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Gellan–thioglycolic acid conjugate: Synthesis, characterization and evaluation as mucoadhesive polymer

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ABSTRACT

Gellan–thioglycollic acid conjugate was synthesized with the objective to improve its mucoadhesive properties. Synthesis of conjugate was confirmed by –SH stretch in the Fourier-transform infrared spectra at 2571 cm^{-1}. It was found to contain 13.92 mM of thiol groups/g of the conjugate. Thiolation of gellan gum was found to slightly increase its degree of crystallinity and decrease its sensitivity to Ca^{2+}-induced gelation. On screening of gellan–thioglycollic acid conjugate for *ex-vivo* ocular tolerance using hen's egg chorio-allantoic membrane test and for biocompatibility by resazurin assay on Vero-cells, it was found to be non-irritant and biocompatible. Metronidazole gels formulated using gellan thioglycollic acid conjugate as bioadhesive agent showed 1.82-fold higher mucoadhesive strength than the gels formulated using gellan gum. Further, the metronidazole gels containing gellan and gellan–thioglycollic conjugate released the drug following first-order and Higuchi's square-root release kinetics. In conclusion, gellan–thioglycollic acid conjugate is a promising bioadhesive excipient.

1. Introduction

Natural gums and mucilages contain large number of hydroxyl, carboxyl and amino functional groups which because of their hydrogen-bond forming ability impart mucoadhesive characteristics on the natural polymers. Their mucoadhesive characteristic can be further improved by conjugation with covalent bond forming thiol-functional bearing compounds (Bernkop-Schnurch, Scholler, & Bieble, 2000). Thiolated polymers apart from improving the mucoadhesive properties have also been reported to improve cohesive properties (Bernkop-Schnurch et al., 2000), impart enzyme inhibitory capabilities (Bernkop-Schnurch & Thaler, 2000) and permeation enhancing effect on the polymer (Clausen & Bernkop-Schnurch, 2000, 2001). Gellan gum is an anionic exopolysaccharide secreted by *Pseudomonas elodea*. Gellan gum undergoes ionic gelation in the presence of cations. It has been used extensively as *in situ* gelling polymer in ophthalmic formulations (Rupenthal Ilva, Green, & Alany, 2011). During earlier study cysteine was conjugated to gellan gum and on the basis of rheological oscillatory measurements it was reported that gellan-cysteine conjugate improves the *in situ* gelling properties (Krauland, Leitner, & Bernkop-Schnurch, 2003).

In the present study thiol-functionalization of gellan gum was achieved by synthesizing gellan–thioglycolic acid conjugate (GTC). The synthesized GTC was characterized by differential scanning calorimetery (DSC), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and scanning electron microscopy (SEM). The number of thiol group substituents was determined by Ellman's method. GTC was screened for *ex vivo* ocular tolerance by hen's egg test on chorio-allantoic membrane (HET-CAM) and for biocompatibility by resazurin assay on Vero cell lines. Thiolated gellan gum was explored for pharmaceutical applications as mucoadhesive agent by formulating bucoadhesive gels using metronidazole as a model drug. The gels were prepared using Carbopol-974P as a gelling agent, and GG or GTC as the bioadhesive agent. The formulated gels were characterized mechanically by texture profile analysis for hardness, cohesiveness and adhesiveness. Mucoadhesive characteristics of formulated gels were determined by modified physical balance method using chicken ileum as the model membrane while the *in vitro* release study was conducted using dialysis membrane.

2. Materials and methods

2.1. Materials

Gellan gum (C.P. Kelcogel, UK, Gelrite®) was gifted by Burzin & Leons, Argenturon (Mumbai, India), Metronidazole was obtained

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as gift sample from GMH Lab Pvt. Ltd. (Baddi, India). Thioglycolic acid (99% AR) and hydrochloric acid were purchased from SD Fine-Chem. Ltd. (Mumbai, India). Ellman's reagent [5,5-dithiobis (2-nitrobenzoic acid)] (DTNB) was purchased from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India). Fresh isolated chicken ileum was procured from the local butcher shop (Hisar, India). Ten days old fertilized hen's eggs were procured as gift samples from Indovax Pvt. Ltd. (Hisar, India). Metronidazole gel (Metrogyl®, Lekar Pharmaceuticals, Ankleshwar, India) was purchased from the local pharmacy store.

2.2. Synthesis of gellan–thioglycolic acid conjugate (GTC)

Synthesis of GTC was carried out by reacting gellan gum with thioglycolic acid in the presence of hydrochloric acid (Kaur, Yadav, Ahuja, & Dilbaghi, 2012). Gellan gum (1 g) was dissolved in 50 ml water aided by stirring and heating. Thioglycolic acid (0.3 ml) and hydrochloric acid (0.3 ml, 7 N) was added to the above solution. This mixture was allowed to react for 3 h at 80 °C under reflux conditions. The reaction mixture was precipitated with methanol (100 ml) and further washing was done with methanol. The resulting precipitate was kept at −80 °C for 4 h, and further dried by lyophilization using lyophilizer (Freeze dryer, Alpha 2-4 LD Plus, Martin Christ, Germany) for 24 h at −90 °C, at 0.0010 mbar.

2.3. Characterization of GTC

2.3.1. Determination of thiol group contents

The contents of thiol groups in GTC were determined by Ellman's method as reported earlier, with slight modification (Sharma & Ahuja, 2011). A 0.5% (w/v) solution of gellan gum (as control) and GTC in aqueous sodium hydroxide (0.5%, w/v) was prepared, and further diluted with phosphate buffer (5 M, pH 8.0) to a concentration of 0.15% (w/v). An aliquot of 5 ml of the above solution was allowed to react with 5 ml of Ellman's reagent (0.3% w/v) for 2 h at room temperature, followed by measurement of absorbance of the reaction mixtures at 450 nm. The number of thiol group substituents per gram of GTC were determined using a calibration curve prepared by reacting standard solutions of thioglycolic acid with Ellman's reagent as detailed above.

2.3.2. Fourier transform infra-red spectroscopy (FT-IR)

The spectra of gellan gum and gellan–thioglycolic acid conjugate were recorded on a Fourier-transform infrared spectrophotometer (IR Affinity-1, Shimadzu, Japan). The data was recorded in the frequency region of 4500–400 cm^{-1}. Sample pellets were prepared with KBr.

2.3.3. Differential scanning calorimetery (DSC)

Thermal characteristics of gellan gum and gellan–thioglycolic acid conjugate were studied employing differential scanning calorimeter (Q 10 TA systems, USA) with nitrogen purge of 50 ml/min. The thermal curves were recorded over a temperature range of 40–250 °C at a heating rate of 10 °C per min employing cell constant calibration method. The calibration of the instrument was done with Indium, having a melting point of 158.26 °C and heat of fusion of 28.80 J/g.

2.3.4. X-ray diffraction (XRD)

The X-ray diffraction patterns were recorded using X-ray diffractometer (Table top XRD, Miniflex 2, Rigaku, Japan). The goniometer was a Miniflex 2 goniometer. The data were collected in the continuous scan mode using a step size of 0.02° (2θ). The scan range was 0–80°. Further parameters of the diffractometer were: Ni filtered Cu-Kα radiation; voltage 30 kV; tube current 15 mA; scan speed 0.05 min^{-1}.

2.3.5. Scanning electron microscopy (SEM)

The photomicrographs of gellan gum and gellan–thioglycolic acid conjugate were obtained using scanning electron microscope (SEMTRAC mini, Microtac, Inc.). The samples were mounted on stub containing double sided adhesive carbon tape. The electron micrographs were taken at an accelerating voltage of 20 kV.

2.3.6. Effect of cations on gelling behaviour of gellan–thioglycolic acid conjugate

Effect of calcium ions on gelling behaviour of gellan and gellan–thioglycolic acid conjugate was investigated employing partial ternary phase diagrams. Briefly, solution of gellan (0.1–1%, w/v) in water and gellan–thioglycolic acid conjugate (0.5–3.5%, w/v) in sodium hydroxide (0.25 N) were prepared. To the above prepared polymer solutions varying concentration of calcium chloride solutions were added and left overnight. The test tubes were observed for their consistency by tilting them at 90° and classified as solutions, viscous solution or gels on the basis of their visual appearance (Rupenthal Ilva et al., 2011).

2.3.7. Ex-vivo ocular tolerance (HET-CAM) study

Gellan gum and gellan-thioglycolic acid conjugate were evaluated for ocular tolerance by estimating their potential irritation in the chorioallantoic membrane (CAM) of hen's egg. The extent of potential irritation caused by the polymer (GG/GTC) in the chorioallantoic membrane of egg was the basis of the study (Luepke, 1985). The potential irritancy was estimated by observing the changes (haemorrhage, vasoconstriction and coagulation) occurring in the membrane within 5 min of application of the formulations (Kaur, Ahuja, Kumar, & Dilbaghi, 2012). HET-CAM study was done on 10 days old fertilized and incubated hen's egg. After 10 days incubation at 37 ± 0.5 °C, the shell was removed. The formulations were added (in triplicate) to the membrane and left in contact with CAM for 5 min. The CAM was examined for irritation effects and potential irritation (PI) was calculated by recording the onset time for each irritation effect.

$$\mathrm{PI}(\%) = \frac{(301-h)\times 5}{300} + \frac{(301-v)\times 7}{300} + \frac{(301-c)\times 9}{300} \quad (1)$$

where, h = onset time (s) for haemorrhage, v = onset time (s) for vasoconstriction, c = onset time (s) for coagulation. The range of PI score were classified as 0–0.9: non-irritant; 1–4.9: slight irritant; 5–8.9: moderate irritant; 9–21: severe irritant.

2.3.8. Cytotoxicity screening

Cytotoxicity of GG and GTC was screened employing Vero cell lines using resazurin assay method. Briefly, Vero cells were placed in 96-well plates in a density of 1×10^5 cells in DMEM (Dulbecco's modified eagle media) culture media having 5% FBS (foetal bovine serum) and incubated for 24 h at 37 °C in 5% CO_2 humidified incubator (Iqbal et al., 2012). Proliferation of cells occurred after 24 h, which were then viewed under microscope. Fifty microliters of samples of GTC and GG (0.05%, w/v) were added to the above cells and incubated for another 24 h at 37 °C and 5% CO_2 incubation. After 24 h, 20 μl of resazurin (1 mg/ml, in DMEM) was then added to each well and were incubated for 4 h at 37 °C and 5% CO_2 humidified incubator. After 4 h the plate was observed in ELISA spectrophotometer at 573 nm. Cytotoxicity (%) was calculated as follows

$$\%\mathrm{Cytotoxicity} = \frac{\mathrm{Abs_u} - \mathrm{Abs_t}}{\mathrm{Abs_u}} \times 100. \quad (2)$$

where $\mathrm{Abs_u}$ is the absorbance of cells not treated with any polymer, $\mathrm{Abs_t}$ is the absorbance of cells treated with GG or GTC.