order to assess the insulin release property, constant flow pump was used to simulate gastrointestinal absorption. AGF and AIF were used as eluent solutions, which were collected after passing through the column with CCMs at predetermined time intervals to obtain the breakthrough curves, respectively. The concentration of insulin was calculated according to the standard curve equation. The cumulative release percentage of insulin was calculated according to the equation as follows:43

\[
\text{cumulative insulin release (\%)} = \left[ \frac{V \sum_{i=0}^{n-1} C_i + V C_m}{m} \right] \times 100\%
\]

where \(M_i\) represents the mass of insulin in insulin−CCMs before release, \(V\) denotes the volume of the eluted water in the collector at a predetermined time interval, and \(C_m\) means the concentration of insulin in the \(n^{th}\) predetermined time interval.

The insulin release data were fitted by the Korsmeyer−Peppas equation to study the kinetics of insulin release from insulin−CCMs:45

\[
M_t/M_\infty = Kt^n
\]

where \(M_t/M_\infty\) is the mass of insulin released from the start of release to time \(t\), \(M_\infty\) means the mass of insulin in insulin−CCMs before release, and \(K\) and \(n\) denotes the rate constant and release exponent, respectively. The mechanism of insulin release depends on the value of \(n\): \(n \leq 0.45\) indicates the Fickian diffusion model, \(0.45 < n < 0.89\) corresponds to the non-Fickian diffusion model, and \(n > 0.89\) corresponds to the case II diffusion model.

4.7. Cytotoxicity of CCMs and Insulin−CCMs. To evaluate the potential toxicity of CMs and CCMs, the MTT assay was performed to measure the cell cytotoxicity of CMs and CCMs in A549 cells.44,45 The A549 cells were seeded into a 96-well plate for 24 h, and then CMs and CCMs with a concentration gradient (0, 50, 100, 150, and 200 \(\mu\)g mL−1) were added to the culture medium and cultured at 37 °C for 24 h. After removing the culture medium, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to A549 cells and cultured for 4 h. Then, 200 \(\mu\)L of DMSO was added to the A549 cells after
the removal of the MTT solution. The crystals were sufficiently dissolved by low-speed shaking. The absorbance was measured at 495 nm with microplate reader (SpectraMax MS, Molecular Devices).46,47

4.8. Circular Dichroism (CD) Tests of Released Insulin.
The activity of released insulin was evaluated by analysis of the structural stability by using CD spectrophotometer according to Hong et al.48 The released insulin and native insulin were measured at 25 °C with a cell path length of 1.0 cm, a bandwidth of 1.0 nm, response time of 0.25 s, and constant nitrogen flow of 5 L min⁻¹. The native insulin was prepared in pH = 7.4 PBS. The samples were scanned from 190 to 250 nm at resolution of 1.0 nm. For CD tests, each sample was tested for three times, and the final data took the average of three measurements.

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Notes
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