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An injectable *in situ* hexagonal mesophase system for local delivery of minocycline hydrochloride: Preparation and pharmacodynamics in rats

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In this study, an optimized *in situ* reversed hexagonal mesophase loaded with minocycline hydrochloride (MH) was developed for the chronic periodontitis treatment. The *in situ* hexagonal liquid crystals (ISH_2) comprised phytantriol (PT), propylene glycol (PG), water and vitamin E acetate (VitEA). The physicochemical properties, in vitro drug release and the therapeutic effects on chronic periodontitis of the formed samples were tested. The injectable liquid crystal-forming systems were characterized by crossed-polarized light microscopy, small angle X-ray scattering, and rheological measurements. The optimal ISH_2 (PT/PG/water/VitEA, 56:27:10:7, w/w/w/w) loaded with 20 $mg \cdot g^{-1}$ MH was proved to be injectable with suitable pH, and was able to sustain the drug release for 10 days. The pharmacodynamic studies of the optimal formula were performed on male SPF rats, the Periocline® ointment was used as a control. The investigated ISH_2 loaded with MH was demonstrated to be effective for periodontal treatment with significantly improved gingival index, pocket depth and alveolar bone loss. The developed ISH_2 may be a promising application for local delivery system of MH in treating periodontal diseases.

1. Introduction

Periodontitis is a chronic inflammatory disease of the periodontal tissues. Periodontal disease is not only the most important cause for loss of teeth in the adult population, but also a risk factor of certain systemic health problems such as cardiovascular disease, diabetes and pregnancy complications (Reich and Hiller 1993; Phipps and Stevens 1995; Agerholm 2001; Gomes et al. 2012). Dental plaques play a key role in periodontitis, while the host immune response stimulated by bacteria is the main cause of periodontal tissue destruction (Mombelli 2003). Therefore, the therapeutic goals in periodontitis are removing dental plaque, eliminating inflammation of the gums, improving the levels of periodontal attachment and regenerating periodontal tissue, as well as avoiding recurrence in long-term maintenance efficacy. At present, removing plaque and calculus mechanically is the common therapeutic method for periodontitis treatment. However, excessive scaling for tooth may cause gum sensitivity. In addition, the irregular structures of the periodontal pocket increase the difficulty levels of scaling operation. The residual pathogenic microorganisms also could cause the recurrence of periodontitis. Thus, periodontitis treatment often combines the basic scraping and medicine therapy (El-Kamel et al. 2007; Venkatesh et al. 2013).

Minocycline hydrochloride (MH) (Fig. 1) is a tetracycline derivative with broad antibacterial spectrum and strong antibacterial effect. MH shows more lipid solubility than tetracycline hydrochloride, thus penetrating directly through the lipid bilayer of the bacterial cell wall (Oliveira et al. 2006; Kassem et al. 2014). Furthermore, MH has significant pharmacological effects on periodontal diseases, including collagenase inhibition, anti-inflammatory action, bone resorption inhibition, and promoting the proliferation of periodontal fibroblasts (Oringer et al. 2002; Panwar and Gupta 2009).

Compared to oral administration, local intra-pocket drug delivery provides an effective drug concentration at periodontal pocket with few side effects. Success of this treatment was measured by its ability to control and prolong the release rate of the drug (Ferreira et al. 2014). The injectable *in situ* liquid crystal systems are good candidates for intra-pocket drug delivery because an intra-pocket syringe allows drugs access to the entire pocket (Vyas et al. 2000). The lyotropic liquid crystal (LLC) is formed spontaneously from the *in situ* liquid crystal system in an aqueous fluid. The formed tortuous networks of aqueous nano-channels in the mesophases

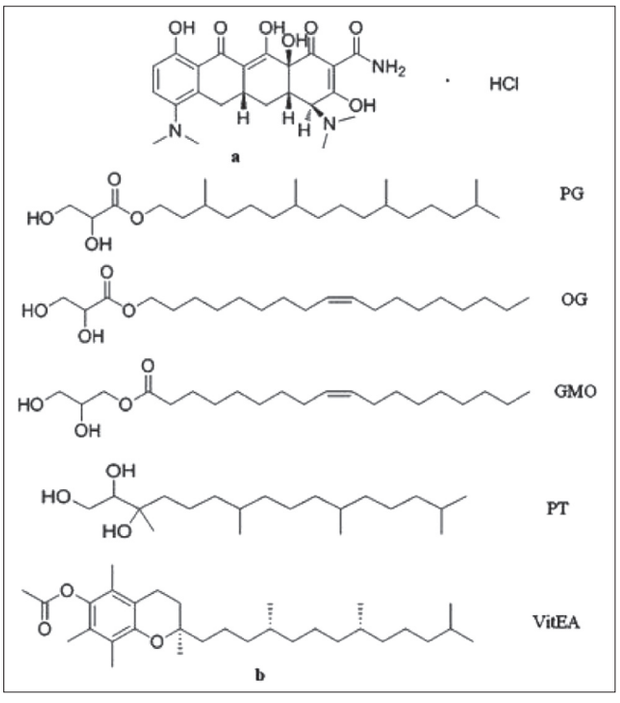

Fig. 1: (a) Chemical structure of minocycline hydrochloride. (b) Chemical structures of liquid crystal-forming materials, phytanyl glycerate(PG), oleyl glycerate(OG), glycerol monooleate(GMO), phytantriol(PT), vitamin E acetate(VitEA).

or mesophase particles play an important role for the sustained release of drugs from liquid crystal systems (Boyd et al. 2006; Fong et al. 2009). LLC composed of amphiphiles can be classified into lamellar, hexagonal and cubic phases based on their assembly shape (Guo et al. 2010; Zabara and Mezzenga 2014). Among them, the reversed hexagonal mesophase (H_2) has been extensively investigated for its ability to control the release rate of numerous drug substances, from low molecular-weight chemicals to macromolecular drugs (Libster et al. 2011; Cohen-Avrahami et al. 2010, 2012; Lopes et al. 2007). The H_2 consisting of an infinite rod-type water channel which arranged in a two-dimensional lattice and separated by lipid bilayers, shows great ability in sustained release (Fong et al. 2009; Lopes et al. 2006).

Various amphiphilic liquid crystal-forming materials, such as glycerol monooleate, oleyl glycerate, phytanyl glycerate, and phytantriol, have been reported (Fig. 1) (Boyd et al. 2006; Misiūnas et al. 2012). The aliphatic alcohol phytantriol (3,7,11,15-tetramethyl-1,2,3-hexadecanethriol, PT), comprising a branched phytanyl hydrophobic tail and a triol hydrophilic headgroup without an ester linkage, is more structurally stable than other materials mentioned above. PT is a promising alternative for the preparation of liquid crystals, and is increasingly employed as a building block of these self-assembling aggregates (Barauskas and Landh 2003; Fraser et al. 2013). The reversed cubic phase (V_2) was formed by PT-water system at physiological temperature. However, the transition of V_2-to-H_2 occurs at a higher temperature. Research indicated that a little vitamin E acetate (VitEA) added to the PT-water system could suppress the transition temperature from V_2 to H_2 (Dong et al. 2006). The transition temperature from V_2 to H_2 may be suppressed at 25 °C by adding 5% (w/w) VitEA to PT.

The aim of this work was developing and evaluating an *in situ* hexagonal mesophase system containing PT, propylene glycol (PG), VitEA, water and the antimicrobial drug (MH), for local treatment of periodontitis. The pharmacodynamic studies of MH loaded-ISH_2 were investigated on animal model of chronic periodontitis with commercially available Periocline® as a control.

2. Investigations, results and discussion

2.1. Characterization of the liquid crystal formulations by CPLM

A co-solvent PG was added into the PT-water system because V_2 was too viscous to be injected directly (Wadsten-Hindrichsen et al. 2007). According to the results from previous studies, the optimal formulation of *in situ* cubic phase (PT/PG/water, 63:27:10, w/w/w) was obtained. However, it was suggested that the selected formulation could be even further optimized to improve the sustained release of MH (Lee et al. 2009). The H_2 which shows great ability in sustained release was utilized to control the release rate of hydrophilic drugs. According to the phase diagram of PT/PG/water, H_2 could not be formed at room temperature (Wadsten-Hindrichsen et al. 2007). VitEA was used as an additive in this study to manipulate the V_2-to-H_2 phase transition, whose structure is similar to PT (Fig. 1) (Dong et al. 2006). By using single factor test, the proportion of VitEA was optimized. The formulations as shown in Table 1 were selected for our studies. The samples with concentrations of VitEA between 7 and 13% (w/w) appeared to be a homogeneous and transparent fluid after 72 h standing. When the concentration of VitEA reached 15%, the samples became heterogeneous and separated into two phases after 72 h. The ISH_2 that showed a dark background under cross-polarized light microscopy (CPLM) was characterized as an isotropic solution phase (Fig. 2). The liquid crystalline gel formed from the in situ hexagonal liquid crystal (ISH_2) in excess water that showed a "fan" type texture, reflecting the formation of a focal conic domain of columns, was characterized as hexagonal phase (Cohen-Avrahami et al. 2010; Rosevear 1954). As shown in Fig. 3, the addition of VitEA affected the texture of H_2, and the texture of F1 was clearest.

Table 1: Compositions of investigated formulations (all quantities are given as percentage by weight)

Formulation	PT (%)	PG (%)	VitEA (%)	Water (%)	MH (mg·g^{-1})
F1	56	27	7	10	20
F2	54	27	9	10	20
F3	52	27	11	10	20
F4	50	27	13	10	20
F5	48	27	15	10	20

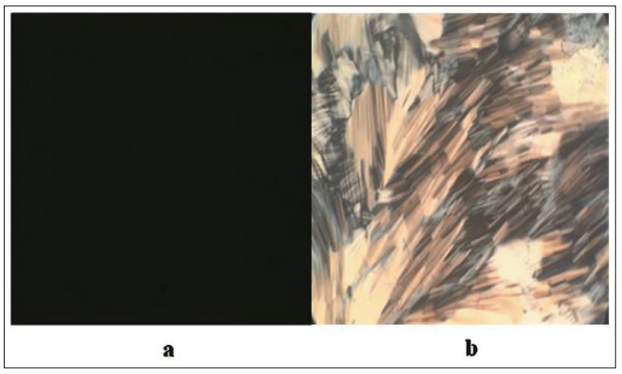

Fig. 2: Images of samples under cross-polarized light microscopy (magnification × 100): (a) 20 mg·g-1 minocycline hydrochloride-loaded in situ hexagonal liquid crystal, (b) the gel formed from 20 mg·g-1 minocycline hydrochloride-loaded in situ hexagonal liquid crystal in excess water.

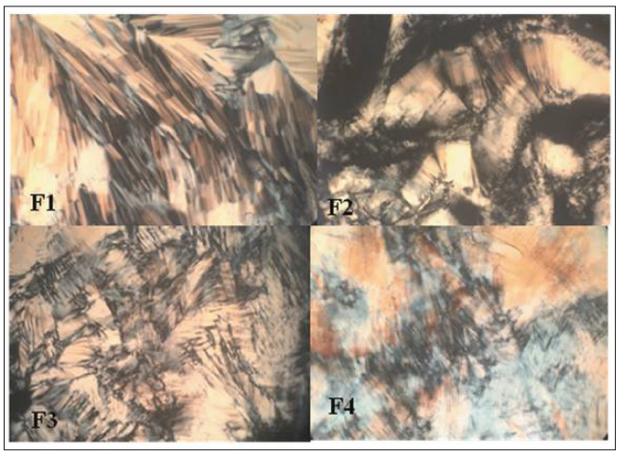

Fig. 3: Images of gels formed from formulations (F1-F4) in excess water under cross-polarized light microscopy (magnification × 100).

2.2. SAXS measurement

The phase structures of liquid crystal formed by ISH_2 in excess water were further confirmed by Small angle X-ray scattering (SAXS) after 72 h. Scattering intensities were plotted versus q value. The scattering vectors were calculated according to the following equation (Fong et al. 2009):

In this equation λ is the wavelength, 2θ is the scattering angle, q is the scattering factor, and S is the scattering vector. The SAXS measurements exhibited multiple peaks with relative positions at ratios of 1: √3: √4, which indicated closely packed cylindrical micelles arranged with 2D hexagonal symmetry, namely hexagonal phase (Phan et al. 2011). Moreover, the presence of 20 mg·g^{-1} MH in the ISH_2 did not affect the phase behaviour (Fig. 4).

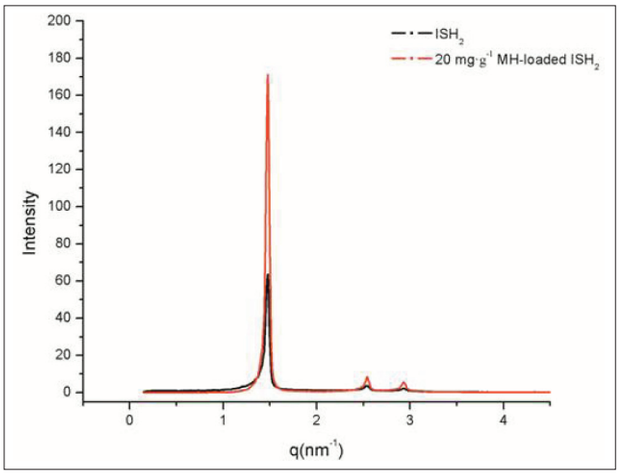

Fig. 4: Small angle X-ray scattering profiles for the gel obtained from F1 in excess water. Formulation without 20 mg·g-1 minocycline hydrochloride was used as control. The graphics show the ratio between the interplanar distances.

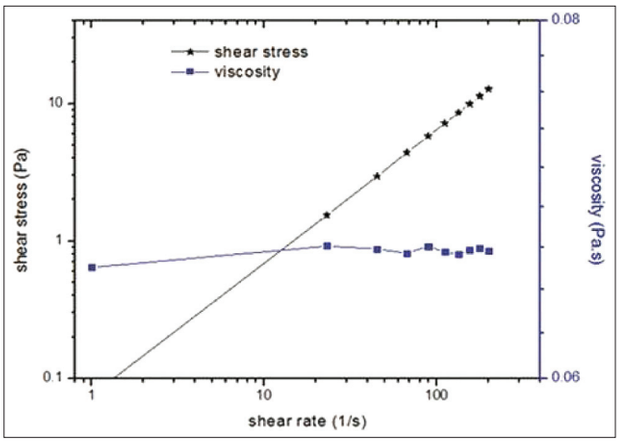

Fig. 6: Flow curves of 20 mg·g-1 minocycline hydrochloride-loaded in situ hexagonal liquid crystal (PT/PG/water/VitEA, 56:27:10:7, w/w/w/w).

2.3. *Rheological measurements*

Rheological methods allow the study of both structural and dynamical properties in a single affordable technique (Sagalowicz et al. 2006). In this work, the rheological properties and viscoelasticity of ISH_2 with 20 mg·g^{-1} MH were investigated before and after implantation of periodontal pocket. The linear viscoelastic region of the formulation was determined before carrying out the oscillatory measurements (Libster et al. 2007). The storage and loss moduli were plotted as a function of strain in the range of 0.01-100% at frequency ω =1 Hz, at temperatures of 25 and 37 °C (data not shown). According to the determined linear viscoelastic region, the viscoelasticity measurements of ISH_2 and liquid crystal gels formed in excess water were generally performed at strain of 0.1% and 0.5%, respectively. Frequency-dependent rheological measurements were performed to characterize the viscoelasticity of the formulations. The storage moduli *G'* (ω) and the loss moduli *G"* (ω) were plotted against the frequency of the applied oscillations (ω) (Siddig et al. 2006). The ISH_2 with 20 mg·g^{-1} MH was found to be more viscous than elastic with time at any specific frequency (*G"* > *G'*), which shows a characteristic of viscoelastic fluids (Fig. 5). Moreover, at low frequencies the liquid crystal gels formed in excess water were displayed that viscosity was the main part (*G"*> *G'*). With an increase in angular frequency, both *G'* and *G"* increased monotonically and, finally, at the crossover point *G'*dominated over *G"*. At frequencies close to the crossover point the H_2 phases reveal viscoelastic behavior that can be classified as "the transition to flow region" according to the terminology suggested by Mezzenga et al. (2005). This means that the H_2 phase behaves as a viscoelastic fluid capable of flowing under shear at low frequencies but exhibits an elastic behavior as the frequency increases.

In addition, to investigate the flow behavior of ISH_2 with 20 mg·g^{-1} MH, a steady-state stress-rate test was performed, as shown in Fig. 6. The stress increased with the shear rate, and viscosity did not change with shear rate. ISH_2 with 20 mg·g^{-1} MH exhibited a Newtonian-like behavior because the shear stress was proportional to the shear rate and the line did not pass through the origin.

2.4. *Physicochemical characterization*

A suitable ISH_2 formulation for periodontal pocket injection requires suitable syringeability, appropriate pH and the ability to form a hexagonal liquid crystalline gel *in situ* rapidly with minimum water absorption (Kumari and Pathak 2012). The physicochemical properties of the formulations F1-F4, such as syringeability, pH value, and phase transformation time (T_p), were characterized and summarized in Table 2.

Table 2: Physicochemical properties of investigated formulations

Formulation	Syringeability	pH	T_p (min)
F1	Injectable	5.37±0.06	≤15
F2	Injectable	5.25±0.05	≤15
F3	Injectable	5.30±0.04	≤30
F4	Injectable	5.18±0.07	≤60

Note: Data are presented as mean ± SD (n= 3).

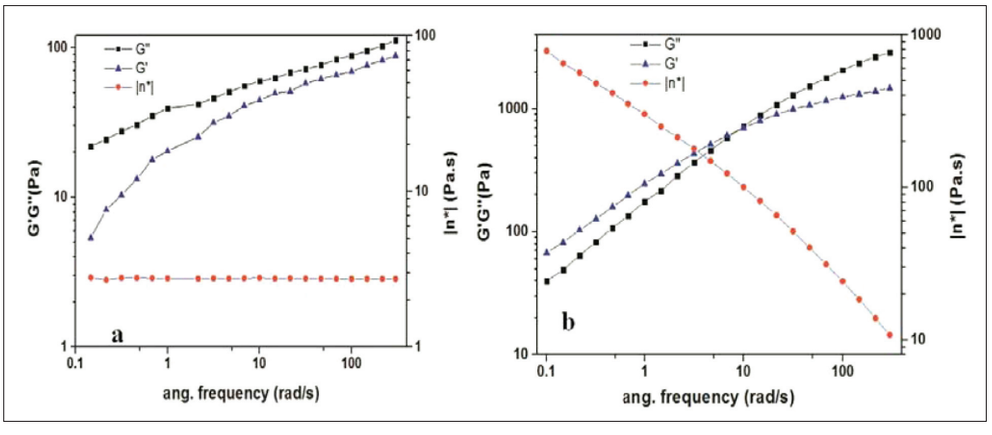

Fig. 5: Storage and loss modulus as a function of shear frequency: (a) minocycline hydrochloride-loaded in situ hexagonal liquid crystal, (b) hexagonal liquid crystal obtained from minocycline hydrochloride-loaded in situ hexagonal liquid crystal in excess water.

As reported in Table 2, all formulations were injectable when evaluated by dental syringes with 0.5 mm internal diameter. In addition, the pH values of all formulations were slightly acidic (pH 5.18-5.37). The effect of compositions on formulation to form hexagonal liquid crystalline gels *in situ* was also evaluated by T_p. F1, F2, F3 and F4 had the same water content (10%, w/w) but with different amounts of VitEA (7, 9, 11 and 13%, w/w). As shown in Table 2, when the water content kept constant, T_p extended with the amount of VitEA increasing. This indicated that F1 and F2 formed hexagonal liquid crystalline gels within the shortest time.

2.5. In vitro release

2.5.1. Influence of VitEA addition

Drug release tests were conducted by placing the formulations in the dialysis bags. As shown in Fig. 7, ISH_2 sustained the drug release for more than 10 days. Remarkably, the inserted panel (from 0-12 h) indicated that the drug release rate was proportional to the amount of VitEA. This could be probably due to the addition of the hydrophobic VitEA. VitEA hindered the process of water absorption, thus impeding the transformation into a hexagonal liquid crystalline gel. As time went on, ISH_2 transformed into hexagonal liquid crystalline gels, and the release rate of MH from the matrixes was mainly dependent on the diameter and tortuosity of water channels. The drug release rate was inversely proportional to the amount of VitEA after 48 h. It is well known that the release rate of hydrophilic drugs from H_2 with rod-like closed water channels is much slower than other types of liquid crystal (Chen et al. 2015). Therefore, phase transition became more complete with time going on, and the release rate of MH was prolonged among formulations containing more VitEA. In the test with the sample containing 7% of VitEA, the steady state was already accomplished at 240 h with more than 95% of the released amount. As a result, the optimal ISH_2 (PT/PG/water/VitEA, 56:27:10:7, w/w/w/w) loaded with 20 $mg \cdot g^{-1}$ MH was obtained.

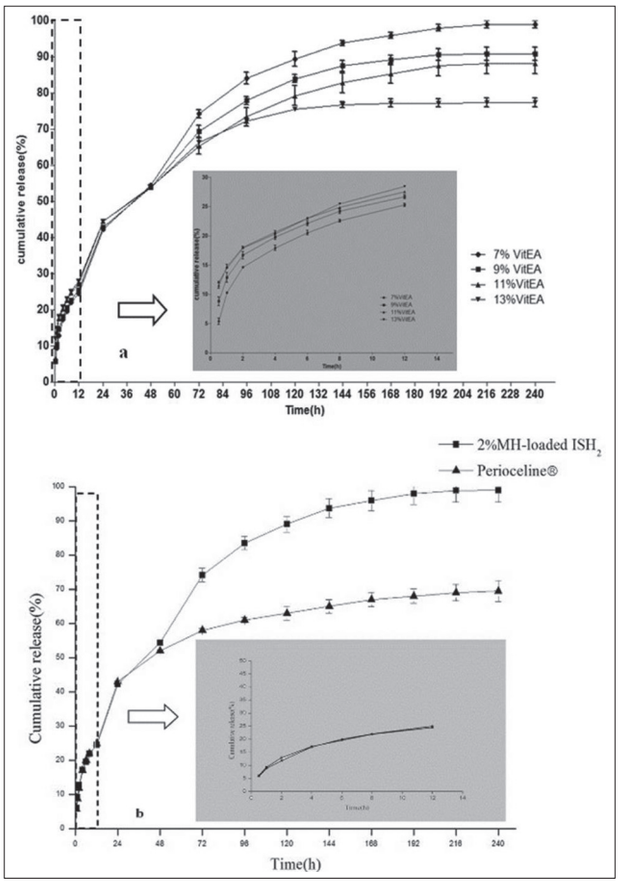

Fig. 7: (a) Effects of VitEA amount on the minocycline hydrochloride release from in situ hexagonal liquid crystal formulations. Inserted panel shows the in vitro release profiles in the first 12 h. (b) In vitro release profiles of 20 mg·g-1 minocycline hydrochloride from in situ hexagonal liquid crystal and Periocline®. Inserted panel shows the in vitro release profiles in the first 12 h. Data are presented as mean ± SD (n = 3).

2.5.2. Comparison of the in vitro drug release behavior of Periocline® and MH-loaded ISH_2

The in vitro release studies were conducted to investigate the differences of release behavior between Periocline® and ISH_2. Fig. 7 illustrates the in vitro release profiles of 20 $mg \cdot g^{-1}$ MH both from Periocline® and ISH_2. Periocline®, a local minocycline device for the treatment of periodontal pockets, is available on the market in the form of an ointment. Both Periocline® and ISH_2 showed a prolonged release rate extended for 10 days, and their daily drug releases were higher than the MIC of MH. During the first 12 h, ISH_2 and Periocline® possessed a similar release rate. The cumulative rates of ISH_2 exceeded those of Periocline®. Approximately 90% MH were released over 240 h from ISH_2, compared to 70% for Periocline®. This may be attributed to the unique internal structure of the hexagonal phase, and MH transported through the outward water channel. While for Periocline®, MH releases were depended on the degradation of the matrixes.

2.6. In vivo pharmacodynamics in rats

Different methods, such as high-sugar diet, ligature of the gingival sulcus and local inoculation of suspected pathogenic bacteria, are reported to induce chronic periodontitis in rats (Shibutani et al. 1997; Keyes and Jordan 1964). Ligation was used in combination with high-sugar diet in the present study to establish an experimental periodontitis model in a short time. Moreover, in order to get more significant pathological characteristics of periodontitis, the induction of experimental periodontitis was taken up to 10 weeks. Normal gums which are close to the tooth surface with probing depth of less than 0.7 mm, are pink and show no edema. Alveolar bones of the health rats tightly surround the root. After 2 weeks, the gums began to present edema, and the color turned into slight red. Four weeks after modeling, bleeding appeared on probing but did not overflow the periodontal pocket. As shown in Fig. 8, edema and a tendency of spontaneous bleeding was obvious when experimental periodontitis rat models was established successfully. The probing depth was up to 2.3 mm, and there was a significant dent in the alveolar bone.

Four groups were selected for evaluation of gingival index (GI), pocket depth (PD) and alveolar bone loss (ABL). Compared to the normal group, there was a significant difference in the gingival index of the model group ($p < 0.01$). The gums of the model group presented severe inflammation, marked redness, edema, and tendency of spontaneous bleeding. After ligature removal a trend of decrease in GI occurred at each follow-up visit, and reached a level of significance at week 4 when compared to week 1 ($p < 0.01$). The gingivae of positive control group and MH-loaded ISH_2 group in the first week of treatment were shallowed, did not show spontaneous bleeding, and bleeding on probing was reduced. A difference between two medicated groups and model group ($p < 0.05$) was seen. After 4 weeks, the gums of two medicated groups were pink, no edema or bleeding on probing was observed, which showed a significant difference to model group ($p < 0.01$) and no significant difference to the normal group ($p > 0.05$). What's more, there was no significant difference between the positive control group and MH-loaded ISH_2 group at week 4 (Table 3). Probing PD measurements demonstrated a reduction in all test groups except the normal group (Table 4). There was a statistically significant decrease in the pocket depth after 1, 2, 3, and 4 weeks when compared to the model group at $p < 0.01$ for both the positive control group and MH-loaded ISH_2 group. There was no statistical significance between the medicated group and the normal group ($p > 0.05$). ABL was found in the rats of all the groups (Fig. 9). However, significant differences in ABL were found between the

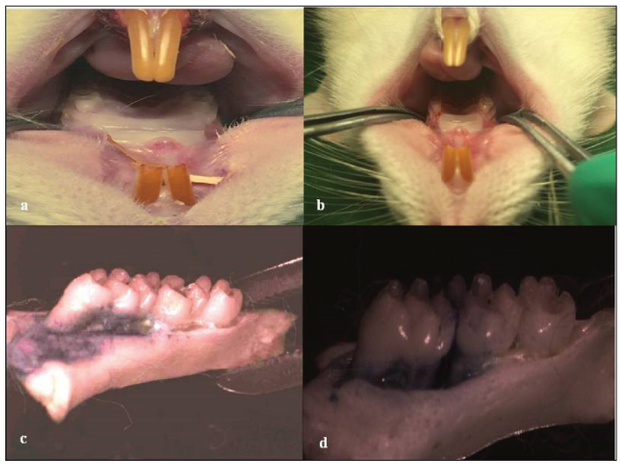

Fig. 8: The photographs of gingival tissue: (a) normal group, (b) model group. The photographs of alveolar bone (magnification × 12.5): (c) normal group, (d) model group.

normal group and the model group ($p < 0.01$) (Table 5). After treatment for 4 weeks, alveolar bone resorption was inhibited and alveolar bones of both groups were reconstructed, which showed a significant difference to the model group ($p < 0.01$) and no significant difference to the normal group ($p > 0.05$). In summary, both Periocline® and MH-loaded ISH_2 had direct effects on GI, the recovery of periodontal pocket and alveolar bone. No significant difference was found between the two groups in GI, PD and ABL ($p > 0.05$).

Table 5: Alveolar bone loss of four groups

Group	ABL
Control	0.437±0.051
Model	1.812±0.076#
Reference	0.507±0.007**
2%MH-load ISH_2	0.510±0.005**

Note: Data are presented as mean ± SD (n= 5 per group).
#p < 0.01 vs. control.
***p < 0.01 vs.* model.

The results of histological observation suggest that the gingival papilla of the normal group was conical, and combined with complete gingival epithelial attaching to cement–enamel junction (CEJ). Gingival collagen fibrils in the connective tissue arranged neatly, and the alveolar bone crest with smooth appearance has no resorption. As for the model group, the epithelium of gingival sulcus was eroded, and migration or dislodgment of the depressed gingival papilla from the CEJ towards the roots was found. Gingival collagen fibrils in the connective tissue arranged disorderly or frac-

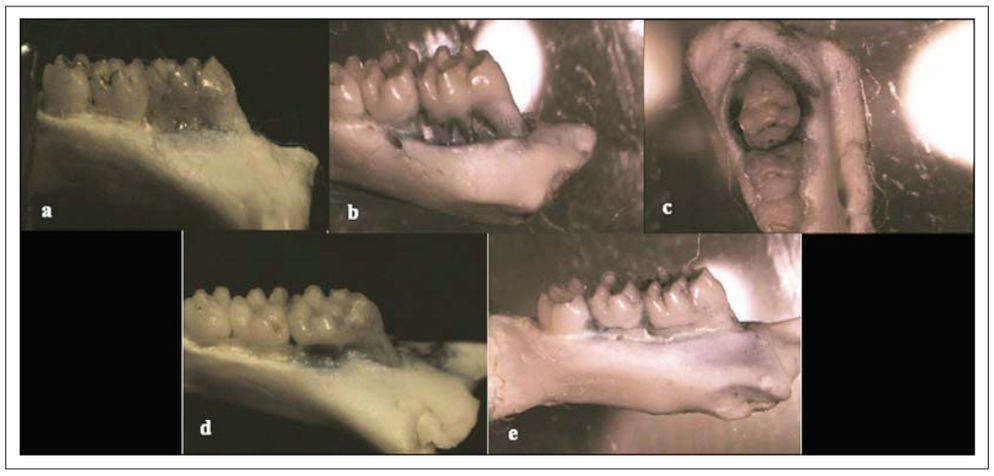

Fig. 9: The observation of alveolar bone (magnification × 12.5): (a) normal group, (b) model group, (c) model group photographed from an aerial perspective, (d) Periocline® group, (e) 20 mg·g-1 minocycline hydrochloride-loaded in situ hexagonal liquid crystal.

Table 3: Scores of gingival index of four groups

Group	1 week	2 weeks	3 weeks	4 weeks
Control	0.500±0.100	0.400±0.000	0.433±0.058	0.467±0.058
Model	3.000±0.100#	2.267±0.058#	1.500±0.100#	1.100±0.100#
Reference	2.800±0.100*	2.067±0.251*	1.267±0.057**	0.633±0.058**
2%MH-loaded ISH_2	2.800±0.092*	2.062±0.112*	1.281±0.046*	0.567±0.033**

Note: Data are presented as mean ± SD (n= 5 per group).
#p < 0.01 vs. control.
**p < 0.05 vs.* model.
***p < 0.01 vs.* model.

Table 4: Pocket depth of four groups

Group	1 week	2 weeks	3 weeks	4 weeks
Control	0.629±0.021	0.653±0.027	0.669±0.031	0.659±0.029
Model	1.873±0.060#	1.756±0.048#	1.667±0.017#	1.516±0.032#
Reference	1.670±0.032**	1.494±0.067**	1.121±0.047**	0.724±0.018**
2%MH-loaded ISH_2	1.619±0.090**	1.493±0.070**	1.168±0.089**	0.747±0.069**

Note: Data are presented as mean ± SD (n= 5 per group).
#p < 0.01 vs. control.
***p < 0.01 vs.* model.

tured. The alveolar bone crest and alveolar bone were all depressed type of bone resorption and destruction, with osteoclasts appeared in the alveolar ridge. In addition, the two medicated groups had different degrees of curative effects after treatment for four weeks. For the Periocline® treatment group, gingival epithelium was proliferative repaired slightly and re-attached to CEJ. Gingival collagen fibers in the connective tissue recovered as neatly arranged, the alveolar bone crest restored smoothly and depressions of alveolar bone resorption disappeared nearly as shown in Fig. 10. As with the Periocline® group, 20 mg·g^{-1} MH-loaded ISH_2 group achieved the same effect on the prosthodontic treatment of periodontal diseases. Therefore, it can be confirmed that 20 mg·g^{-1} MH-loaded ISH_2 has an effective therapeutic effect on the gingival epithelial, connective tissue recovery, alveolar ridge and alveolar bone repair.

2.7. Conclusion

Results of this work revealed that MH-loaded ISH_2 containing PT could be used for periodontal pocket injections. The ISH_2 was formulated using PT, PG and water for the core components as well as VitEA for additives. CPLM, SAXS and rheological measurements revealed that ISH_2 showed a typical characteristic of hexagonal liquid crystalline in excess water. The in vitro release tests proved that the MH-loaded ISH_2 had higher cumulative releases than Periocline® for 10 days. Finally, the in vivo pharmacodynamic studies indicated that ISH_2 has similar therapeutic effects for chronic periodontitis compared to Periocline®. Consequently, the PT-based ISH_2 is considered as an ideal administration approach for periodontal pocket injection. It is also expected that the developed ISH_2 in liquid form can be applied for other drugs via injection to sustained delivery.

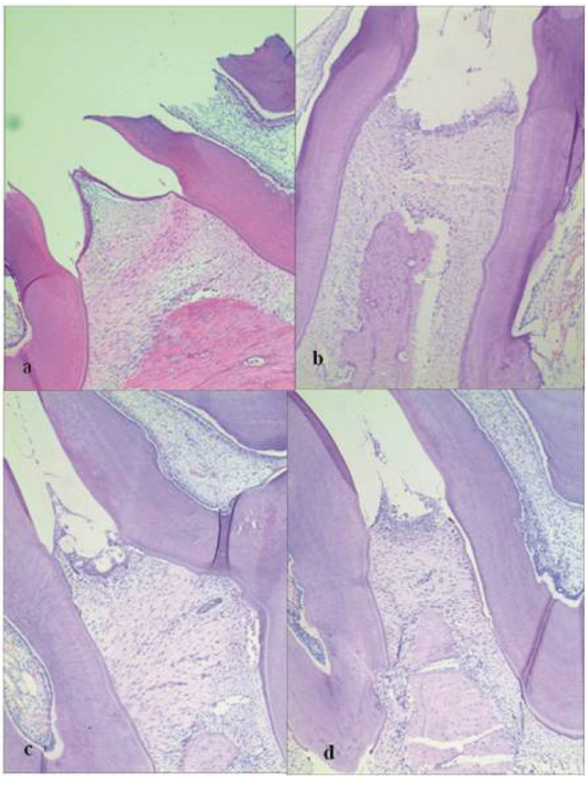

Fig. 10: Micrographs of rat periodontium sections embedded in paraffin and stained with hematoxylin and eosin (magnification × 100): (a) normal group, (b) model group, (c) Periocline® group, (d) 20 mg·g-1 minocycline hydrochloride-loaded in situ hexagonal liquid crystal.

3. Experimental

3.1. Materials

Phytantriol (3,7,11,15-tetramethyl-1,2,3-hexadecanetriol, purity > 95%) and vitamin E acetate (VitEA, purity > 98%) were purchased from Tokyo Chemical Industry Co., Ltd. (Shanghai, China). Propylene glycol was supplied by Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Minocycline hydrochloride was obtained by Wuhan Sheng Tianyu Biotechnology Co., Ltd. (Wuhan, China). Periocline® was gained by New-Era Co., Ltd. (Japan). Phosphate buffer solution (PBS) sachets (pH 7.4, 0.01 M) were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). Chloral hydrate was supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sodium hydroxide was obtained by Chinasun Specialty Products Co., Ltd. (Changshu, China). All other reagents were of analytical or pharmaceutical grade. The water used was distilled and passed through a Milli-Q water purification system (Millipore, Bedford, USA).

The animals were male specific pathogen free (SPF) rats (3 months old, about 300±20 g body weight, certificate no. SCXK (Jiangsu) 2011-0003) which were purchased from Changzhou Cavens Lab Animal Co., Ltd.

3.2. Preparation of in situ hexagonal mesophase

The phase diagram of PT/PG/water and the optimal formulation of *in situ* cubic phase (PT/PG/water, 63:27:10, w/w/w) were presented in our previous study. On the basis of preliminary work, ISH_2 was prepared using PT, PG, VitEA and water. Different amounts of PT and VitEA were gently melted at 60±0.5 °C. Meanwhile, an aqueous solution of MH was prepared by dissolving the drug in PG and then mixed with PT and VitEA. After that, an appropriate quantity of preheated water at the same temperature was added, and the samples were vortex-mixed homogeneously for 3 min. The resulting formulations were finally sterilized by filtration through a 0.45-μm filter and kept in closed vials to equilibrate in the dark at 25 °C for 72 h before measurements.

3.3. Visual observation and cross-polarized light microscopy

The samples were characterized by visual observation and CPLM (CK-500, Caikon, China) at room temperature. Likewise, ISH_2 in excess water were inserted between two glass microscope slides and characterized via the same method.

3.4. Small angle X-ray scattering

Scattering experiments were performed using a SAXSess mc^2 SAXS (Anton Paar, Austria) with X-ray source of wavelength λ=0.154 nm, and operated at 40 kV and 50 mA. The temperature was maintained at 37 °C, and samples were equilibrated at the particular measurement temperature for 15 min before making 15 min exposure. Using aluminum foil as the carrier, the scattering information is recorded by the image plate. The scattering of aluminum was removed, and scattering intensities were plotted versus q value, which enabled the identification of peak positions.

3.5. Rheological measurements

Rheological signatures carried out in this study were performed with an AR-2000ex rheometer (TA Instruments, USA). A cone-plate sensor was used with an angle of 1◦ and the diameter of 20 mm. Two different experiments were carried out: steady flow (Flow-Sweep mode) and oscillation (Frequency-Sweep mode). Steady flow measurements were performed to investigate whether the samples were Newton fluid in the shear rate range of 1-200 s^{-1} at 25 °C. In the oscillation experiments, the storage moduli *G'*, loss moduli *G"* and complex viscosity η* were measured in the frequency range of 0.01-100 rad·s^{-1}. The test temperature of ISH_2 and the samples in excess water were 25 °C and 37 °C respectively. All measurements were carried out after the linear viscoelastic range was determined.

3.6. Physicochemical characterization

3.6.1. Evaluation of syringeability

Syringeability is regarded as the ability of a preparation to be successfully administered by syringe with an appropriate needle (Réeff et al. 2013). In this study, syringes (New-Era Co., Ltd., Japan) specially designed for periodontal pocket local delivery were chosen to evaluate the syringeability of the formulations at room temperature. The inner diameter of injection head is 0.5 mm, and the evaluation index of syringeability was divided into injectable and non-injectable. All analyses were carried out in triplicate.

3.6.2. Investigation of phase transformation

After intra-pocket injection, ISH_2 forms a hexagonal liquid crystalline gel when absorbing gingival cervical fluid. The minimum volume of water and gelation time were determined using the magnetic stirring method as reported before (Yuan et al. 2012). According to the results of a pre-experiment, the viscosity of the hexagonal phase was not enough to stick the rotor. Therefore, the ability of phase transformation was observed in rats. The chronic periodontitis rats were randomly divided into five groups with three rats in each group. ISH_2 containing 20 mg·g^{-1} MH was injected into the periodontal pocket of each rat under 5% (w/v) chloral hydrate anesthesia. The phase transformation was investigated by periodontal probe at 10, 15, and 30 min, and 1, 2 h after the injection. Only if ISH_2 gathered into a mass, phase transformation was complete. All analyses were carried out in triplicate.

3.6.3. Determination of pH value

Generally, for parenteral formulations, the acceptable pH range is 4-9 (Ghosh and Jasti 2004). The pH values of chosen samples were determined by a SevenMuti type multi-tester (Mettler Toledo, Shanghai, China). All analyses were carried out in triplicate.

3.7. In vitro release test

Drug release in vitro was determined by a dialysis membrane diffusion method (Chang and Bodmeier 1998). The formulations (0.5 g) in triplicate were placed separately in 6-cm dialysis bags (14,000 Da MWCO). The dialysis bags were then closed and immersed into 6 mL of PBS (pH 7.4) in glass bottles, which were further placed in a horizontal shaker (ZD-88B, Bolaite, China) (37.0±0.5 °C, 60 rpm). Sink conditions were provided throughout the experiments. At predetermined time points of 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 h, all the release medium was withdrawn and replaced with fresh medium. The release medium samples were filtered through 0.45-μm millipore filters and analyzed by an ultraviolet spectrophotometer (UV 1800 Shimadzu, Japan). The amount of MH released was analyzed using a validated UV method at wavelength of 274 nm.

3.8. Pharmacodynamics studies

All the animal studies were approved by the Animal Ethical Committee of Anhui University of Chinese Medicine, and experiments were conducted in accordance with the guidelines of the Laboratory Animal Center of Anhui University of Chinese Medicine. Animals were housed under a 12-h light and dark cycle with free access to food and water, and were acclimatized for at least one week before the study.

0.5 g Periocline® (reference) or ISH_2 containing 10 mg MH was injected into the periodontal pocket of rats (five animals per group). Rat chronic periodontitis model was established by silk thread ligation with 10 weeks (Shibutani et al. 1997). The rats were anesthetized with 5% (w/v) chloral hydrate (350 $mg \cdot kg^{-1}$) on the fixed plate, 4-0 surgical suture was used to establish the passage into the first molar and second molar space, and then double ring ligature the tooth neck towards the gingival sulcus as much as possible. Meanwhile, the sucrose solution (100 $g \cdot L^{-1}$) was chosen as drinking water to assist model establishment (Keyes and Jordan 1964). Check 3 times a week for the situation of the fixed thread, immediately tire silk thread again if loosen. A total of 10 weeks was spent to establish experimental chronic periodontitis model in rats. After the induction of experimental periodontitis, silk thread was removed, then the periodontal index was checked and the tooth was washed with saline. Two groups of rats were tested by local application of either MH-loaded ISH_2 or Periocline® into the periodontal pockets. The applications were done every week for 28 days. All groups were fed normally. Assessment of some parameters was carried out at the baseline. These parameters were gingival index, pocket depth, and alveolar bone loss. Gingival index measurement was done according to Löe (1967). The tissues around each tooth were divided into four gingival scoring units. A periodontal probe was used to assess the bleeding potential of the tissues. Each gingival unit was assessed according to the criteria described in Table 6.

Table 6: Criteria for the gingival index system

Score	Characteristics of gingival
0	Normal gingival
1	Mild inflammation, slight changes in color, slight edema, and no bleeding upon probing
2	Moderate inflammation, redness, edema, glazing, and bleeding upon probing
3	Severe inflammation, marked redness, edema, ulceration, and tendency to spontaneous bleeding

Pocket depth is a measurement of the severity of the periodontal disease; it was calculated in millimeters using a sterile metered periodontal probe immediately before drug application at six sites (distal, mid and mesial aspects for both buccal and lingual sites) of each tooth weekly (Kõll-Klais et al. 2009). The mean value of six points was recorded as pocket depth of the tooth.

To evaluate the extent of alveolar bone loss, we used a modified protocol reported by Madeira et al. (2012). Rats were sacrificed after four weeks-application, alveolar bone tissues with three molars from maxillae were taken. The alveolar bone tissues were exposed overnight to sodium hydroxide (1 M), and mechanically defleshed. To distinguish the CEJ, maxilla was stained with 0.3% (w/v) methylene blue. The palatal faces of the molars were photographed at ×12.5 magnification with a stereomicroscope (Olympus BXS1, Germany) and a digital camera (Olympus DP70, Germany). Quantitative analysis was used for the measurement of the distomedial area between the CEJ and the alveolar bone crest. Measurements were recorded at 6 sites (distal, mid and mesial aspects for both buccal and lingual sites), the average value of six points was recorded as alveolar bone loss. Four animals per group were analysed in this study.

Histologic analysis was carried out by hematoxylin and eosin (H&E) staining. Rats were euthanized, and the tissues obtained were then fixed in 1% (w/v) paraformaldehyde, for 24 h at room temperature. The maxilla specimens were demineralized in home-made decalcifying solution for four weeks, dehydrated in graded ethanol, and embedded in paraffin. Serial sections (5 μm) were stained for H&E and analysed under light microscopy. The alveolar bone crest between the first and the second molars was observed.

3.9. Statistical analysis

Statistical analysis was carried out by SPSS statistical software (18.0 version, SPSS Inc., Chicago, IL). Each experiment was performed in quintuplicate and all data were expressed as mean ± standard deviation (SD). One-way analyses of variance were performed for evaluation of the results. *P* values less than 0.05 were considered to be statistically significant.

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