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Preparation and characterization of gellan gum/glucosamine/clioquinol film as oral cancer treatment patch

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ABSTRACT

To administer cancer drugs with improved convenience to patients and to enhance the bioavailability of cancer drugs for oral cancer therapy, this study prepared gellan gum/glucosamine/clioquinol (GG/GS/CQ) film as the oral cancer treatment patch. GG/GS/CQ film fabricated through the EDC-mediated coupling reactions (GG/GS/CQ/EDC film). The film of the physicochemical properties and drug release kinetics were studied. The effectiveness of GG/GS/CQ/EDC film as oral cancer treatment patch were evaluated with the animal model. The results confirmed that CQ can be incorporated via EDC-mediated covalent conjugation to gellan gum/glucosamine. Mechanical testing revealed that the maximum tensile strength and elongation percentage at break were 1.91 kgf/mm^2 and 5.01% for GG/GS/CQ/EDC film. After a drug release experiment lasting 45 days, 86.8% of CQ was released from GG/GS/CQ/EDC film. The Huguchi model fit the GG/GS/CQ/EDC drug release data with high correlation coefficients ($R^2 = 0.9994$, respectively). The effect of the CQ dose on oral cancer cells (OC-2) was tested, and the IC_{50} of CQ alone and CQ with 10 μM $CuCl_2$ were 9.59 and 2.22 μM, respectively. The animal testing indicated that GG/GS/CQ/EDC film was decreased epidermal growth factor receptor (EGFR) expression and suppress tumor progression. These findings provide insights into a possible use for GG/GS/CQ/EDC film for oral ca in clinical practice. The GG/GS/CQ/EDC film is suitable as the dressing for use in the treatment of early-stage cancer or as wound care after surgery in late-stage of oral cancer treatment.

1. Introduction

Every year, nearly half of a million patients worldwide are diagnosed with oral cancer, and approximately 150,000 oral cancer patients die each year [1]. The normal oral mucosa is generally pink in color. Leukoplakia and erythroplakia are the two most common potentially malignant disorders of the oral cavity and are also considered precancerous conditions. Leukoplakia is a condition in which thick white and gray patches form inside the mouth or on the tongue as a result of chronic irritation. In erythroplakia, abnormal red areas or red spots form on the mucous membrane lining the mouth, an area that often bleeds easily [2,3]. The treatment for oral cancer is dependent on the stage of development of the cancer. Staging for oral cancer follows a classification into stages 0–4 [4]. In stage 0, tumor cells are localized inside the oral mucosa epithelium; in stages 1–2, the diameter of the tumor is between 2 cm and 4 cm, and there are no metastases to the neck lymph node. However, in stages 3–4, the diameter of the tumor is >4 cm, or metastases are found in the neck lymph node and other tissues. Chemotherapy and radiotherapy are usually used in early-stage oral cancer treatment, whereas surgery is the primary treatment in late-stage oral cancer. An improvement of patient convenience is necessary for treatment with cancer drugs. The study aimed to design a gellan gum/glucosamine (GG/GS) oral cancer treatment patch, which includes the chemotherapy drug clioquinol (CQ), and that can be used not only in early-stage oral cancer therapy but also as a wound care dressing after surgery in late-stage oral cancer treatment.

>100 chemotherapy or chemo drugs are used to treat cancer such as doxorubicin, cisplatin, paclitaxel et al. [5]. Clioquinol (CQ) has been used for many years as an antimicrobial agent and more recently as a potential for cancer therapies. Clioquinol, a lipophilic compound capable of forming stable complexes with copper ions, is a potent proteasome inhibitor and inducer of apoptosis [6]. The following research suggests that copper can be used as a novel selective target for cancer therapies. Daniel et al. [7] have demonstrated that CQ induces cell death in malignant cells by inhibiting the proteasome through a dual copper-dependent and -independent mechanism. In addition, Mao and Schimmer [8] also demonstrated that CQ delays the growth of tumors in mouse

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models of malignancy. Moreover, CQ has been used in the treatment of many types of human cancer, including prostate, breast, colon, lung, and brain cancer [9,10]. Thus, we believe that CQ may be a novel anti-oral cancer agent that could be repurposed for this new indication. CQ given directly to the oral cancer area is quickly diluted by oral fluid and cleared. To enhance the bioavailability of CQ for oral cancer treatment, we proposed GG/GS film as the CQ delivery carriers. In the future, the film can be used for oral cancer therapy application.

Nature polymers have attracted attention as matrix materials for controlled release [11,12] and gene delivery applications [13]. Polymers derived from microbial source [14–16] are excellent candidate biomaterials due to their exceptional biodegradability and biocompatibility. There are two major microbial macromolecules, polyesters and polysaccharides, are used in drug delivery [17] and other medical applications [18]. Gellan gum (GG) is an anionic heteropolysaccharide produced by *Sphingomonas elodea*. Based on its excellent biocompatibility and nontoxicity and its special physicochemical properties, gellan gum has been widely used as wound dressings and as drug delivery materials in previous studies. We previously utilized gellan gum as a delivery material for anti-inflammatory agents to prevent tissue adhesion in the postoperative abdomen. In addition, we prepared gellan gum films as wound dressings, demonstrated its good biocompatibility, and showed that it was capable of accelerating wound repair [19]. Due to the brittleness of gellan gum film fabricated through EDC (1-ethyl-3-(3-dimethylamino-propyl) carbodiimide) cross-linking [20], we used a mixture of gellan gum with glucosamine as the CQ delivery agent to improve the mechanical properties of gellan gum film. In this study, CQ can be incorporated via EDC-mediated covalent conjugation to gellan gum/glucosamine film (GG/GS/CQ/EDC film), the film of the physicochemical properties and drug release kinetics were studied [21,22]. Finally, we developed animal models for oral cancer and used this model to investigate the effectiveness of GG/GS/CQ/EDC film as oral cancer treatment patch.

2. Experimental methods

2.1. Materials

Gellan gum, Glucosamine hydrochloride, and MTT reagent were obtained from Sigma. 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) was purchased from Acros. Clioquinol was purchased from Alfa Aesar. Cell culture medium (DMED), 10× trypsin-EDTA, fetal bovine serum were purchased from Gibco. Zoletil 50 was obtained from Virbac. OCT-Polyethylene glycol was obtained from Leica. All chemicals used in this study were of reagent grade. The animal experiments in this study were approved by the Chung-Shan Medical University Experimental Animal Center. A rabbit anti-EGFR polyclonal antibody (St John's Laboratory; subclass IgG) was used at a 1:200 dilution. Immunohistochemistry (IHC) detection kits were purchased from Enzo Life Sciences (product: HighDef™ red IHC chromogen (AP)).

2.2. Fabrication of GG/GS/CQ/EDC film

The GG/GS/CQ film was fabricated by mixing 28 mL of an aqueous solution of gellan gum (1%), 7 mL of an aqueous solution of glucosamine (1%) and 50 mg of CQ (dissolved in 2 mL of DMSO) in a glass dish (diameter = 10 cm). This solution was evaporated at 37 °C and 1 atm for 3 days to obtain a dry GG/GS/CQ film. The GG/GS/CQ film was then cross-linked by immersing them into DDW (distilled deionized water) containing 15 mM EDC for 24 h at room temperature. The cross-linked films were washed with DDW three times to remove residual EDC and then dried at room temperature [23]. After crosslinking, the film was referred to as GG/GS/CQ/EDC, respectively.

2.3. Characterization of the GG/GS/CQ/EDC oral cancer treatment patch

We used an FTIR-L396A (Perkin-Elmer) to analyze the properties of the chemical functional groups of the GG, GG/CQ, GG/CQ/EDC, GG/GS/CQ and GG/GS/CQ/EDC films. The analysis of the gel content and water content of the GG/GS/CQ/EDC film was performed as follows. The GG/GS/CQ/EDC films were dried (2 cm × 2 cm pieces). The dry weight (Wd) was measured, and then the dried films were swelled in phosphate buffered saline (PBS) at 37 °C for 24 h. The wet weight (Ww) of the film was determined after wiping off excess water using filter paper. The film was dried again in an oven for 24 h at 50 °C, and its subsequent weight was recorded as Wrd. The gel content and water content ratio were calculated as follows [19]:

$$\text{Gel content}\% = (\text{Wrd}/\text{Wd}) \times 100$$

$$\text{Water content}\% = (\text{Wrd} - \text{Wd})/\text{Wrd} \times 100$$

2.4. Mechanical property measurements

GG/GS/non-crosslink, GG/GS/EDC, GG/GS/CQ/EDC films were cut into 1 cm × 5 cm pieces. We then used an H1-KS testing machine (Tinius Olsen) with a crosshead speed of 5 mm/min to measure the mechanical properties of these films and to automatically record the mechanical parameters.

2.5. In vitro release studies

In vitro drug release studies were performed in 15 mL tubes. The GG/GS/CQ/EDC films (1 × 1 cm^2) were placed into the tubes and immersed in 1 mL of phosphate buffer (0.02 M, pH 7.2). Samples ($n = 5$) were incubated at 37 °C with shaking for 45 days. At defined time points, 1 mL of the release buffer was withdrawn and replaced with fresh buffer. The CQ content was determined spectrophotometrically at 255 nm. The kinetics of CQ release from GG/GS/CQ/EDC film was determined by finding the best fit of the dissolution data to one of five distinct models as previously described: Zero-order, First-order, Second order Hixson-Crowell and Higuchi as follows [24,25] (Table 1):

2.6. Effect of CQ dose on cytotoxicity in oral cancer cells (OC-2)

Human oral cancer cells (OC-2) were plated in 96-well plates with 5000 cells per well. Following overnight incubation, cells were treated with 2, 5, or 10 μM of CQ with or without 10 μM of $CuCl_2$ for 24 h. DMSO-treated cells served as a control. To quantify the cell viability, the medium was replaced with 150 μL of medium containing 10% MTT (Sigma-Aldrich). After 1 h of incubation at 37 °C, the MTT solution in wells was removed, and the formazan crystals within cells were solubilized with 100 μL of DMSO [26]. The absorbance of each sample at 595 nm was measured by an enzyme-linked immunosorbent assay

Table 1
Mathematical models used to describe drug dissolution curves.

Zero-order	$Q_t = Q_0 + K_0 t$ where Q_t is the amount of drug dissolved in time t, Q_0 is the initial amount of drug in the solution (most times, $Q_0 = 0$) and K_0 is the zero order release constant expressed in units of concentration/time.
First-order	$\log Q_t = \log Q_0 - kt/2.303$ where k is the first order rate constant, and t is the time
Second order	$Q_t/Q_\infty(Q_\infty - Q_t)k_2 t$ where k_2 is the second order rate constant, Q_∞ is the amount of drug dissolved at infinite time
Hixson-Crowell	$Q_0^{1/3} - Q_t{}^{1/3} = k_s t$ where k_s is a constant incorporating the surface-volume relation.
Higuchi	$Q_t = k_H t^{1/2}$ where k_H is the Higuchi dissolution constant