Carbohydrate Polymers 136 (2016) 516–526

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Characterization and biocompatibility of injectable microspheres-loaded hydrogel for methotrexate delivery

Qifeng Dang [a], Chengsheng Liu [a,*], Yujie Wang [a], Jingquan Yan [b], Huiqin Wan [c], Bing Fan [d]

[a] College of Marine Life Science, Ocean University of China, 5 Yushan Road, Qingdao 266003, PR China
[b] School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao 266003, PR China
[c] Shandong Qingdao Health School, 66 Fuzhou Road, Qingdao 266071, PR China
[d] Qingdao Aorun Biotechnology Co. Ltd., Room 602, Century Mansion, 39 Donghaixi Road, Qingdao 266071, PR China

ARTICLE INFO

Article history:
Received 21 August 2015
Received in revised form
13 September 2015
Accepted 23 September 2015
Available online 28 September 2015

Chemical compounds studied in this article:
Chitosan (PubChem CID: 71853)
α-Glycerophosphate (PubChem CID: 14754)
β-Glycerophosphate (PubChem CID: 6101544)
Lactic acid (PubChem CID: 612)
Giemsa stain (PubChem CID: 13735)
Hematoxylin (PubChem CID: 442541)
Eeosin (PubChem CID: 11048)
Methanol (PubChem CID: 887)
Sodium chloride (PubChem CID: 5234)

Keywords:
Chitosan
Microspheres-loaded hydrogel
Characterization
Biocompatibility
Drug release

ABSTRACT

Injectable thermosensitive hydrogels have widely been studied as drug delivery systems for their minimally invasive administration and localized drug release. However, burst drug release limits clinical applications of such hydrogels. A double-component injectable formulation (microspheres-loaded hydrogel, CMs-CS-HG) was thus fabricated to eliminate the limitation. Gelation temperature, gelation time, complex viscosity and syringeability tests for CMs-CS-HG demonstrated excellent injectability. After injection, the drug-loaded chitosan-based microspheres (CMs) were localized within the hydrogel, leading to localized drug release. Moreover, CMs-CS-HG had good hemocompatibility and histocompatibility, and had non-genotoxicity and non-cytotoxicity to Kunming mice. In addition, both *in vitro* and *in vivo* methotrexate (MTX) releasing efficiencies were evaluated, demonstrating long-term sustained MTX release from MTX-loaded CMs-CS-HG. These results showed the double-component CMs-CS-HG not only maintained good injectability and biocompatibility but also prolonged drug-releasing time in comparison with the single-component CS-HG or CMs, suggesting that CMs-CS-HG may be a promising drug delivery system.

1. Introduction

Injectable thermosensitive hydrogels are intelligent materials that usually undergo a sol–gel phase transition in response to changes of temperature (Dang et al., 2012a; Lv et al., 2014). Both natural and synthetic polymer-based injectable thermosensitive hydrogels have widely been investigated in controlled drug delivery systems because of their outstanding performance, such as syringeable solutions at room temperature, semisolid gels at physiological temperature, minimal invasion during administration, and localized drug release at administrating sites (Wang et al., 2015; Xie et al., 2015). Especially, the natural polymer-based injectable thermosensitive hydrogels have increasingly received considerable attention mainly due to their relative safety (Zhang et al., 2011; Zhou, Jiang, Cao, Li, & Chen, 2015). Among such natural polymers, chitosan (CS) and its derivatives are particularly highlighted due to their well-demonstrated advantages, including biocompatibility, biodegradability, nontoxicity and nonimmunogenicity (Rinaudo, 2006). CS-based injectable hydrogels and their potential applications to drug delivery have extensively been reported by numerous literatures (Islam, Riaz, & Yasin, 2013; Peng et al., 2013; Sun et al., 2010). However, burst release of loaded drugs

* Corresponding author.
E-mail address: liucsouc@126.com (C. Liu).

http://dx.doi.org/10.1016/j.carbpol.2015.09.084
0144-8617/

is becoming a new challenge when the CS-based injectable hydrogels are used as drug-delivery systems, just as some other hydrogels reported (Posadowska et al., 2015; Zhang, Jo Lewis, & Chu, 2005).

We have previously reported a single-component injectable thermosensitive hydrogel (chitosan-α,β-glycerophosphate hydrogel, CS-HG) whose correlative parameters, including gelation temperature, complex viscosity and pore size, can be controlled as requested (Dang et al., 2011, 2012b). Further experiments show CS-HG is of good biocompatibility and biodegradability (Dang et al., 2012a, 2015). For this single-component hydrogel, we have no reports on drug-releasing properties yet, but one published literature shows burst release of drug loaded is also inescapably becoming a critical limitation of using CS-HG as a drug delivery system (Zhou et al., 2015). Therefore, exploiting new-type CS-HG-based formulations, not only with the excellent original properties of CS-HG, but also with the great ability to keep long-term sustained-release of drugs loaded, are urgently necessary.

On the other hand, we have also reported another single-component formulation, CS-based microspheres (CMs), with long-term sustained drug-releasing ability (Wang et al., 2009; Zhou et al., 2014). However, the particle-based drug carriers, like microspheres, can migrate away from their targeting sites after injection or embedment *in vivo*, causing failure in localized drug release (Ozeki et al., 2012) and impairment of biocompatibility (Hoare & Kohane, 2008). Thus, for this single-component CMs, further research to solve the above issues is of practical significance.

A new strategy, designing a novel double-component injectable formulation (CMs-loaded CS-HG, CMs-CS-HG) based on CS-HG embedded with CMs, is proposed in our laboratory. In contrast to the single-component CS-HG or CMs, the double-component CMs-CS-HG is supposed to simultaneously provide for triple abilities: localized drug delivery, long-term sustained drug release, and good biocompatibility, which are mainly due to the simultaneous incorporation of CMs and CS-HG. As stated above, CS-HG can transform itself into semi-solid gel in physiological environment after injection, which localizes the drug-loaded CMs around the administrating target, and the loaded drug in CMs can be continually released from CMs-CS-HG. Furthermore, comparing with CMs, the double-component CMs-CS-HG may extend drug-releasing time, which mainly attributes to CS-HG that endows an auxiliary barrier to drug release. In addition, the formation of semi-solid gel may also improve the biocompatibility by preventing the CMs from diffusing throughout tissue *in vivo*. Other similar formulations, which fabricated by embedding or integrating particle-based drug carriers (*e.g.*, liposomes, microspheres, vesicles, or nanoparticles) into injectable hydrogels based on both natural and synthetic polymers, have been demonstrated to be effective in sustained drug release at the target sites (Lopez-Noriega et al., 2014; Ozeki et al., 2012; Posadowska et al., 2015; Zhang et al., 2005). So far, however, no reports on the double-component CMs-CS-HG as a drug delivery system have been found yet. It is thus necessary to perform more investigations on such type of formulation under various conditions in order to accelerate its clinical applications.

This paper mainly focuses on the potential of this new-type double-component CMs-CS-HG as a drug delivery system. Accordingly, the gelation temperature, gelation time, complex viscosity, and syringeability of CMs-CS-HG were investigated to verify whether its correlative parameters change or not in the presence of CMs, and further confirm its feasibility as an injectable formulation. Furthermore, *in vivo* injection experiment was carried out to assess *in situ* gelling and localization properties of CMs-CS-HG. Moreover, bone marrow micronucleus test, hemolysis analysis, hematologic and plasma biochemical parameters assay, and histological observation were employed to justify the biocompatibility of CMs-CS-HG. In addition, both *in vitro* and *in vivo* drug-releasing efficiencies of CMs-CS-HG were evaluated using methotrexate (MTX) as a model drug.

2. Materials and methods

2.1. Materials and animals

Chitosan (CS) was extracted from *Fenneropenaeus chinensis* (Osbeck, 1765) shells by ourselves. MTX and Fetal Calf Serum (FCS) were purchased from Sigma–Aldrich (Sigma Co., St. Louis, USA). All the other chemical reagents used were of analytical grade and commercially obtained from Sinopharm Chemical Reagent Co. Ltd. Millipore (Milli-Q) type I water with a resistivity of 18 MΩ was used throughout.

Adult SPF Kunming mice and New Zealand White rabbits were purchased from Qingdao Food and Drug Administration (Shandong, China). The animals were kept under standard environment (12 h light/12 h dark circle, 22 ± 2 °C, 50 ± 10% relative humidity, water and diet *ad libitum*) up to 7 days before experiments. All animal experiments were conducted following the NIH guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev. 1985) and EU Directive 2010/63/EU for animal experiments.

2.2. Deacetylation degree and molecular weight of chitosan

The deacetylation degree of CS was ascertained according to the method of acid–base titration and FTIR spectrum. The molecular weight of CS was determined using an Ubbelohde Viscometer. All these methods are traditional methods used in our laboratory and has been described in our previously published literature (Liu et al., 2006).

2.3. Preparation of microspheres-loaded hydrogel formulation

The injectable chitosan-α,β-glycerophosphate hydrogel (CS-HG) was prepared as described in our previous publication (Dang et al., 2011). In present work, 0.18% (w/v) of CS solution (with 0.08 mol/L of lactic acid aqueous solution as solvent) and 50% (w/v) of aqueous α,β-GP solution were used, and the ratio of CS solution to α,β-GP solution was 9.0:1.0 (v/v). MTX-loaded CS-HG (MTX-CS-HG) was prepared by adding MTX (64.9 mg) into CS solution (18 mL) under stirring. The following steps were the same as the forenamed preparation method of CS-HG.

CS-based microspheres (CMs) were prepared using Water-in-Oil emulsion technique (Zhou et al., 2014). CS solution (6%, w/v) was prepared by dissolving 600 mg CS in acetic acid aqueous solution (2%, v/v) as water phase, then was dropped into 60 mL liquid paraffin (oil phase) containing 0.4 mL Tween-80 and 0.4 mL Span-80. The mixture was stirred at 800 rpm for 30 min using a RW20 Overhead Stirrer (IKA-Werke GmbH & Co., Staufen, Germany). Next, 2 mL glutaraldehyde was drop-wise added into the reaction system and stirred for 60 min. The CMs was separated, washed with deionized water, and successively dehydrated in ethanol, and then freeze dried. MTX-loaded CMs (MTX-CMs) was prepared by mixing MTX into CS solution (CS:MTX = 3:1, w/w) as water phase. The following process was the same as the above-described preparation of CMs.

The microspheres-loaded hydrogel formulation (CMs-CS-HG or MTX-CMs-CS-HG) in sol phase was prepared by dispersing 500 mg CMs or MTX-CMs into 20 mL CS-HG solution. CMs-CS-HG or MTX-CMs-CS-HG in gel phase was obtained by elevating temperature to the gelation temperature.