Synthesis of oxidized glycerol monooleate-chitosan polymer and its hydrogel formation for sustained release of trimetazidine hydrochloride

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In this paper, a lipid material glycerol monooleate was used as the starting material to synthesize the oxidized glycerol monooleate (OGMO). OGMO was subsequently linked to chitosan (CS) via imine bonds (–C=NC=) to obtain a new chitosan-based polymer (OGMO-CS), which can form hydrogels rapidly in aqueous media. Scanning electron microscopy, swelling behavior studies and degradation kinetics studies were performed to demonstrate the effect of this synthetic modification on the hydrogels formation of chitosan network and in vitro drug release. The effects of OGMO-CS type, dry hydrogels percentage, release media and drug loading on the sustained release of the model drug trimetazidine hydrochloride were evaluated. The release profiles of the hydrogels could be described by the Peppas–Sahlin mechanism, a combination of Fickian diffusion and Case-II relaxation. Based on the fact that numerous pharmaceutical lipids are available, the present study may pave the way for other lipids to be employed as modifiers of chitosan for more innovative chitosan derivatives with versatile properties and pharmaceutical applications.

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1. Introduction

Hydrogels are three-dimensional cross-linked polymer networks that exhibit the ability to swell in aqueous solutions to many times their original volume (Peppas et al., 2000). Their affinity to absorb water is attributed to the presence of hydrophilic groups such as –OH, –CONH2, –COOH and –SO3H within the molecular structure (Peppas and Khare, 1993). Hydrogels will swell instead of being dissolved in aqueous solutions due to the critical cross-links present in the hydrogel structure. Fully swollen hydrogels have excellent biocompatibility and low toxicity because of their similar physical properties to living tissues (Kim et al., 2008).

Chitosan (CS) is a cationic polysaccharide composed of randomly distributed β-(1→4)-linked d-glucosamine and N-acetyl-d-glucosamine units (Chandy and Sharma, 1990; Kato et al., 2003). It has a highly stable crystalline structure that can only be dissolved in dilute acids (pH ≤ 5.0) through the protonation of the free amino groups but cannot form hydrogels spontaneously in the acidic solution. The reactive amino groups and the hydroxyl groups in CS can be either physically associated (Berger et al., 2004) or chemically cross-linked (Hennink and van Nostrum, 2012) to some functional reagents such as PEG (Kulkarni et al., 2005), Konjac Glucomannan (Yu et al., 2007), sodium hexametaphosphate (Gupta and Jabrail, 2006), starch (Tang et al., 2003), hyaluronic acid (Tan et al., 2009) or carboxylic compounds (Lu et al., 2007) to obtain many new derivatives, which possess versatile properties and have been investigated as drug delivery vehicles.

In the present study, a rarely reported lipid modifier for CS was investigated to offer the groundwork for further lipids use in modifying CS, based on the present knowledge on the diversity and safety of lipids, as well as their comprehensive applications in pharmaceutics (Akoh, 2005; Wasan, 2006). Glycerol monooleate (GMO) was used as a novel starting material for the preparation of CS-based pharmaceutical hydrogels. GMO is a polar amphiphilic lipid that can form liquid crystalline phases depending on the water content and temperature (Engstrom, 1990). In the presence of excess water, GMO forms gels with high viscosity, known as the cubic phase. However, the water uptake of GMO is very slow, with a swelling ratio of lower than 1.5% in 12 h. Its swelling is strongly dependent on the temperature and the initial water content of GMO (Lee et al., 2003). Once contact with water, the surface of GMO produces a viscous gel, which prevents further immersion of water and makes GMO very difficult to be handled. All of these restrict the use of GMO and its formation of hydrogels.
Ganguly et al. used CS and GMO to prepare an in situ gel with and without a cross-linker (glutaraldehyde) (Ganguly and Dash, 2004). It is conceivable that it is hard to prepare the gel due to the gel formation of GMO alone in aqueous media and the resultant high viscosity, even with the help of a prolonged period of sonication. Besides, this system would produce reversible gel formation and imprecise control of drug release because the network formation by this interaction was purely physical (Bhattarai et al., 2010). With the addition of glutaraldehyde, the gel properties and drug release were enhanced (Ganguly and Dash, 2004). However, this toxic cross-linker may be of great concern for oral delivery (Bhattarai et al., 2010).

In this paper, we synthesized oxidized glycerol monooleate (OGMO) from GMO and subsequently used OGMO as a novel modifier to prepare new OGMO-CS polymers with different degrees of substitution and OGMO-CS based hydrogels. The effect of OGMO-CSs on the physicochemical and release profiles of the new matrix was also investigated. Trimetazidine hydrochloride (TMH), an effective anti-ischemic agent which has the marketed sustained release tablets (Marzilli, 2003), was used as the model drug.

2. Materials and methods

2.1. Materials

Chitosan (CS, MW = 1 × 10⁴, degree of deacetylation = 85.46%) was purchased from Jinan Haidebei Marine Bioengineering Co., Ltd. (Shandong, China). Glycerol monooleate (GMO) was kindly gifted by Danisco A/S Co., Ltd (Copenhagen, Denmark). Trimetazidine hydrochloride (purity of 99.7%) was purchased from Hubei-Sihuan Pharmaceuticals Co., Ltd. Sodium periodate was provided by Guangdong Guanghua Chemical Factory Co., Ltd. (Guangdong, China). Sodium triacetoxymethylhydride (STAB-H, analytical grade) was purchased from Henan Wanxiang Technology & Trade Co., Ltd. (Henan, China). Acetic acid, N,N-dimethylformamide (DMF), ethanol, sulfuric acid, hydroxylammonium chloride and other agents were analytical grade.

HepG2 cell lines were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. HepG2 cells were grown in the Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated calf serum (Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 U/ml penicillin G and 100 U/ml streptomycin (pH7.4), in a water jacketed CO₂ incubator with a humidified atmosphere of 5% CO₂ at 37 °C.

2.2. Synthesis of oxidized glyceryl monooleate (OGMO)

OGMO was synthesized by oxidizing GMO with sodium periodate (Scheme 1a), a compound with high selectivity for oxidative cleavage of vicinal glycols (Perlin, 2006). GMO (10 g) was first dissolved in absolute ethanol to obtain 50 ml of solution. 25 ml of distilled water was added. After stirring for 1 h, 0.5% sulfuric acid solution was added drop-wise to adjust the pH to 4.5. 50 ml of sodium periodate aqueous solution (18%, w/v) was added to the mixture and stirred at 45 °C for 3 h in the dark. OGMO was then collected by separating the oily upper phase from the reaction solution.

2.3. Synthesis of oxidized glyceryl monooleate-modified chitosan (OGMO-CSs)

OGMO-CSs were obtained by introducing OGMO into the N-terminal of glucosamine units of chitosan through one-pot reductive amination (Scheme 1b). STAB-H, a mild and selective reducing agent, was used for the reductive amination of ketones and aldehydes (Abdel-Magid and Mehrman, 2006) in the synthesis process. 2 g of CS was suspended in 100 ml of acetic acid solution (1%, v/v) and stirred until it was completely dissolved. On the basis of the molecular weight (10000), the number of monomers (59) and the deacetylation of CS (85.46%), 2 g of CS is equivalent to 0.01 mol of amine group. Sodium hydroxide (0.5 mol/l) was added slowly to adjust the pH (approximately 12), resulting in the precipitation of expanded CS. The oyster white suspension was then filtered. The expanded CS on the filter membrane was subsequently transferred into a flask containing 50 ml of DMF. OGMO was added to the flask and the pH of the mixture was adjusted to approximately 6.5 using acetic acid. After stirring for 6 h at ambient temperature, STAB-H was added slowly (the molar ratio of STAB-H to OGMO = 1.4:1). The reaction continued for another 24 h. The filtered cake, OGMO-CS, was collected and fully washed with absolute ethanol to completely eliminate the free OGMO. About 0.33 g (equivalent to 0.001 mol aldehyde group), 0.65 g, 0.97 g and 1.62 g of OGMO were used in the synthesis to obtain OGMO-CS1, OGMO-CS2, OGMO-CS3 and OGMO-CS4, respectively, by varying the molar ratios of amine group in CS to aldehyde group in OGMO from 10:1, 5:1, 10:3:2:1.

2.4. Characterization of OGMO, OGMO-CSs

2.4.1. Determination of the oxidized degree of OGMO

The oxidized degree (OD) of OGMO was determined by measuring the molar weight of the aldehyde groups applying the hydroxylamine hydrochloride potentiometric titration, which has been proved to be reliable without using a standard (Zhao and Heindel, 1991). After drying in a vacuum oven at 25 °C to constant weight, hydroxylamine hydrochloride (8.75 g) was dissolved in 100 ml of 70% ethanol solution and 3.0 ml of methyl orange reagent (0.05%) was then added. The solution was diluted to 500 ml with 70% ethanol solution to obtain hydroxylamine hydrochloride solution (0.25 mol/l). 0.45 g of OGMO was dissolved in 25 ml of the hydroxylamine hydrochloride solution and stirred at room temperature in the dark for 2 h. Afterwards, each sample was titrated with standardized sodium hydroxide solution until the red-yellow end point was achieved. Simultaneously, pH changes were recorded. The equivalent volume could be determined more precisely by the first derivative of the titration curve. The oxidation product was measured in triplicate. The OD of OGMO was calculated by:

\[ \text{OD} = \frac{V_{\text{NaOH}} \times N_{\text{NaOH}} \times 10^{-3} \times MW_{\text{OGMO}}}{W_{\text{sample}}} \times 100 \]

where \( V_{\text{NaOH}} \) and \( N_{\text{NaOH}} \) are the equivalent volume and the concentration of the standardized sodium hydroxide solution, respectively. \( MW_{\text{OGMO}} \) is the molecular weight of OGMO. \( W_{\text{sample}} \) is the weight of the oxidation product added in the hydroxylamine hydrochloride potentiometric titration.

2.4.2. Elemental analysis

The degree of substitution (DS) of OGMO-CSs was estimated using the automatic elemental analyzer Vario EL Elementar Analysensysteme GmbH., Hanau, Germany.

2.4.3. Fourier transforms infrared spectroscopy (FTIR)

FTIR was performed to confirm the formation of a new modified chitosan by OGMO. The FTIR data of OGMO-CSs were collected by using Tensor-27 Infrared Spectroscopy (Bruker, Germany) over the wavenumber range of 4000–400 cm⁻¹. Each sample was mixed with dry KBr (at a ratio of 1:10) and pressed into a transparent disc prior to FTIR spectroscopic analysis.

2.4.4. ¹H NMR spectroscopy

¹H NMR spectra of CS, OGMO and OGMO-CSs were recorded on a Bruker AVII 500 MHz NMR spectrometer (Bruker Biospin, Germany).
Germany), operating at 500.13 MHz proton frequency. CS was dissolved in CD$_3$COOD/D$_2$O. GMO and OGMO-CSs were dissolved in DMSO-d$_6$. All NMR experiments were performed at 25°C.

2.4.5. X-ray powder diffraction (XRPD)

The crystalline forms of chitosan and OGMO-CSs were examined over a diffraction angle (2θ) range of 5–40° by X-ray diffraction (XRD) using a Bruker D8 advance X-ray diffractometer (D8 Advance, Bruker AXS Inc., Germany) with Cu-Kα radiation (λ = 1.5418 Å). The operation voltage and current were 40 kV and 40 mA, respectively.

2.5. Preparation of OGMO-CSs hydrogels

About 0.5 g of OGMO-CSs with different DS was added into 10 ml of acetic acid solution (0.5%, v/v). The hydrogels formed after vortex mixing for 1 min were lyophilized to obtain the dry hydrogels prior to further characterizations. Different amounts of CS were also
added to acetic acid solutions (0.5%, v/v) for hydrogels formation comparison.

2.6. Characterization of OGMO-CSs hydrogels

2.6.1. Scanning electron microscopy (SEM)

Microstructure and morphology evaluation of OGMO-CSs hydrogels were performed by scanning electron microscope (SEM) (Hitachi S3400, Tokyo, Japan). The samples were examined on a brass stub using carbon double-sided tape. Fractured samples were glued and mounted on metal sample plates. The samples were gold coated (thickness ≈ 15–20 nm) with a sputter coater (Fison Instruments, UK) using an electrical potential of 2.0 kV for 10 min. An excitation voltage of 20 kV was used in the experiments.

2.6.2. Swelling behavior studies

The swelling behaviors of OGMO-CSs hydrogels were evaluated in simulated gastric fluid (SGF, 0.1 mol/l hydrochloric acid solution, pH about 1.2), phosphate buffers (pH 4.5 and pH 6.8) at 37 °C. The pre-weighed dry hydrogels were immersed in the swelling medium. At each time point, excess water on the surfaces of swollen hydrogels was removed carefully by filter papers and the swollen gels were weighed. Each type of OGMO-CSs hydrogels was measured in triplicate. The swelling ratio (SR) of hydrogels at each time point was calculated by

\[
SR(t) = \frac{W_t - W_d}{W_d}
\]

where \( W_t \) and \( W_d \) are the weight of swollen gels at time \( t \) and the dry gels at \( t = 0 \), respectively.

2.6.3. Degradation kinetics studies

In order to study the degradation of the hydrogels and explore the mechanism of the sustained release of drug from the hydrogels, in vitro degradation experiments were carried out. The pre-weighed dry hydrogels were incubated in tubes containing 10 ml of SGF at 37 °C. Excess SGF was removed from the tube at fixed time intervals. The hydrated hydrogels were dried in a vacuum oven at 25 °C and the dried hydrogels were weighed. The degradation process was monitored by comparing the weight loss of dry hydrogels before and after the degradation experiments (Lu et al., 2010). Each type of OGMO-CSs dry hydrogel was measured in triplicate. The degradation ratios (DR) were calculated by

\[
DR(t) = \frac{W_t - W_d}{W_0} \times 100
\]

where \( W_0 \) and \( W_t \) are the weight of the dry hydrogels before and after degradation, respectively.

2.7. In vitro release study

To study the in vitro release of TMH from hydrogels, various formulations containing dry hydrogels and TMH were prepared as listed in Table 1. The types and percentages of dry hydrogels as well as drug loading were studied. TMH was completely dissolved in water to get a solution of 10–50 mg/ml. 2 ml of TMH solution was pipetted into a 20 ml flat-bottomed tube containing the pre-weighed dry hydrogels (0.07–0.12 g). The mixture was then vortexed gently for 5 min to form homogeneous initial hydrogels which occupied the bottom of the tube. The surface area exposed to the release medium was 1.77 cm² for all samples. To simulate the conditions in gastro-intestinal tract, 20 ml of release medium (pH=1.2, 4.5 or 6.8) was added into each tube. The tubes were then placed in a shaking incubator (37 °C) at 100 rpm to initiate the release (Lu et al., 2010; Gao et al., 2010).

At each predetermined time interval, 0.5 ml of the supernatant was withdrawn and diluted to 10 ml with distilled water for subsequent analysis. 0.5 ml of the fresh release medium was added into the tubes for maintaining a constant volume of the release medium. The amount of TMH released from the hydrogels was determined using UV–Visible spectrophotometer (TU1900, Beijing Purkinje General Instrument Co., Ltd., China) at 270 nm. All experiments were repeated six times.

2.8. MTT assay

MTT assay was performed for investigating the effect of OGMO-CSs on HepG2 cell viability and evaluating their cytotoxicity (Zhang et al., 2013). HepG2 cells (1 × 10⁶ per well in a 96-well plate) were treated with OGMO-CSs solutions (50, 100, 500 and 1000 µmol/l in 0.1% acetic acid solution, n=6) for 24 h, respectively. Then, 20 µl MTT (5 mg/ml) was added into each well and incubated for 4 h at 37 °C. The supernatant was discarded and 150 µl DMSO was added. The mixture was gently shaken on a micro-vibrator for 10 min to achieve a complete dissolution. The absorbance was then recorded at 570 nm using multimode microplate reader (Infinite 200 Pro, Tecan, Switzerland). T-test was used to compare the DS of OGMO-CSs.

3. Results and discussion

3.1. Synthesis of OGMO and OGMO-CS

In this study, lipid GMO was for the first time used as the starting material to form a novel lipid covalent modifier for CS. The vicinal hydroxyl groups of GMO were cleaved with periodate oxidation to achieve an aldehyde derivative, OGMO before subsequent hydrogel formation.

Several molecules have been used to make covalent hydrogels of chitosan. The possible linking chemistry and reaction conditions (reagents, time, temperature, etc.) are crucial for the covalent hydrogel formation of chitosan (Bhattarai et al., 2010). Considering the chemical features of CS, primary amines in its backbones were generally used as the target functional group. In the present study, once OGMO was synthesized, the composite CS-OGMO polymers with four CS/OGMO ratios were prepared by a reaction of

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Dry hydrogel</th>
<th>The weight of dry hydrogel (g)</th>
<th>The concentration of the TMH solution (mg/ml)</th>
<th>The volume of the TMH solution added (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IH1</td>
<td>OGMO-CS1</td>
<td>0.12</td>
<td>30</td>
<td>2.0</td>
</tr>
<tr>
<td>IH2</td>
<td>OGMO-CS1</td>
<td>0.12</td>
<td>30</td>
<td>2.0</td>
</tr>
<tr>
<td>IH3</td>
<td>OGMO-CS1</td>
<td>0.17</td>
<td>30</td>
<td>2.0</td>
</tr>
<tr>
<td>IH4</td>
<td>OGMO-CS2</td>
<td>0.07</td>
<td>30</td>
<td>2.0</td>
</tr>
<tr>
<td>IH5</td>
<td>OGMO-CS2</td>
<td>0.12</td>
<td>30</td>
<td>2.0</td>
</tr>
<tr>
<td>IH6</td>
<td>OGMO-CS2</td>
<td>0.12</td>
<td>30</td>
<td>2.0</td>
</tr>
<tr>
<td>IH7</td>
<td>OGMO-CS1</td>
<td>0.17</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>IH8</td>
<td>OGMO-CS1</td>
<td>0.12</td>
<td>50</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Schiff base between the reactive amino groups of CS backbone and the aldehyde groups of OGMO. This formed irreversible inter- or intra-molecular bridges between the CS chains.

The OD of OGMO was measured by the hydroxylamine hydrochloride potentiometric titration curves. The marked deflection proved the existence of aldehyde groups in the product. The volume of sodium hydroxide solution needed to reach the end point was the value corresponding to the peak of the first derivative. The mean OD of OGMO was calculated to be \(88.99 \pm 2.03\%\), representing the content of OGMO in the oxidized product. A small amount of un-oxidized GMO in the product had no influence on the next synthesis process.

### 3.2. Characterization of OGMO-CS

#### 3.2.1. The degree of substitution

The DSs of OGMO-CSs were calculated using the C to N molar ratio obtained from element analyses (Liu et al., 2012). Because every unit of CS contains one nitrogen atom, an increase in C to N molar ratio of the OGMO-CS product indicates an increase in the number of carbon atoms per unit of OGMO-CS. More precisely, the DS was estimated from the increase of molar ratio divided by 20 because each OGMO unit contains 20 carbons. The increase in molar ratio was obtained by calculating the difference in C to N molar ratios of OGMO-CS and CS. The DS values of four OGMO-CSs were calculated to be 8.17%, 13.06%, 18.75% and 27.12%, respectively. A characteristic peak at 1597 cm\(^{-1}\) (stretching vibration of C=O, respectively. A characteristic peak at 1597 cm\(^{-1}\)) is attributed to the secondary amines generated by reductive amination. The peaks between 1250 cm\(^{-1}\) and 1000 cm\(^{-1}\), attributed to the hydroxyl group, exhibited no changes. It has been proven that OGMO was linked to the primary amino groups but not the hydroxyl groups of CS. Comparing the FTIR spectra of OGMO and CS, the bands present in the FTIR spectra of OGMO-CSs suggest that the reactions occurred between the aldehyde groups of OGMO and the primary amine moiety of CS.

#### 3.2.2. FTIR analysis

The spectra of OGMO-CS1 and OGMO-CS2 with significantly lower DS and more primary amines possessed greater capability of protonation in acid media and caused the repulsion between the protonated amines, which resulted in stretching of the long chain of OGMO. These were beneficial to both the inter-chain hydrophobic associations for

#### 3.2.3. \(^1\)H NMR analysis

\(^1\)H NMR spectra of CS, OGMO and OGMO-CS4 are shown in Fig. 2. The amide peak at 7.0 ppm (14H), 1.2 ppm (11H), 2.0 ppm (12H), 2.3 ppm (10H) and 5.3 ppm (13H) are attributed to the C20-alkyl and the peaks at 1.8 ppm (7H), 2.9 ppm (7H) are ascribed to the CS backbone. The principal peaks appear only in the \(^1\)H NMR spectrum of OGMO-CS4. This proves that reductive amination has occurred between the OGMO and CS.

#### 3.2.4. XRPD

The X-ray diffractograms of neat chitosan and four OGMO-CSs are compared (Data not shown). A strong crystalline peak at 2\(\theta=20.14\) in the X-ray diffractogram of neat CS corresponds to crystal form II with constrained chain conformation (Gao et al., 2010). However, for all OGMO-CSs, these crystalline peaks became relatively obtuse and broad with the increase in DS and almost disappeared in the diffractogram of the OGMO-CS4. This indicates that the hydrogen bonds in neat CS were partially destroyed through the modification by the OGMO, thus forming a descending fraction of crystalline phase and an ascending fraction of amorphous phase.

### 3.3. The formation mechanism of OGMO-CSs hydrogels

Numbers of the primary amines, hydroxyl groups and long hydrophobic chain of the C20-alkyl present in the structure of OGMO-CSs are shown in Scheme 1. The nitrogen atoms in primary amines of OGMO-CSs can be predominantly protonated in the acidic solution to form positively charged ammonium group, making the new polymer more hydrophilic. Because the amino groups are positively charged, the like-charged amines repel each other, prompting the long-chain in OGMO-CS to stretch. Subsequently, the stretched chain makes it possible for the C20-alkyl to form inter-chain hydrophobic associations (Philippova et al., 2001). The OGMO-CSs hydrogels were achieved by the combined action of the hydrophobic associations of the C20-alkyl, electrostatic forces and the hydrophilicity of protonated amines, satisfying the two demands for the formation of hydrogels in absence of chemical cross-linked agent: strong inter-chain interactions for network formation, and sufficient number of hydrophilic groups within the polymer structures to promote the access and residence of water molecules inside the networks (Peppas and Huang, 2004).

It was found that 70 mg of OGMO-CS1 and OGMO-CS2 formed hydrogels rapidly in 20 ml of acetic acid solution (0.5%, v/v). However, hydrogels of OGMO-CS3 and OGMO-CS4 were not observed. OGMO-CS1 and OGMO-CS2 with significantly lower DS and more primary amines possessed greater capability of protonation in acid media and caused the repulsion between the protonated amines, which resulted in stretching of the long chain of OGMO. These were beneficial to both the inter-chain hydrophobic associations for
cross-linked network and the ability of water diffusion into the network which facilitated hydrogels formation. As the DS increased, and the corresponding protonated amines decreased, the repulsion between the protonated amines became too weak to provide the adequate energy for long chain stretching. Along with the increase of intra-chain hydrophobic associations, hydrogels formation was further prevented.

For comparison, pure CS was unable to form any hydrogels in acetic acid solution. Even at a high CS concentration of 6% and 8%, CS could only be partially dissolved to form a suspension. It was reported that CS could only form hydrogels via either physical association with help of anionic molecules (Shu and Zhu, 2002), polyelectrolytes (Tsuchida and Abe, 1982) or polymers (Khan et al., 2009), or chemical cross-linkage. Although CS alone had more primary amines than OGMO-CSs available for protonation, it lacked hydrophobic long-chain in its structure. Therefore, the cross-linked networks in aqueous media were not available. The absence of pure CS hydrogels demonstrated that the inter-chain hydrophobic association might have been the key contribution to the hydrogels formation of OGMO-CSs in acidic solution. However, the protonation of N in primary amines of CS structure was also critical for facilitating the stretch of long chain introduced by GMO. The hydrogels formation of OGMO-CSs in acidic environment was crucial for oral dosage form because the gastric fluid would be the first medium that the polymeric carrier comes in contact. In the following studies, only OGMO-CS1 and OGMO-CS2 were used as the carrier for TMH due to the absence of hydrogels formation with OGMO-CS3 or OGMO-CS4.

3.4. Characterization of OGMO-CSs hydrogels

3.4.1. SEM observations

The morphology of OGMO-CS1 or OGMO-CS2 was highly porous as a result of the formation of ice crystals during the freeze drying process (Noble et al., 1999), as shown by SEM in Fig. 3. OGMO-CS1 hydrogels had smaller pore sizes (about 50–100 μm, Fig. 3a) and formed a denser network than OGMO-CS2 hydrogels (about 130–200 μm, Fig. 3b). The pore size and the density of a porous network were affected by the DS. Lower DS could produce a more cross-linked network and result in smaller pores in hydrogels.

The weakened stretch of long chain and the resultant more intra-chain hydrophobic association inside the polymeric matrix caused the density of cross-linked network to decrease. This was reflected in the larger pores in the OGMO-CS2 hydrogels and its relatively loose internal structure. Both OGMO-CSs maintained a honeycomb-type porous morphology that resembles chitosan gels (Freier et al., 2005). It was previously reported that the concentration of the initiator used in cross-linking had the similar outcome (Yu et al., 2007). This inner structural feature of the hydrogels might be responsible for offering special passageways for water ingression and solute diffusion.

3.4.2. Swelling behavior

The swelling profiles of OGMO-CS1 hydrogels and OGMO-CS2 hydrogels are presented in Fig. 4. Both hydrogels showed pH-dependent swelling patterns. The increase of pH of swelling media was accompanied with a decrease in the swelling ratio of
OGMO-CS1 hydrogels. As described in the formation of OGMO hydrogels, the effect of pH was inevitable on the swelling profiles of OGMO-CSs.

It was also evident that the swelling ratios of OGMO-CS2 in all media were significantly lower than those of OGMO-CS1. The lower swelling ratios of OGMO-CS2 illustrated that its network had a poorer capacity for the access and residence of water molecules within the polymer network due to the increased hydrophobicity. The hydrophilicity of chitosan came from the hydrophilic groups, such as $-\text{OH}$ and $-\text{NH}_2$ of the chitosan chain. The decrease in the $-\text{NH}_2$ group content enhanced the hydrophobicity of the chitosan network. Analogously, an increase in $-\text{NH}_2$ group content in OGMO-CS1 might possess higher hydrophilicity of the cross-link network. One classical example would be the formation of chitosan network cross-linked by sulfosuccinic acid, which demonstrated hydrophilic performance (Tsai and Wang, 2008).

### 3.4.3. Degradation kinetics

OGMO-CS1 had a higher degradation rate than OGMO-CS2 in SGF at 37°C (Data not shown). Generally, the degradation rate of biomaterials was related to the steric hindrance, which could delay the in vitro penetration of media as well as the steric effects of the linked chain among CS or modified CS molecules (Cao et al., 2005). It has been reported that the high stability of chitosan-alginate scaffolds was mainly attributed to the interaction between the amino groups in chitosan and the carboxyl groups in alginate that prevented the protonation of amino groups on chitosan (Li et al., 2005).

![Fig. 3. SEM images of the hydrogels based on OGMO-CS1 (a) and OGMO-CS2 (b).](image)

In this study, although no amine group of CS interacted directly with the functional groups on OGMO, the increase in consumption of amino groups of CS during synthesis would still impair the extent of amino group protonation. Both OGMO-CSs presented slow degradation patterns in SGF. OGMO-CS2, which had less protonation, had a relatively lower degradation rate compared to OGMO-CS1. This comparison was similar to that in their swelling behaviors.

### 3.5. Drug release

The in vitro release profiles of TMH from different initial hydrogels in three release media are shown in Fig. 5. The release rate of TMH from hydrogels increased with the increase of pH of release medium. The cumulative release of TMH from IH2 (OGMO-CS1) was $54.52 \pm 1.61\%$ and $94.96 \pm 1.25\%$ at 70 h in SGF and pH4.5 PBS, respectively. Conversely, $98.53 \pm 0.81\%$ of TMH was released at 22 h in pH6.8 PBS. In the acidic solution, the initial hydrogels could swell quickly and continuously, increasing the inner density of cross-linked networks. As a result, longer distance and more time were necessary for the solvent to diffuse into or out of these networks of the hydrogels. This subsequently slowed down the release of TMH. In the strongly acidic medium (pH1.2), OGMO-CS1 had the highest swelling ratio (Fig. 4). Similar results were obtained for IH5 (OGMO-CS2) initial hydrogels, but with noticeably faster release relative to IH2 hydrogels in the same release medium. As shown in Fig. 5a, the cumulative TMH release from IH5 is $77.54 \pm 1.89\%$ at 70 h in SGF, compared to $54.52 \pm 1.61\%$ for IH2. This was in coincidence with the higher cross-linking degree in the OGMO-CS1 networks generated by the larger swelling ratio.

However, this result was not exactly coincident with the difference in degradation test. Theoretically, OGMO-CS2 hydrogels with lower degradation rate might result in a slower release. This inconformity made it clear that degradation was not the principle mechanism for TMH release from OGMO-CS hydrogels and had little impact on the TMH release, partly due to the low degradation rates of both hydrogels. After incubation in SGF for 70 h, the degradation rates of OGMO-CS1 and OGMO-CS2 hydrogels were about 11% and 8%, respectively.

The effect of dry hydrogels amount on TMH release was also investigated by comparing the release profiles of hydrogels using OGMO-CS1 (IH1, IH2 and IH3) in SGF (Fig. 5b). It was noted that the increase in dry hydrogel percentage resulted in an obvious decrease of the cumulative TMH release, which was further confirmed by the release profiles of IH4, IH5 and IH6 (using OGMO-CS2). It was the strengthened cross-linking degree of hydrogels networks in aqueous media along with the increased amount of the dry hydrogels that led to the decline of TMH diffusivity from hydrogels.

![Fig. 4. The swelling profiles of OGMO-CS1 and OGMO-CS2 in simulated gastric fluid (pH 1.2) and phosphate buffers (pH 4.5 and 6.8) at 37°C.](image)
Effect of TMH loading on the release behavior in SGF was studied as well, using OGMO-CS1 hydrogels as the sustained release matrix in the preparation of IH2, IH7 and IH8. The release mechanism of TMH was investigated by DDSolver software (Zhang et al., 2010) and presented in Fig. 6. It was assured that OGMO-CS polymer inside the hydrogels began its relaxation and swelling from the first sampling point, with a Fickian contribution of about 75%. During the entire release stage, Fickian diffusion had a relatively larger contribution to the release rate. There was a large gradient of solvent penetration within the system. TMH was gradually released along with the permeation of solvent diffusion rate was much slower than the polymer relaxation allowed an easier penetration of the aqueous media and the solvent.

As a system in which a drug may undergo diffusion out of the polymer having the properties of swelling and chain relaxation, the fraction of drug released ($M_t/M_{\infty}$) on time $t$, was investigated according to the following mathematical models:

Ritger–Peppas equation (Ritger and Peppas, 1987): $\frac{M_t}{M_{\infty}} = k t^n$

Peppas–Sahlin equation (Peppas and Sahlin, 1989): $\frac{M_t}{M_{\infty}} = k_1 t^m + k_2 t^{2m}$

where $k$ is a constant incorporating characteristics of the polymeric network system and the drug; $n$ is the diffusional exponent; $m$ is the purely Fickian diffusion exponent for a device of any geometrical shape; $k_1 t^m$ is the Fickian contribution; $k_2 t^{2m}$ is the Case-II relaxational contribution (Zarzycki et al., 2010).

The release parameters and two important statistical parameters (coefficient of determination, $r^2$; akaike information criterion, AIC) for determining the release mechanism were statistically analyzed by DDSolver software (Zhang et al., 2010) and presented in Table 2. The first 60% of all release curves was used for statistical analysis. Peppas–Sahlin model was better for describing the release mechanism for all formulation in SGF based on the statistically higher $r^2$ and lower AIC. This result indicates that the release of drug from hydrogels was controlled by the combination of Fickian diffusion and Case-II relaxation (Peppas and Sahlin, 1989).

To evaluate the comparative contribution of two mechanisms to the release, the percentage of drug release due to the Fickian mechanism ($F$) and the ratio of relaxational contribution ($R$) over Fickian contribution ($R/F$) were calculated as follows (Peppas and Sahlin, 1989):

$$F = \frac{1}{1 + \left(\frac{k_2}{k_1}\right)^{2m/n}}$$

$$R = \frac{k_2}{k_1} t^{2m/n}$$

The $R/F$ values of IH2, IH7 and IH8 versus time are plotted in Fig. 6. It was assured that OGMO-CS polymer inside the hydrogels began its relaxation and swelling from the first sampling point, with a Fickian contribution of about 75%. During the entire release stage, Fickian diffusion had a relatively larger contribution to the release based on the $R/F$ of less than 1. High mobility of polymer chains allowed an easier penetration of the aqueous media and the solvent diffusion rate was much slower than the polymer relaxation rate. There was a large gradient of solvent penetration within the system. TMH was gradually released along with the permeation of solvent.

Table 2. Parameters for release model and statistics of various hydrogels in simulated gastric fluid (pH 1.2).

<table>
<thead>
<tr>
<th>Samples and release media</th>
<th>Ritger–Peppas model</th>
<th>Peppas–Sahlin model</th>
<th>Higuchi model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k$</td>
<td>$n$</td>
<td>$r^2$</td>
</tr>
<tr>
<td>IH2 in pH1.2</td>
<td>9.30</td>
<td>0.41</td>
<td>0.992</td>
</tr>
<tr>
<td>IH7 in pH1.2</td>
<td>10.38</td>
<td>0.40</td>
<td>0.983</td>
</tr>
<tr>
<td>IH8 in pH1.2</td>
<td>8.20</td>
<td>0.44</td>
<td>0.995</td>
</tr>
</tbody>
</table>
with versatile properties and pharmaceutical applications. Based on the fact that numerous currently available pharmaceutically active ingredients (API) when contacting the drug core, the desired release profile could be achieved. Moreover, the hydrophilicity of protonated amines. By modulating the DS of the polymer blend (Sharma and Kanchan, 2013).

Due to its swelling behavior and the ability to sustain the release of pharmaceutical active ingredients (API) when contacting the aqueous media, OGMO-CS can be used in various ways as the drug delivery system. It can be mixed homogeneously with API and other inactive ingredients to prepare the matrix-type tablets by granulation method. It can also be filled into capsules after hydration and lyophilization to obtain the dry hydrogels, as shown in this study for TMH. Other ways to use OGMO-CS polymer for more APIs are still under investigation.

3.6. MTT assay for cell viability

As shown in Table 3, the cell viability of HepG2 was more than 99% at an OGMO-CS concentration of 50 μM/l and was above 95% even if the polymeric concentrations were 1000 μmol/l. T-test indicates that the cell viability of four OGMO-CSs was not significantly different (P > 0.05). The MTT assay demonstrates the bio-safety of synthesized OGMO-CSs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td>OGMO-CS1</td>
<td>100%</td>
</tr>
<tr>
<td>OGMO-CS2</td>
<td>99%</td>
</tr>
<tr>
<td>OGMO-CS3</td>
<td>99%</td>
</tr>
<tr>
<td>OGMO-CS4</td>
<td>100%</td>
</tr>
</tbody>
</table>

4. Conclusion

In the present study, lipid GMO was for the first time to be exercised as the starting material in the preparation of oxidized GMO which was subsequently employed for the synthesis of a novel chitosan-based polymer, OGMO-CS. Among the four OGMO-CSs synthesized, the OGMO-CS with lower DS had the capability of forming hydrogels rapidly in acidic solution and retarding the drug release. The formation of OGMO-CS hydrogels was primarily attributed to both the inter-chain hydrophobic associations and the hydrophilicity of protonated amines. By modulating the DS of OGMO-CS, the desired release profile could be achieved. Moreover, based on the fact that numerous currently available pharmaceutical lipids, the present study may pave the way for other lipids to be used as the modifiers for more innovative chitosan derivatives with versatile properties and pharmaceutical applications.

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References


