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Fabrication and Characterization of a Glucose-sensitive Antibacterial Chitosan-Polyethylene Oxide Hydrogel

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Abstract

A novel glucose-sensitive chitosan-polyethylene oxide (CS/PEO =1:0.5~1:2.5) hydrogel with controlled release of metronidazole (MNZ) was obtained by chemical cross-linking and immobilization of glucose oxidase (GO_x). The hydrogel was characterized by Fourier-transformed infrared spectroscopy (FTIR), compressive mechanical test, rheological analysis, cytotoxicity test, and antibacterial test against *Porphyromonas gingivalis*. The study found that the CS-PEO composite hydrogel possessed significantly better mechanical properties and biocompatibility than a single-component hydrogel. This might result from the physical cross-linking and formation of semi-interpenetrating network (semi-IPN). In addition, this novel hydrogel has self-regulate ability to release MNZ in response to the environmental glucose stimulus. Specifically, it released more drugs at higher glucose concentration, thus can lead to a greater ability to inhibit *Porphyromonas gingivalis*. This study has demonstrated the glucose-sensitive antibacterial hydrogel has a great potential as a new therapeutic material for treatment or prevention of periodontitis in diabetic patients.

Graphical Abstract

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Keywords

glucose-sensitive; hydrogel; chitosan; polyethylene oxide; controlled drug release; periodontitis

1. Introduction

Diabetes mellitus (DM) has become a challenging problem in today's oral therapy because it can easily cause complications of chronic periodontitis and wound inflammation [1, 2]. Therefore, it is very important to effectively monitor and control oral bacterial infection in diabetic patients. Unfortunately, the conventional methods, including routine oral administration of antibiotic (e.g. amoxicillin) and wound rinse, have been proved to be inefficient in many clinical cases [3, 4], because of the inflexible drug delivery formulation. Moreover, oral administration of drugs may also cause side effects to diabetic patients [5, 6]. Glucose-sensitive hydrogel is considered as an ideal system for site-specific controlled drug delivery mainly because of its novel self-regulated property [7]. Theoretically, this type of materials always has a 'smart sensor' built inside, which could sense and judge the stimulus caused by the change of diabetics' glycemic concentration and further activate (swell or shrink) the special 3D structure of the hydrogel's cross-linked network to control drug release at predetermined rates and predefined time. In addition, this kind of hydrogels also plays a key role to separate the drug from ambient hostile medium before its release [8].

Although glucose-sensitive hydrogels have enormous potentials, their application has been limited largely due to some inherent drawbacks such as poor mechanical conformability, unfavorable biocompatibility, and low controlled drug release ability [9]. In this study, for the first time, we have developed a glucose-sensitive chitosan-polyethylene oxide (CS-PEO) hydrogel with controlled release of metronidazole (MNZ)-a highly effective antibiotic to treat periodontitis [10]. Chitosan (CS) was used because of its good biocompatibility and biodegradability as well as inherent antimicrobial properties [11-13]. In addition, polyethylene oxide (PEO), even with the relatively high molecular weight, is regarded as a safe, biocompatible material and an effective enhancement to improve the mechanical and biological properties of composite hydrogels [14-16]. In this study, the experimental hydrogel has been tested for its physicochemical and mechanical properties,

biocompatibility, drug release ability in response to different glucose concentrations (GC) and antibacterial activity against *Porphyromonas gingivalis* (*P.gingivalis*) which is regarded as one of the major bacteria to be associated with human periodontitis [17].

2. Materials and Methods

2.1 Materials

Chitosan (CS: Molecule weight: 100,000-300,000 Da, practical amine: 7-12%) was purchased from Polysciences, Inc., USA. polyethylene oxide (PEO, Molecule weight: 400,000 Da), glucose oxidase (GO_x, from *Aspergillus niger*, 50KU), glutaraldehyde (GA, 25% aqueous solution) and metronidazole (MNZ) were all purchased from Sigma-Aldrich, USA. Osteoblasts used in cells culture study were derived from rats. For antibacterial activity, *P. gingivalis* ATCC 33277 was grown in Trypticase soy broth (BBL™, BD Company, USA), supplemented with 0.1g (per 100 ml) yeast extract (BD Company, USA), 0.5 mg hemin (Sigma-Aldrich, USA) and 0.1 mg vitamin K (Menadione, Sigma-Aldrich, USA).

2.2 Fabrication of Hydrogel

2.2.1 Preparation of CS-PEO film—Pre-weighted CS was dissolved in 0.5M acetic acid aqueous solution to prepare 1.5% weight/volume (w/v) CS solution. After 2h stirring, PEO was added to form the blend with pre-determined weight ratio (CS/PEO = 1:0.5~1:2.5). Then, the mixture was stirred constantly until these two components completely dissolved and formed homogeneous solution. After that, the solution was poured onto glass surface and dried in vacuum oven at 60°C. The CS-PEO films with thickness of 0.8 mm to 1.0 mm were obtained after 24h drying.

2.2.2 Preparation of cross-linked CS-PEO hydrogel film—The preparation of cross-linked CS-PEO hydrogel film was carried out similarly as described above. The only difference was that after obtaining the homogeneous CS-PEO blend, a certain amount of cross-linking agent (GA) was slowly dripped into this solution under constant stirring. Then, the solution was transferred onto a glass surface to form the CS-PEO hydrogel at room temperature. The resulting product was obtained by putting the hydrogel into the vacuum oven at 60°C overnight.

A simple stability experiment was conducted to investigate the cross-linking reaction between GA and CS. The CS-PEO film and CS-PEO hydrogel film cross-linked by GA were immersed in acetic acid aqueous for 24 hours, respectively. The photos at 4 different time points including 0, 1, 6 and 24 h were recorded. In addition, the possible cross-linking reaction between PEO and GA was also investigated using a similar method. First, 2 wt% PEO aqueous solution was prepared. After that, 2.5% (v/v) GA was slowly dripped into this solution under constant stirring. The photos of this blend at the beginning (0 h) and after 24 h were recorded to show if there was chemical cross-linking reaction between PEO and GA.

2.2.3 Preparation of glucose-sensitive CS-PEO hydrogel loaded with MNZ

First, the CS-PEO blend was obtained using the method mentioned in section 2.2.1. Then,

0.5% (w/v) MNZ was added into this solution. After the drug was completely dissolved, cross-linking agent GA (0.5 - 2.5%, v/v) was slowly added into this mixture under constant stirring. Next, the solution was poured into a home-made cylinder mode (diameter: 25mm, height: 20 mm) to form the hydrogel at room temperature. The hydrogel was washed three times with deionized water to remove extra GA and acetic acid. Finally, the samples were immersed in GO_x solution (phosphate buffer pH=5) for 24h and kept at -40°C [18].

2.3 Characterization of hydrogel

2.3.1 Fourier Transform Infrared Spectroscopy (FTIR) analysis—FTIR analysis was carried out using Thermo-Nicolet Nexus 670 spectrometer (Thermo Electron, USA) to identify the chemical transformation of our obtained CS-PEO film and cross-linked CS-PEO hydrogel film, respectively. All the specimens were recorded from 400 to 4000 cm^{-1} through 64 scans with resolution of 8 cm^{-1} .

2.3.2 Solubility test—CS-PEO hydrogel film with the CS/PEO weight ratio of 1:1.5 was prepared and dried using the method described in section 2.2.2. After that, each dry film was adjusted to the same mass (1 g) and then all the films were divided into three groups: the first group was immersed in phosphate buffer (pH = 7.4) and then placed into a shaker with continuously shaking at 107 cycles per minute under 37°C (type I); the second group was immersed in the same phosphate buffer and continuously agitated at 400 rpm by magnetic stirring under 37°C (type II); the final group was immersed in 50% aqueous methanol solution (v/v) with the constant 400 rpm magnetic stirring under 60°C (type III). After 24h stirring, all the samples were taken out, dried in oven and measured for the dry weight. Each type of samples had three replicates.

2.3.3 Compressive Mechanical Test—At the beginning of this test, the cylindrical samples were prepared with the diameter 25 mm and height 20 mm ($n = 8$). The test was conducted using the Instron 5566 universal testing machine (Instron Co., St Paul, USA) at crosshead speed of 0.5 mm/min.

2.3.4 Rheological analysis—The dynamic rheological properties of our hydrogel blend were determined using the ARES rheometer (TA Instruments-Waters LLC, New Castle, DE). 300 μl pre-gel blend (CS, PEO and GA at pre-determined ratio listed in Table.1) was quickly dripped onto the peltier plate. Storage modulus (G'), loss modulus (G'') were investigated as a function of time. The gel time (T_{gel}) of each sample was also recorded as the time when $G' = G''$. The initial measuring conditions were set to 0.5 mm gap, 1 Hz oscillation frequency and 25°C .

2.3.5 Binding Capacity (BC) of GO_x —The relationship between GO_x 's binding capacity and its initial concentration was characterized using a simple spectrometric method [18]. Briefly, after the hydrogel was immersed in GO_x solution for 24 hours, the enzyme's final concentration was measured by UV-visible spectrometer (Lambda 40, PerkinElmer Instruments, USA) at 280 nm using a standard calibration curve. Then, the BC was calculated using the following formula:

$$BC = \frac{C_i - C_f}{V_i} \times 100\%$$

C_i : The initial concentration of GO_x solution;

C_f : The final concentration of GO_x solution;

V_i : The initial volume of the immersed hydrogel;

2.3.6 Biocompatibility test

MTT assay: Considering the intrinsic cytotoxicity of GA [19], the cell viability of CS-PEO hydrogel sample was evaluated using the classical methylthiazolyl tetrazolium (MTT) method. Briefly, CS-PEO hydrogel film sample was primarily placed in a 48-well plate, then, 200 μ l pre-cultured osteoblasts suspension was added into each well followed by adding dukbecco's modified eagle medium (DMEM) after 4 hour's cultivation. The selected time points in this assay were 1, 2 and 3 days. At each determined point, 1mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide culture medium solution was dripped into each well. After 4 more hours' more cultivation, the cell culture was transferred to a 96-well plate and dimethyl sulfoxide (DMSO) was added. The optical density (OD) at 570 nm was recorded using enzyme-linked immune adsorbent assay (ELISA) plate reader (ELX800, Bio-TEK, USA). In this study, CS hydrogel film with the same amount of GA was prepared as another experimental group. Pure CS film was selected as negative control and cell's culture medium with DMEM as background. Each kind of samples had three replicates. Cell viability was calculated using the following formula:

$$\text{Cell Viability (\%)} = \frac{N_e - N_b}{N_c - N_b} \times 100\%$$

N_e —— the OD value of experimental sample.

N_c —— the OD value of negative control group.

N_b —— the OD value of background.

Calcein Staining: After cultivating the cells on the films at determined time points, samples were taken out and incubated in polyanionic dye calcein (calcein-AM, Sigma-Aldrich, USA) solution with the concentration of 1×10^{-6} M. Then, the samples were washed with PBS before fluorescence microscopy (DMIL, Leica, Germany) observation.

2.3.7 Swelling study—The swelling property of our hydrogels at different glucose concentration (GC) were investigated using a classical method [20]. Briefly, each pre-weighted hydrogel was immersed in phosphate buffer (pH=7.4) at selected GC (50 mg/dl, 100 mg/dl, 140 mg/dl, 200 mg/dl and 250 mg/dl) based on the World Health Organization diabetes diagnostic criteria [21]. At each determined time point including 1 day, 2 days and 3 days, the swollen samples were taken out, carefully removed the excessive surface water and weighted. The swelling ratio of each sample was calculated using the following formula:

$$\text{Swelling Ratio} = \frac{W_s - W_o}{W_o} \times 100\%$$

W_s : The weight of swollen sample.

W_o : The original weight of the sample.

2.3.8 Scanning Electron Microscope (SEM) analysis—The morphology of cross-section of the swollen hydrogel was observed by SEM (Hitachi S-2700, Hitachi-High Technologies America, Pleasanton, CA). The obtained hydrogels were dried using vacuum-freeze-drying method [22]. Briefly, the samples were placed in liquid nitrogen for 30 minutes and then cut to expose their cross-sections. After that, transferred those samples into the chamber of vacuum-freeze-drying equipment (Labconco corporation, USA) to remove the inner water at -40°C . After 5 hours dehydration, the samples were coated with carbon. The accelerating voltage of SEM analysis was 20 kv and the magnification was 100x.

2.3.9 Drug Release—First, pre-weighted glucose powder was added into phosphate buffer ($\text{pH} = 7.4$) to prepare the drug release medium with different GC (0 mg/dl, 50 mg/dl, 100 mg/dl, 140 mg/dl, 200 mg/dl and 250 mg/dl). Then, each hydrogel was immersed in a test tube containing 40 ml medium ($n=3$). These tubes were continuously agitated at 37°C and 107 cycles per minute. At every predetermined interval, 1.0 ml of supernatant was collected and 1.0 ml fresh glucose solution was added at the same time. The amount of released metronidazole was determined using the UV-visible spectrometer at 312 nm. Specifically, considering the glycemic concentration in human body is not always constant, glucose or phosphate buffer was added to adjust the system's original GC at a certain time point to investigate the drug release performance of CS-PEO hydrogel when the environmental GC suddenly changed.

2.3.10 Antibacterial activity test

Minimum inhibitory concentration (MIC): The MIC of MNZ against *P. gingivalis* was measured by classical broth micro-dilution method [23]. Briefly, prepared trypticase soy broth was added to a 96-well plate, 75 μl for each well. After that, 75 μl 1mg/ml MNZ solution was added to the wells of first column. The following step was transferring 75 μl mix dilution from the prior columns to the next columns in order to make sure the drug concentrations decreased by 50% successively. Next, 75 μl *P. gingivalis* suspension was added into each well and the plate was then put into an anaerobic incubator at 37°C for 48 hours. MIC was determined as the lowest concentration of MNZ at which there is no visible bacteria growth.

Preparation of aliquots: First, glucose solutions with determined concentrations (0 mg/dl, 50 mg/dl, 140 mg/dl and 250 mg/dl) were prepared and added into test tubes (2 ml for each tube), respectively. After that, the drug loaded samples ($n = 3$) were immersed into this solution followed by the continuous agitation at 37°C and 107 cycles per minute. At each time point including 1 day, 2 days and 3 days, 2 ml Aliquot was collected from each tube and followed by adding fresh glucose solution with same volume. In addition, to test the

possible interference from the components of CS-PEO hydrogel (e.g., unreacted GA) that could also inhibit the bacterial growth, a blank group - pure CS-PEO hydrogel was included for comparison. All the obtained aliquots were kept at 4°C until next experiment.

Measurement of antibacterial activity: Overall, 15 different kinds of aliquots were obtained and we used a simple and classical method to investigate the antibacterial activity of each aliquot. Briefly, 75µl liquid broth was transferred into each well of a 96-well plate. Then, 75µl aliquot obtained from blank group was dripped into the first well. After uniformly mixing, 75µl obtained solution from the prior well was transferred to the latter well to decrease its concentration by 50% successively. After ten times dilution of each sample, 75µl *P. gingivalis* suspension was added into every well. The plate was then put into the aerobic incubator at 37°C for 48 hours. The pure aliquot was used as background and *P. gingivalis* inoculated in broth without aliquot as the negative control. The antibacterial activity of this sample was measured by recording its optical density at 600nm using the same method mentioned in the cell culture study. Similarly, the aliquots from 0 mg/dl, 50 mg/dl, 140 mg/dl, 250 mg/dl after 1 day release and blank group, 0 mg/dl, 50 mg/dl, 140 mg/dl, 250 mg/dl after 3 days release were also tested using the same method. Each sample had 3 replicates.

2.4 Statistic analysis of data

The mean standard deviations of all measurements were calculated using the related function of Microsoft Excel 2013. The significant differences between two groups were determined using one-way ANOVA method. Difference in values with $p < 0.05$ was considered statistically significant.

3. Results

3.1 Optimization of the CS-PEO hydrogels fabrication

3.1.1 The cross-linking property and stability of hydrogel—Figure 1 shows the CS-PEO film and hydrogel's solubility in acetic acid aqueous, respectively. With the increase of time, CS-PEO film was quickly dissolved after 1 h immersion. On the contrary, even after 24 h immersion, the hydrogel film still remained intact, which has proved the cross-linking process actually occurred with the help of glutaraldehyde.

Figure 2(a) shows the state of PEO and GA blend at different time points. Obviously, PEO and GA blend still kept the original liquid state after 24h mixing which has strongly proved that there was no cross-linking reaction between these two components. Figure 2 (b) illustrates the dry weight of the CS-PEO hydrogel films under three different conditions. First, in view of the cross-linked CS and pure CS are difficult to dissolve in distilled water or methanol, therefore we could gain the amount of dissolved PEO by calculating the dry weight loss. In general, there was only a few PEO dissolved when the hydrogel film was immersed in phosphate buffer at 37°C. Furthermore, milder experimental conditions (type I) can lead to a fewer loss. However, when the film was immersed in the methanol solution at 60°C, nearly all the PEO was dissolved which could mainly be attributed to the fracture of intermolecular hydrogen bond between CS and PEO.

3.1.2 FTIR analysis—Figure 2(c) shows the FTIR spectrums of CS-PEO and CS-PEO hydrogel film, respectively. For CS-PEO film, there were two characteristic absorption peak at 1582 cm^{-1} and 841 cm^{-1} assigned to the primary amine, respectively. However, in the spectrum of CS-PEO hydrogel film, the above two peaks weakened significantly. At the same time, another new peak appeared at 1643 cm^{-1} , which corresponded to the formation of C=N group, the typical Schiff's base structure resulted from the cross-linking reaction between amine group and aldehyde group [24]. Besides, no obvious characteristic peaks were found at $1140\text{--}1190\text{ cm}^{-1}$ that would have been resulted from the acetal or hemiacetal reaction between GA and PEO, indicating that the cross-linking reaction may occur only between CS and GA. However, some broad peaks ranged from $1080\text{--}1100\text{ cm}^{-1}$ were observed in both spectrums, which suggested some intermolecular interaction (e.g., hydrogen bond) between CS and PEO according to the previous report [25].

3.1.3 Compressive Mechanical Test—In order to analyze the function of additive PEO in this system and further determine the optimal preparation conditions of our hydrogels, compressive mechanical test was carried out to measure the compressive strength and elastic modulus of those samples with different weight CS/PEO ratios. The results are shown in Figure.3. In general, with the increase of PEO content, the compressive strength exhibited a dramatic uptrend until the CS/PEO ratio reached to 1:2, followed by a slight decrease (from 43.09 KPa at 1:2 to 36.88 KPa at 1:2.). Besides, the elastic modulus of these samples showed a continuous increasing trend (but with less amount) from the ratio of 1:0 to 1:2.5.

3.1.4 Rheological analysis—Table.1 summarizes the samples' storage modulus (G'_{2500}) and loss modulus (G''_{2500}) when the rheology experiment proceed to 2500 s. Meanwhile, the gel time (T_{gel}) defined as the crossover of G' and G'' of each sample was also recorded. For the first five samples with the same CS/PEO weight ratio at 1:1.5, both G'_{2500} and G''_{2500} increased with the increasing of GA during gelation process. In addition, T_{gel} declined dramatically from over 1900 s to less than 200 s. Interestingly, similar trend could be observed when we fixed the GA volume and increased the CS/PEO ratio from 1:0 to 1:2.5, suggesting that PEO may have similar cross-linking function to GA during the hydrogel's gelatin process

3.1.5 Glucose oxidase (GO_x) binding capacity—Figure. 4 shows the bonded GO_x amount as a function of its initial concentration. In general, there was a steady increase of the bonded enzyme when its initial concentration changed from 0.5% to 2%. After that the increase started leveled off. This phenomenon is mainly due to the combination between GO_x and hydrogel's surface. It can be gradually impaired by the formation of GO_x - GO_x layer when GO_x reached a high concentration. Similar results about the physical adsorption of macromolecule substance such as proteins or enzymes were also reported in other studies [26, 27]. Therefore, the optimum initial GO_x concentration was selected at 2% (w/v).

3.2 Biocompatibility test: *in-vitro* cell culture study

The biocompatibility of the CS and CS-PEO hydrogels at determined time points were investigated by cell culture study of osteoblasts' viabilities. The results are shown in Figures 5 and 6. The fluorescence images at magnification of 40x illustrate the cell growth on these

two samples after 1 day or 3 days, respectively. Compared with the CS hydrogel sample, more osteoblasts grew on the surface of CS-PEO hydrogel after the same cultivation time, which strongly suggested that the hydrogel could improve cell proliferation with the help of additive PEO. Meanwhile, osteoblasts grown on CS-PEO hydrogel are larger and more spread out than those on CS gel after culture for 1 or 3 days, which implies that CS-PEO gel can provide a more favorable adhesion between cells and material surface. Overall, the cell viability of CS-PEO at each time point was significantly higher than that of pure CS hydrogel which indicated that the additive PEO could reduce the hydrogel's cytotoxicity and improve biocompatibility. However, compared with the negative control, CS-PEO still presented a certain degree of cytotoxicity which has proven that the undesirable influence of GA was difficult to eliminate.

3.3 Swelling study

Typical swelling behaviors of CS-PEO hydrogels in the solutions with different glucose concentration (GC) at each time point are shown in Figure 7(a). The swelling ratio of each sample presented fastest growth at the first 24 h and significantly slowed down at the latter two time points. Compared with the swelling performance at different GC, greater swelling ratio was observed at higher GC, which suggested glucose has acted as a key factor to stimulus the swelling of hydrogels.

The average pore sizes shown in Figure 7(b) have further demonstrated the significant effect of glucose on the swelling. Specifically, at lower GC (< 140 mg/dl), the pore size increased from $180\text{ }\mu\text{m}$ to about $300\text{ }\mu\text{m}$ when GC increased from 50 mg/dl to 140 mg/dl . At higher GC ($>140\text{ mg/dl}$), the pore size increased significantly from $300\text{ }\mu\text{m}$ to nearly $700\text{ }\mu\text{m}$ when GC increased from 140 mg/dl to 250 mg/dl .

The following SEM images illustrate the morphology of each sample's cross-section after 3 days' swelling. A steady and firm network at relatively low GC could be obtained. However, when GC reached to a higher level, some obvious fracture part (yellow arrow) appeared in the network which means very high GC level may have negative effect to the hydrogel structure because of the acceleration of the degradation process.

3.4 Drug Release

Figure 8(a) shows the release profiles of metronidazole (MNZ) from the immersed hydrogels at various GC. In general, a burst release could be observed at first, afterwards, the release rate gradually decreased and finally reached the plateau within 48 hours. Moreover, higher GC led to more drug release (from less than 15% at 0 mg/dl to over 85% at 250 mg/dl) which has also proved our hydrogel's glucose sensitivity.

Furthermore, the drug release performance of the hydrogel in response to the sudden change of surrounding GC was also investigated (as shown in Figure 8(b)). Within the first 4 hours, the sample at 200 mg/dl had a higher release rate than the one at 140 mg/dl . However, after GC decreased to 140 mg/dl , the release rate decreased sharply and quickly reached to the plateau (about 65%); In contrast, when GC increased from 140 to 200 mg/dl , the initial relatively low drug release rate increased gradually, and finally reached a higher total drug release percentage at about 78%. This phenomenon indicated that our drug release system

possessed self-regulated ability that it can automatically adjust the drug release rate based on the environmental glucose concentration.

3.5 Antibacterial activity investigation

The minimum inhibition concentration (MIC) of MNZ against *P. gingivalis* growth in this study was measured at 3.91 mg/L. Figure 9 shows the change of OD600 nm of those 10 pre-determined samples' drug release aliquots after repeatedly dilutions, which can quantitatively reveal each sample's antibacterial activity. First, the OD values of the aliquot collected from the blank group have kept a relatively high level regardless of the dilution time or time point. It implies that the any possible chemicals released from the blank hydrogel have little effect to inhibit the *P. gingivalis* growth. Moreover, compared with the rest of 4 aliquots collected after 1 day drug release, the OD values obtained from the samples immersed in the highest GC (250 mg/dl) maintained a relatively low level even after 10 times of 1:1 dilution (no bacterial growth). However, a dramatic increase of OD could be observed for the 140 mg/dl sample after 9th dilution, 50 mg/dl sample after 8th dilution and 0mg/dl sample after 7th dilution. This phenomenon reconfirmed that our synthesized hydrogel could release more MNZ at the medium with higher GC because of its glucose-sensitivity. Besides, it also has shown that it can effectively inhibit the growth of *P. gingivalis* even the released drugs were diluted several times. For the aliquots collected after 3 days, the samples exhibited lower antibacterial activity after fewer (1:1) dilutions (2 for 0 mg/dl, 3 for 50mg/dl, 4 for 140 and 250 mg/dl respectively) because the relatively low drug release in the 3rd day of each sample and the drug concentrations in the further diluted aliquots cannot meet the requirement of MIC. Combining with the results of *in-vitro* drug release, theoretically, after 3 days release, the drug concentrations were 2.38mg/L for the 0mg/dl aliquot after 2nd dilution; 3.19 mg/L for the 50 mg/dl aliquot after 4th dilution; 3.64 mg/L for the 140 mg/dl aliquot after 5th dilution and 3.47 mg/dl for the 250 mg/dl after 5th dilution. This phenomenon has implied that so far the antibacterial activity of our hydrogels may only sustain a relatively short time interval.

4. Discussion

In this study, a novel glucose sensitive hydrogel with controlled release of metronidazole (MNZ) was fabricated and characterized in order to reduce the risk of periodontitis in diabetic patient with high glucose concentration (GC). One important finding is that PEO has a significant impact on the mechanical and rheological properties of the hydrogel and CS/PEO ratio need to be optimized. We believe that PEO here only has two states: a small amount of PEO is free and the majority is physically cross-linked with CS. As shown in Figure 2, we have found no evidence of chemical cross-linking reaction between PEO and GA based on the following two results: a) after 24 h mixing, the PEO- GA blend remained in liquid state; b) no obvious acetal or hemiacetal characteristic peaks was observed in FTIR spectrum of CS-PEO hydrogel film.

Therefore, we believe that there was a large amount of physical cross-linking existing in the hydrogel and finally formed a special structure called semi-interpenetrating network (semi-IPN). Semi-IPN is a structure formed by one cross-linked network and another linear

polymer chain, which has special functions that the original hydrogel lacks [28]. Therefore, preparation of semi-IPN hydrogel could be a preferable method to satisfy the divergent requirements in terms of both ingredients' functionalities. In this study, as shown in scheme 1, despite of the basic cross-linked Schiff base structure brought by CS and GA, linear PEO with relatively long polymer chain was also incorporated into CS matrix before cross-linking reaction. Because of its high viscosity, the chain's movement was restricted, resulting in additional physical entanglement. Moreover, secondary interactions such as strong hydrogen bonds appeared and participated in the hydrogel formation. Therefore, the compressive strength and elastic modulus increased as the CS-PEO ratio increased from 1:0 to 1:2 the cross-linked network was strengthened. In addition, the crystallinity development of PEO may also be a reason for impaired compressive mechanical property of this hydrogel according to a previous report [29].

Another positive effect of semi-IPN in this study is the enhancement of the cross-linking property. This property is mainly determined by the ratio of the polymer repeating units and the cross-linking agent [30]. In this study, incorporating PEO can improve the hydrogel's storage modulus and loss modulus and decreased the gel time simultaneously. The similar result could be obtained when just increased the amount of cross-linking agent GA. In other words, the additional PEO may have physical cross-linking effect. This is because PEO can lead the two adjacent CS molecular chains linked together by formation of physical entanglement and secondary interactions (hydrogen bond) to build another type of cross-linking unit, which could further strengthen hydrogel's entire network. Such a CS-PEO semi-IPN is significant and very useful because it provides an alternative method to form strong hydrogel and thus it can minimize the intrinsic cytotoxicity of GA. As shown in cells culture study, the additive PEO effectively improves the whole system's biocompatibility. However, the negative effect of adding too much PEO into CS matrix was also observed. First, we noticed that when PEO content reached a relatively high level (>1:2), the whole system's compressive strength slightly decreased. This is because PEO cannot maintain a uniform distribution at such a high concentration due to its high viscosity. Besides, phase separation between these two ingredients will also result in stress concentration or the leaching of the water soluble PEO from the hydrogel that could finally reduce its mechanical property [31, 32]. Moreover, according to the previous report, higher cross-linking density will lead to smaller swelling ratio, which will largely impair the system's drug release ability [33]. Therefore, in this study, the final weight ratio of CS and PEO at 1:1.5 and the volume ratio of GA at 150 μ l/10ml were selected as the ideal preparation conditions.

Another important aspect in this study is the mechanism of stimulating the drug release by this hydrogel's glucose-sensitive ability. Scheme 2 describes the mechanism of glucose-sensitive drug release process. After the enzyme was completely absorbed into the hydrogel, the surrounding glucose and enzyme began to react and the product gluconic acid gradually reduced the medium's pH. Because of the cationic properties of Schiff's base in hydrogel's cross-linking structure, a great amount of H^+ penetrated into the hydrogel and protonated the amine nitrogen on chitosan to form $-NH^+$. After a short while, higher osmotic pressure inside the hydrogel would form to absorb the outside water into its inner network, causing the hydrogel to swelling and stimulate the drug release. Besides, the repulsive force between $-NH^+$ can also enlarge the distance of adjacent CS molecule chains to activate the hydrogel's

swelling behavior. Therefore, GC played a key role in initiating and catalyzing the whole process. In this study, as shown in swelling study, because of the relatively low acidity of gluconic acid ($pK_a = 3.86$), when the samples were immersed at a lower GC such as 50mg/dl or 100mg/dl, there were not enough glucose to react with enzyme and cannot produce sufficient H^+ to finish the following protonation. In other words, the surrounding pH was difficult to decrease to a sufficient level to cause the material to swell completely. There are three kinds of driving force that can influence the hydrogel's drug release: (1) the free diffusion of the drug, (2) the biodegradation of the hydrogel, and (3) the volume change of the hydrogel. When GC was at a low level, the swelling of the hydrogel was negligible, and therefore, the dominating force of the drug release was its diffusion and material's degradation, which could lead to only a small amount of release. However, when the GC gradually increased and reached to the threshold (140 mg/dl in this study), the drug release was mainly driven by the swelling of the material, which can utilize the water movement to significantly increase the drug release efficiency. This conclusion can also be proved by the results from 2 days and 3 days release, after 1 day's pronounced swelling performance, the absorption of water reached the balance, the amount of drug release (at 250 mg/dl) was only less than 3% for the last 24 hours in response to the swelling ratio of 12%.

One important result obtained from the antibacterial activity experiment is that a higher GC could increase the hydrogel's ability to inhibit the growth of *P. gingivalis* (even after several times of dilution), indicating that our samples possess favorable glucose sensitivity that has the potential to reduce the risk of periodontitis in diabetic patients. However, we have also noticed that the ordinary oral therapy is always a relatively long-term process but so far, our samples cannot maintain this antibacterial activity more than a few days. Therefore, further studies and improvements are needed to control the initial drug release burst and to achieve a sustained long-term drug release process.

Conclusion

In this study, a novel glucose sensitive CS-PEO hydrogel loaded with metronidazole was fabricated and characterized by physicochemical and biological methods. The results suggest that this kind of composite hydrogel possessed better mechanical properties and biocompatibility than "single-component" CS hydrogel. Meanwhile, it can self-regulate the metronidazole release according to the ambient glucose stimulus, thus achieve optimal antibacterial activity (*Porphyromonas gingivalis* as subject) at high glucose concentration. This work is just a preliminary research to prove a new concept of a new material to reduce periodontitis in diabetic patients during the oral therapy. Further improvements such as better glucose sensitivity and improved long-term drug release rate will be our next target. In addition, through further modification, this kind of hydrogel may be not only a simple controlled drug release system, but also a potential scaffold to repair and regenerate the periodontal tissues.

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Highlights

1. A new glucose-sensitive chitosan-polyethylene oxide (CS-PEO) hydrogel system for controlled release of antibiotics was developed;
2. The mechanical property and biocompatibility of the hydrogel was enhanced with increasing PEO concentration;
3. This hydrogel showed higher drug release and greater ability to inhibit *Porphyromonas gingivalis* at higher glucose concentration;

Figure 1.
Images of CS-PEO film and CS-PEO hydrogel film after immersion in acetic acid aqueous for different time.

Figure 2.

Stability and FTIR spectra of hydrogel. (a) Images of the mixed PEO -GA solution at 0 h and 24 h. (b) The dry weight of CS-PEO hydrogel films after immersion and agitation at different conditions for 24 h: Type I: in phosphate buffer at 37°C and shaking at 110 cycles per minute; Type II: in phosphate buffer under 37°C and 400 rpm magnetic stirring; Type III: in 50% (v/v) aqueous methanol solution under 60°C and 400rpm magnetic stirring. (c) FTIR spectra of chitosan-PEO film and cross-linked chitosan-PEO hydrogel film.

Figure 3. Compressive strength (a) and elastic modulus (b) of chitosan-PEO hydrogels with different weight ratios.

Figure. 4.
The amount of bounded GO_x in the hydrogel as a function of initial GO_x concentrations.

Figure 5. The fluorescence images of osteoblasts growth on CS hydrogel film ((a) and (c)) and CS-PEO hydrogel film ((b) and (d)) after 1 day or 3 day's cultivation. (magnification: 40x).

Figure 6. Cell viabilities of chitosan hydrogels and chitosan-PEO hydrogels at different time. The symbol* represents the significant difference ($p < 0.05$).

Figure 7.

(a) Swelling ratios of samples immersed in glucose solutions with different glucose concentration at different time points; (b) average pore sizes of samples immersed in the different glucose concentrations for 3 days; The SEM images of samples immersed in the different glucose concentration solutions for 3 days (c) 0 mg/dl; (d) 50 mg/dl; (e) 100 mg/dl ; (f) 140 mg/dl; (g) 200 mg/dl and (h) 250 mg/dl; yellow arrow represented the fracture part of the hydrogel network (the magnification was 100x).

Figure 8.

(a) The cumulative drug release of hydrogel samples (CS/PEO=1:1.5, MNZ= 0.5% w/v) immersed in glucose solutions with different glucose concentrations at different time points;
 (b) The cumulative drug release of samples immersed in the medium with the changed glucose concentration from 140mg/dl to 200mg/dl or from 200mg/dl to 140mg/dl after 4 hours drug release.

Figure 9.

The optical density values at 600 nm of the *P. gingivalis* inoculated in aliquot collected from blank group and various drug-release aliquots after different times of (1:1) dilution. The drug release aliquots were obtained by immersing the MNZ-loaded CS-PEO hydrogels in the solutions with different glucose concentrations (0-250 mg/dl) after (a) 1 day and (b) 3 days drug release.

Scheme 1.

Two interactions including Schiff base and hydrogen bonding during the formation process of chitosan-PEO hydrogel.

Scheme 2.

The mechanism of chitosan-PEO hydrogel's glucose sensitivity and controlled drug release process.

Table.1 G'_{2500} , G''_{2500} , and T_{gel} values of chitosan-PEO samples with different weight ratio or GA volume recorded by rheology analysis.

CS:PEO (w/w)	GA* (ml)	G'_{2500} (Pa)	G''_{2500} (Pa)	T_{gel} (s)
1:1.5	50	28.34±2.49	6.26±1.61	1927.3±70.42
1:1.5	100	37.62±2.71	5.43±0.87	1174.7 ± 82.48
1:1.5	150	81.76±6.43	8.53±2.31	672 ± 50.71
1:1.5	200	120.88±5.73	12.46±4.38	320.7±39.58
1:1.5	250	275.10±22.25	29.83±6.27	198.7±10.26
1:0	150	32.90±3.49	9.20±1.20	925±53.29
1:0.5	150	48.29±6.73	8.07±2.51	840.7±68.61
1:1	150	67.89±4.88	7.52±1.71	739.3±39.14
1:2	150	90.62±7.92	11.64±3.01	608.3±40.79
1:2.5	150	96.74±5.64	19.25±4.92	579.7±29.81

GA*: the volume of GA in 10 ml CS/PEO solution.

G'_{2500} and G''_{2500} : the storage modulus and loss modulus of CS/PEO gels at 2500s;

T_{gel} : time for initiation of gelation is indicated as the time when $G'=G''$;