Chung Shan Medical Journal 2018; 29:17-26

Original Article

Preparation and characterization of gellan gum film as a carrier of 5-Chloro-8-hydroxy-7-iodoquinoline (CQ) for oral cancer therapy

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In this study, gellan gum (GG) film was prepared with chemotherapy drug 5-Chloro-8-hydroxy-7-iodoquinoline (CQ) as an oral cancer treatment patch. Covalent conjugation of CQ and GG was via 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC)-mediation (GG/CQ/EDC film). The thickness of GG/CQ/EDC film was 22 ± 4 μm with 87.7% gel content and 96.1% water content. Mechanical testing revealed maximum tensile strength and elongation percentage at break of 1.98 kgf/mm^2 and 4.24%, respectively. Drug release experiment lasted for 45 days, and the amount of CQ released was 82.4%. Huguchi model fit the GG/CQ/EDC release data with high correlation coefficient (R^2=0.9990). From the results of animal testing, GG/CQ/EDC film can decrease epidermal growth factor receptor (EGFR) expression and suppress tumor progression. These findings provide insight into the potential use of GG/CQ/EDC film for oral cancer therapy in clinical practice.

Key words: Gellan gum, 5-Chloro-8-hydroxy-7-iodoquinoline, oral cancer treatment patch

Introduction

Oral cancer is the sixth most common cancer worldwide [1]. Treatment is dependent on stage of development. Stages 1 and 2 usually involve a small tumor, and cancer cells have not spread to the lymph nodes. Stages 3 and 4 are considered advanced stages. Tumors are large and cancer cells have usually spread to the lymph nodes or other parts of the body [2]. Chemotherapy and radiotherapy are usually used in the early stages. Surgery is the primary form of treatment in late-stage oral cancer. The aim of this study was to design gellan gum (GG) oral cancer treatment patch, with chemotherapy drug (5-Chloro-8-hydroxy-7-iodoquinoline, CQ), which can be used in early stages of oral cancer therapy and as wound care dressing after surgery in late stages of oral cancer therapy. CQ is a potent proteasome inhibitor and apoptosis inducer. Daniel et al. [3] demonstrated that CQ induces cell death in malignant cells by inhibiting the proteasome, through a dual copper-dependent and independent mechanism. In addition, Mao et al. demonstrated that CQ delays the growth of tumors in mouse models of malignancy [4]. CQ has been used in many types of human cancers, including prostate, breast, colon, lung, and oral cancers [5-7]. In addition, copper chelation has been shown to result in MMP-9 reduction in a murine model of multiple sclerosis with CQ and to protect against matrix degradation and cancer invasion [8].

CQ can be administered directly to the oral cancer area. It is quickly diluted by oral fluid and

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cleared. To enhance bioavailability of CQ for oral cancer treatment, we proposed GG as the CQ delivery carrier. In this study, GG polymer covalently conjugated with CQ via 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) mediation during fabrication of GG/CQ/EDC film as oral cancer treatment patch. In the future, this film may be applied to oral cancer therapy.

GG is an exopolysaccharide (EPS), also known as polysaccharide S-60. It is a gelling agent produced by a non-pathogenic strain of Sphingomonas elodea ATCC 31461 [9]. Natural GG is a linear electronegative EPS. The main chain of GG consists of four repeating carbohydrates, including two D-glucose carbohydrates, one L-rhamnose, and one D-glucuronic acid. Based on its excellent biocompatibility, nontoxicity and special physicochemical properties, GG has been widely used as wound dressing and for drug delivery [10-12]. Previously, we prepared GG film as wound dressing and demonstrated that it has good biocompatibility and is capable of accelerating wound repair [13]. The aim of this study was to develop GG as anti-cancer drug carrier. Dynamic release behavior of CQ from the GG film was analyzed in vitro. The anti-cancer capability of the GG/CQ/EDC film was evaluated in vivo.

Methods

2.1 Fabrication of the GG/CQ/EDC film

To prepare the GG/CQ film, 0.35 g of GG (Sigma G1910) were dissolved in 35 mL of deionized water (DDW) and heated at 85°C–90°C until a transparent solution was produced. CQ (50 mg) was dissolved in 2 mL of DMSO and mixed with GG solution. This mixture was poured onto a glass dish (diameter = 10 cm) and evaporated at 37°C and 1 atm for 3 days to obtain a dry GG/CQ film. The GG/CQ film was then cross-linked by immersing into DDW containing 15 mM of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma 1769) [13] for 24h at room temperature. The cross-linked film was washed with DDW three times to remove residual EDC and then dried at room temperature. After crosslinking, the film was expressed as GG/CQ/EDC.

2.2 Characterizations of GG-based films

We used FTIR-L396A (Perkin-Elmer) to analyze the properties of the chemical functional groups of GG, GG/CQ and GG/CQ/EDC films. Analyses of the gel content and water content of these GG-based films were performed as follows: The GG-based films were dried (2 cm x 2 cm pieces), weighed (Wd), and then swelled in phosphate buffered saline (PBS) at 37°C for 24 h. The wet weight (Ww) of the film was determined after removing excess water using filter paper. The film was dried again in an oven for 24 h at 50°C and weighed (Wrd). The gel content and water content ratios were calculated as follows [14]:

Gel content % = (Wrd/Wd) x 100

Water content %= 【(Wrd-Wd)/Ww】x 100

2.3 Mechanical property measurement

GG--based films were cut into 1cm×5 cm pieces. Then, H1-KS testing machine (Tinius Olsen) with crosshead speed of 5mm/min was used to measure the mechanical properties of these films and to automatically record the mechanical parameters [15].

2.4 In vitro release studies

In vitro drug release studies were performed in 15 ml tubes. The GG/CQ/EDC films (1x1 cm^2) were placed into the tubes and immersed in 1 ml of phosphate buffer (0.02 M, pH 7.2) with 0.1% tween 80. Samples (n = 4) 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. CQ content was determined spectrophotometrically at 255 nm. The kinetics of CQ release from GG film were determined by finding the best fit of the dissolution data to five distinct models, Zero-order, First-order, Second-order,Hixson-Crowell, and Higuchi, as follows [16,17]:

2.5 In vivo evaluation of GG/CQ/EDC film for oral cancer treatment

Male Syrian golden hamsters aged 4 weeks were obtained from the National Laboratory Animal Center (Taipei, Taiwan). A total of 10 male hamsters were randomized into two groups (control group and experimental group) of 5 hamsters each. Model of 7,12-dimethyl-1,2-benz[a]

Table 1. *Mathematical models used to describe drug dissolution curves*

Zero-order	$Q_t = Q_0 + K_0t$ 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	$Q_t/Q_\infty(Q_\infty - Q_t)\ k_2t$ where k_2 is the second-order rate constant, Q_∞ is the amount of drug dissolved at infinite time.
Hixson-Crowell	$Q_0^{1/3} - Q_t^{1/3} = kst$ where k_s is a constant incorporating the surface-volume relation.
Higuchi	$Q_t = k_Ht^{1/2}$ where k_H is the Higuchi dissolution constant.

anthracene (DMBA)-induced hamster cheek pouch carcinogenesis was modified from Salley[18]. The animals were treated by painting the entire mucosal surface of the left cheek pouch three times per week for 14 consecutive weeks with a 0.5% solution of DMBA dissolved in mineral oil. After 14 weeks, 100% oral tumor formation with severe histopathological abnormalities was observed. The tumor and surrounding tissue were removed. The wound was then covered with GG/CQ/EDC film. Hamsters in the control group were not given oral cancer treatment patch. The experimental hamsters were sacrificed on the 7th day after surgery. The injured site with film was removed and fixed in 10% formalin solution. The tissues were processed by the standard procedure for histological and immunohistochemical (IHC) analyses. In this study, we selected epidermal growth factor receptor (EGFR) expression as the marker for evaluating the growth of cancer. 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)).

3 Results and Discussion

3.1 FTIR characterization of GG-based films

Figures 1-3 are the FTIR spectrograms of GG, GG/CQ and GG/CQ/EDC films. In the FTIR spectrum of GG (Fig 1), the peaks at 3335, 2896, 1605, 1401 and 1015 cm^{-1} are attributed to the stretching vibrations of –OH, stretching aliphatic –CH, asymmetric COO^-, symmetric COO^- and hydroxylic C-O bonds, respectively [12]. In the FTIR spectrum of GG/CQ (Fig 2), CQ reveals one important peak at 1480 cm^{-1} assigned to the stretching vibration of C=C, C=N of heterocyclic aromatic ring [19,20]. The peak at 1203 cm^{-1} is due to the aliphatic C-N stretch in CQ. The N-H groups of CQ absorption stretching frequencies are at 3400-3250 cm^{-1} and overlap with the –OH of GG. The FTIR spectrum of GG/CQ via EDC crosslinking shows two new absorption peaks at 1720 and 1556 cm^{-1} (Fig 3). The absorption peak at 1720 cm^{-1} indicates that carboxyl groups on β-D-glucuronic acid (Glcp A) of GG generate an ester bond with –OH groups and confirms that the GG cross-linking reaction is successful. The absorption peak at

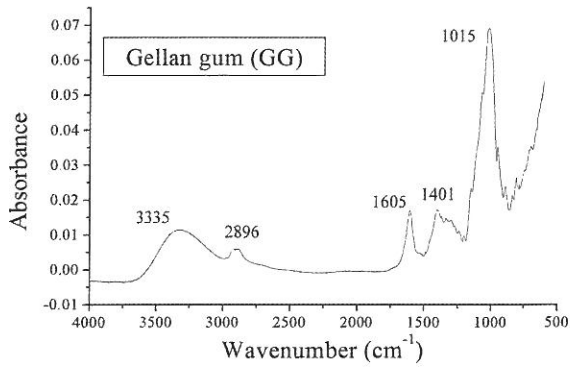

Fig. 1 FTIR spectra of GG film.

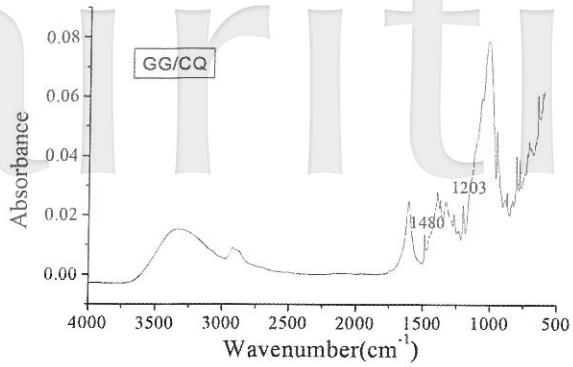

Fig. 2 FTIR spectra of GG/CQ film.

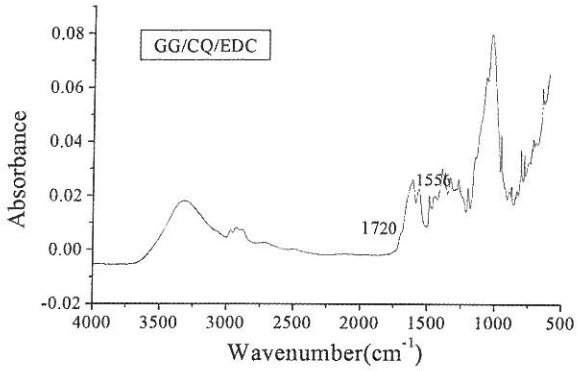

Fig. 3 FTIR spectra of GG/CQ/EDC film.

1556 cm^{-1} indicates that carboxyl groups on β-D-glucuronic acid (Glcp A) of GG generate an N=O bond with –NH groups of CQ and confirms that CQ covalent conjugation with GG is via EDC-mediation.[21]. Covalent bonding of CQ to GG carriers allows for release over extended time periods, which reduces drug loading dose and prevents adverse side effects.

3.2 Physical properties of GG-based films

By applying the EDC-mediated cross-linking process, we fabricated and studied the physical properties of GG-based films. The average thickness of these films was 22 ± 4 μm, and they were soft, flexible, transparent, and able to be fixed in position. The gel content of a film is related to its crosslinking density [22]. The gel contents of GG/EDC and GG/CQ/EDC films were 79.5±1.3 and 87.7±0.5, respectively. The results showed that CQ increases cross-linking of GG polymer, as CQ forms covalent bonds with GG, increasing interpenetrating polymer network structure formation. Water content is a basic property of polymer film. In general, the water content of a polymer film can be considered the sum of strong affinity water and weak affinity water in the film. A film with high water absorption may absorb excess water and expand, causing deformation and making it unsuitable as an oral cancer treatment patch. Table 1 shows the water contents of various GG-based films. With an increase in GG crosslinking, the capacity of water absorption is reduced. The capacity of water absorption of GG film is closely related to its porosity. Some studies have indicated that polymer films with higher porosity have increased water storage space and, thus, a higher capacity for water absorption [23]. In this system, CQ increased GG crosslinking. However, there was slight decrease in the water absorption capacity of the film. The addition of CQ to GG did not affect the porosity of the films, as CQ is of low molecular weight.

In clinical use, the most important mechanical properties of oral cancer treatment patches are tensile strength and elongation percentage. Mechanical testing revealed (Table 1) that the maximum tensile strength and elongation at break of the non-crosslinked GG film are 2.18 kgf/mm^2 (21.37 Mpa) and 3.54%, respectively. The maximum tensile strength and elongation at break of the EDC-crosslinked GG film were 2.61 kgf/mm^2 (25.59 Mpa) and 5.35%, respectively, representing increases in tensile strength and elongation. Vijayabaskar, Tikku, Bhowmick, and Anil [24] indicated that the tensile strength of a polymer closely correlates with density of crosslinking. Crosslinking causes increases in tensile strength and elongation [25]. As shown in Table 1, in the presence of CQ, tensile strength and elongation of the GG/CQ crosslinked film progressively decreased (1.98 kgf/mm^2 and 4.24%). CQ is a hydrophobic drug. The addition of CQ to GG hydrogel might decrease the hydrophilic susceptibility of GG, as well as tensile strength and elongation, resulting in mechanical resistance [26].

Oral cancer treatment patches are not currently

Table 2. *Physical properties of Gellan gum-based films.*

Sample	Gel content (%)	Water content (%)	Mechanical Test		
			Maximun force (gf)	Maximun strength (kgf/mm^2)	Elongation of break (%)
GG/non-crosslink	-	-	1312±6	2.18±0.01	3.54±0.53
GG/EDC	79.5±1.3	97.6±0.5	1571±63	2.61±0.10	5.35±0.45
GG/CQ/EDC	87.7±0.5	96.1±0.4	1192±70	1.98±0.11	4.24±0.58

available. Thus, there is no standard reference for their physical and chemical properties. In this study, we completed efficacy test and pharmacokinetic study of GG-based oral cancer treatment patch in animal model. The physical properties of the film can be used as a reference for future clinical applications.

3.4 In vitro release studies

The in vitro release studies for GG/CQ/EDC film were performed using phosphate buffer (0.02 M, pH 7.2) with 0.1% tween 80 as a representative medium. On a spectrophotometer, the amount of CQ released was measured by absorbance at 255 nm. Fig 4 plots the percentage release of CQ from GG/CQ/EDC film versus time. The experimental results showed that the amount of CQ released from GG/CQ/EDC increases with release time. After the release experiment, which lasted for 45 days, the amount of CQ released from GG/CQ/EDC film was 82.4%. CQ was directly administered to the oral cancer area, quickly diluted by oral fluid and cleared. In this study of CQ covalent conjugation with GG, the percentage of CQ released from the matrix was approximately 6-8% (8.0-10.8 μM) every other day with sustained release for up to 45 days.

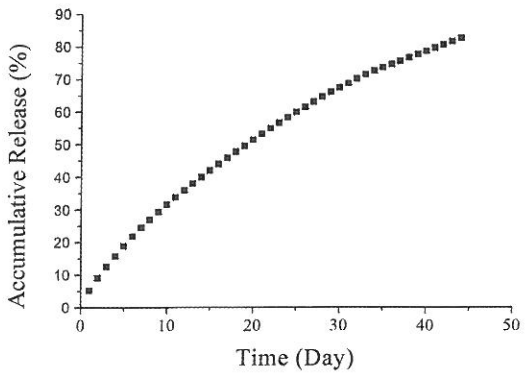

Fig. 4 Release profile of CQ from GG/CQ/EDC film in PBS at 37°C.

Drug release modeling and determination of the critical parameters of carrier systems are important for understanding and elucidating mechanical and drug transport properties. This system used GG as a carrier of CQ. GG is a biodegradable polysaccharide polymer with water-absorbing and swelling properties. Diffusion, swelling and erosion mechanisms are the most important rate-controlling mechanisms of controlled-release products. To better characterize drug release from polymeric systems, five common pharmacokinetics models were used to analyze the release kinetics of CQ from GG film. The model that best fit the CQ release data was selected based on correlation coefficient (R^2). The results are shown in Table 3. Results indicated that the Huguchi model fit

Table 3. ***The results of kinetic models applied to the release of CQ from GG/CQ/EDC film.***

	GG/CQ/EDC film
Zero-order	Fits linear equation y=0.06609+0.00860x R^2=0.9865
First-order	y=0.04382x-2.48046 R^2=0.8859
Second-order	y=13.194141-0.32624x R^2=0.6456
Hixson-Crowell	y=0.4348+0.00822x R^2=0.9354
Huguchi	y=0.07274x-0.06715 R^2=0.9990

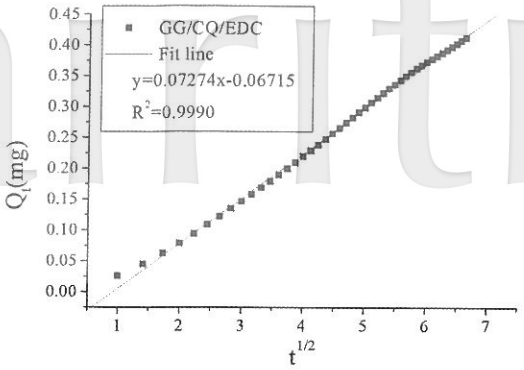

Fig. 5 Huguchi model fits the release of CQ from GG/CQ/EDC film.

the GG/CQ/EDC release data (Fig 5) with high correlation coefficient (R^2=0.9990). In 1961, the first example of a mathematical model for describing drug release from planar matrix system was proposed by Huguchi. This model is based on the hypotheses that (i) initial drug concentration in the matrix is much higher than drug solubility; (ii) drug diffusion takes place only in one dimension; (iii) drug particles are much smaller than system thickness; (iv) matrix swelling and dissolution are negligible; (v) drug diffusivity is constant; and (vi) perfect sink conditions are always attained in the release environment [27, 28]. Therefore, this study confirmed that CQ release from GG or GG/GS films is at a constant rate of diffusion, without burst effect, and these systems can achieve prolonged therapeutic action in vivo.

3.5 Histological and immunohistochemical analyses

EGFR is the cell-surface receptor for members of the epidermal growth factor family (EGF-family) of extracellular protein ligands [29, 30]. The EGFR signaling pathway is one of the most important pathways for regulating growth, survival, proliferation, and differentiation in mammalian cells. Overexpression of EGFR has been linked to oncogenic transformation, invasive growth, metastasis and angiogenesis in multiple cancers. The results of histological and immunohistochemical analyses for control (without treatment patch) and GG/CQ/EDC film-

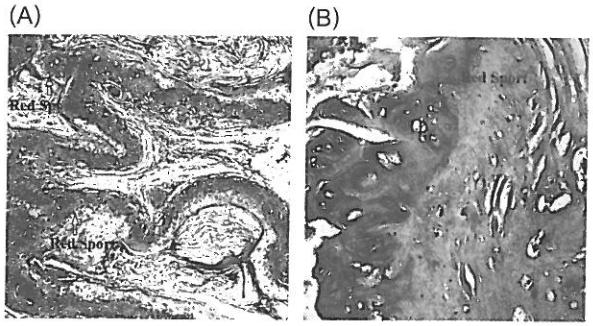

Fig. 6 Immunoreactivity of EGFR in (A) control (without treatment patch), (B) GG/CQ/EDC film-treated group.(200X)

treated groups are shown in Fig 6. Control and experimental groups presented with oral squamous cell carcinoma (OSCC) and cancer cells showed continual unregulated proliferation with infiltration into the subcutaneous tissue [31-33]. To determine the molecular mechanism of GG/CQ/EDC film for tumor growth inhibition, EGFR expression in a Syrian golden hamster model of cancer tissue was analyzed. In this study, cancer with EGFR positive immunoreactivity was labeled in red. In control group (Fig 6A), the immunohistochemical reactivity of EGFR significantly increased in the cell membrane and cytoplasm of oral squamous cells, but EGFR expression was lower in cancer cells in experimental group (Fig 6B). In this study, EGFR was shown to be a promising target for cancer therapy [34]. The anti-tumor activity of CQ was not due to the potentiation of inhibition of EGFR or downstream signaling of EGFR [35]. Rather, CQ markedly activated apoptosis in the tumor cells. From the results of animal testing, GG/CQ/EDC film can suppress OSCC progression. The results also confirmed that the release dose of CQ from GG film meets the requirement for in vivo applications.

Conclusion

Worldwide, the incidence of oral cancer is about 2% - 4% of all cancer cases with high death rate. In this study, GG/CQ/EDC film was prepared and tested on early-stage cancer and as wound care dressing after surgery in late-stage cancer. Developing GG-based patches as replacements for

chemotherapy and radiation therapy in oral cancer was not the main purpose of this work, but rather improving convenience of treatment involving cancer drugs and enhancing bioavailability of CQ for oral cancer therapy. The results showed that GG/CQ/EDC film possesses the capacity to inhibit oral cancer progression in animal model and the potential for clinical application.

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