Crosslinked Chitosan/PVA Film, Saturated with 5-Fluorouracil for The Prevention of Proliferative Vitreoretinopathy

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ABSTRACT
5-Fluorouracil (5-FU)-loaded chitosan (Ch) film for chemotherapy were prepared applying a superhydrophobic surface-based encapsulation technology. The aim of this study was to develop polymeric film with glutaraldehyde (GA) of controlled drug delivery systems for 5-FU as a model drug for the treatment of proliferative vitreoretinopathy. Polymer film of chitosan and polyvinyl alcohol (PVA) in 75:25 ratios were prepared and treated with GA. FTIR spectra of 5-FU, Ch/5-FU and Ch/PVA film loaded 5-FU were studied. Physical characteristics such as thickness and swelling coefficient of the film were performed. The thermal of the Ch/PVA film was studied with thermogravimetric analysis. The drug loading efficiency, film size and chemical compositions of the film loaded drug were confirmed by UV–vis spectrophotometer and Fourier transform infrared spectroscopy. In vitro release kinetics of drug from the polymeric films was investigated to determine the drug release properties. In vivo study of PVR was showed the efficacy and no toxicity of this formulation. Further uses of the film loaded 5-FU-fluorouracil may provide an efficiency deliverable for ophthalmic administration.

Keywords: 5 - fluorouracil, chitosan, polyvinyl alcohol, glutaraldehyde, proliferative vitreoretinopathy

INTRODUCTION
5-Fluorouracil (5-FU) is a cytotoxic agent that is a pyrimidine analog known for its widespread use as potentially effective drugs against postsurgical proliferative vitreoretinopathy. However, due to toxicity its clinical use is limited substantial evidence has demonstrated that 5-FU could also harness the host immune system to prevent cancer progression. The ability of 5-FU to reduce fibroblast proliferation and subsequent scarring has made it one of the most often used antimitaboles in ophthalmology1-5. Chitosan (Ch) is a widely used polysaccharide in pharmaceutical formulations due to its combination properties such as bioactivity, biocompatibility, biodegradability, water binding capacity and nontoxicity6,7. Chitosan has shown many applications in medicine, biomaterials and drug controlled release systems8-11. Poly vinyl alcohol (PVA) is a water-soluble synthetic polymer. Because of the film-forming ability, biocompatibility, nontoxicity, high hydrophilicity and good chemical stability the studies on diffusive permeability of solutes in PVA films have been reported12,13. Intermolecular interaction between chitosan and PVA in the blends and Ch/PVA blend has good mechanical properties have been reported14-18. The preparation methods of Ch/PVA films treated with glutaraldehyde (GA) have been studied19. In this study the blended films were characterized by FTIR. The thermal

of the Ch/PVA film was studied with thermogravimetric analysis (TGA). The aim of the present work is to investigate release of 5-FU from crosslinked Ch/PVA film, the properties and structures of the films.

MATERIALS AND METHODS
Experimental
Chitosan 70% deacetylation, with molecular weight of 3.5-250 kDa was purchased from Sigma-Aldrich (USA), sodium hydroxide (NaOH), hydrochloric acid were used without further purification. PVA with molecular weight of 90 kDa was purchased from Sigma-Aldrich (USA) and was used without further purification. Pharmaceutical grade fluorouracil - 5-fluoro-2,4-(1H, 3H)-pyrimidinedione - was produced by "Grindeks" (Latvia). Sodium chloride, potassium chloride and calcium chloride was purchased from Biopharma (Ukraine). Glutaraldehyde and distilled water were used in this experiment. Glutaraldehyde was used as cross-linked agent.

Preparation of Ch/PVA blended blank film
Initially 1,5% w/w of chitosan solutions were prepared in 1% (v/v) hydrochloric acid and 8% PVA solutions were prepared by dissolving in 80°C distilled water with stirring for 4-5 h. The weight ratio between Ch and PVA is 75:25. This solution was further cross-linked with 0.05
Table 1: The experimental groups and periods of enucleation.

<table>
<thead>
<tr>
<th>Type of surgery</th>
<th>Group</th>
<th>7th day</th>
<th>14th day</th>
<th>28th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD - partial pars plana vitrectomy + polymer film loaded 250 mg/g of 5-FU</td>
<td>1 main</td>
<td>3 eyes</td>
<td>3 eyes</td>
<td>3 eyes</td>
</tr>
<tr>
<td>OS - partial pars plana vitrectomy + polymer film loaded 500 mg/g of 5-FU</td>
<td>2 main</td>
<td>3 eyes</td>
<td>3 eyes</td>
<td>3 eyes</td>
</tr>
<tr>
<td>Intravitreal 19 G knife injured</td>
<td>control</td>
<td>2 eyes</td>
<td>2 eyes</td>
<td>2 eyes</td>
</tr>
</tbody>
</table>

Table 2: 5-FU release from the cross-linked Ch/PVA film.

<table>
<thead>
<tr>
<th>Total volume, ml</th>
<th>Time, h</th>
<th>Concentration, µg/ml</th>
<th>Amount of 5FU taken, µg</th>
<th>Amount of 5FU missing in the sample, µg</th>
<th>Amount found, µg</th>
<th>Total amount of 5FU, µg</th>
<th>Cumulative release of 5FU, %</th>
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</thead>
<tbody>
<tr>
<td>70</td>
<td>1</td>
<td>26,749</td>
<td>0</td>
<td>0.000</td>
<td>1872,43</td>
<td>1872,43</td>
<td>11.534</td>
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<tr>
<td>70</td>
<td>5</td>
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<td>53,498</td>
<td>3428,25</td>
<td>3481,746</td>
<td>21.457</td>
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<tr>
<td>70</td>
<td>10</td>
<td>108,963</td>
<td>97,95</td>
<td>53,498 by 97,95</td>
<td>7627,41</td>
<td>7778,858</td>
<td>47.039</td>
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<tr>
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<td>15</td>
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</table>

Figure 1: TGA curves for the crosslinked Ch/PVA films

wt% of GA. The film was cast onto glass moulds by casting method and solvent evaporation technique. Prepared film was characterized for swelling coefficient. Polymer composite film was carefully removed from the cup and placed in a sterile plastic bag. The dry films were immersed in 5% NaOH solution to neutralize hydrochloric acid residues, and then washed with ethanol to remove excess NaOH. After rinsing with excess distilled water, the films were air dried for 24 h. Prepared film was characterized for swelling coefficient. Implants of size 8 × 1 × 0.35 mm³ were formed from polymer film.

Thickness uniformity test
Film thickness was measured using Micrometer (MK 102, Russia), with the unit measured count of 0.01 mm.

Thermal properties of the films
Thermal properties of the films were investigated by thermogravimetric analysis (TGA) on the instrument Mettler Toledo TGA/SDTA 851 (Switzerland). TGA was carried out in the temperature range from 50 to 800°C at a heating rate 5°C/min.

Fourier transform infrared spectroscopy (FTIR) measurement
FTIR spectra of samples palletized with KBr were analyzed at room temperature using a Nicolet 5700 IR spectroscopy (USA). The peak variation of adsorption between 4000 and 500 cm⁻¹ were detected.

Design of in vivo tests
Biomedical tests were conducted in accordance with the order of the Chairman of the Pharmaceutical Control of Health Ministry of RK from 13 November 2013 protocol No. 2 "On approval of the list of recommended drugs to conduct biomedical tests". 10 chinchilla rabbits (range of weight from 2.5 to 3.5 Kg) at the age of 4-6 months were used. Surgery was performed on the operating microscope "LOMO". Series of histologic cuts were carried out with hematoxylin and eosin staining, according to...
Wang – Gizon and threechrome Masson methods. The paraffin sections were tested with a microscope Leica DM4000 using a phase-contras filter, followed by photo graphing with camera Leica DFC320.

Evaluation of in vitro 5-FU release studies
In order to determine the prolonged properties in vitro the release of 5-FU from cross-linked Ch/PVA films was investigated. The drug release was determined using UV/VIS spectroscopy (Evolution 300, USA) at 266 nm in quartz cuvettes with thick of 10 mm at 37°C in Ringer-Locke solution. Ringer-Locke solution - a standard isotonic solution 6.5 g NaCl, 0.42 g KCl, 0.25 g CaCl₂ and 1 mole of sodium bicarbonate is dissolved in one Liter of distilled water.

Design of in vivo tests
The research was approved by ethical committee of Semey State Medicine University. Implantation into the intravitreal implant of Ch/PVA film loaded 5-FU was produced in amounts of 250 mg/g and 500 mg/g, with deducing them from the experience with subsequent enucleation in different periods (7th, 14th and 28th days). Distribution eyes of experimental animals in groups and period enucleation was presented in Table 1.

RESULTS AND DISCUSSION

Thermal properties of the films
In figure 1 the TGA curves for the crosslinked Ch/PVA films were shown. The initial decomposition temperature of Ch/PVA films was at about 295 ± 5 °C. Compared with the Ch/PVA film to the Ch/PVA film treated with GA, there was no significant effect on initial decomposition temperature. The maximum peak temperature of the crosslinked Ch/PVA films shifted from about 300°C to 400 °C. The maximum decomposition temperatures of the crosslinked Ch/PVA films are higher than films without GA treatment. The TGA curves level off at about higher than 500°C. Compared to the Ch/PVA films, the wt. % loss was lower for the crosslinked Ch/PVA films.
crosslinking with GA would result in the increase of the maximum decomposition; whereas the thermal stability of Ch/PVA film is reduced when it is crosslinked with GA.

**IR spectra analysis**

FTIR spectra of 5-FU, the crosslinked Ch film loaded 5-FU and the crosslinked Ch/PVA film loaded 5-FU were displayed in Figure 1a, b, c, respectively. The main peaks were observed in 5-FU spectrum at 3084 cm$^{-1}$ (N–H stretching), 1689 cm$^{-1}$ (C=O stretching), 1250 cm$^{-1}$ (C=C stretching) and 1180 cm$^{-1}$ (C–F bond) (Fig. 2a). The absorbance at 3127 cm$^{-1}$ in the Ch film loaded 5-FU spectrum showed the peaks of the O–H stretching from chitosan and the N–H stretching from 5-FU that superimposed with each other. The C=O stretching at 1683 cm$^{-1}$ which occurred in Ch film spectrum also revealed the presence of Ch and 5-FU that the lie over each other. The absorbance at 1247 cm$^{-1}$ (C=O stretching) and at 1172 cm$^{-1}$ (C–F bond) detected the occurrence of 5-FU in film spectrum (Fig. 1b). In the Ch film treated with GA the amount of –NH$_2$ group on the film decreased, which resulted from the reaction between GA and –NH$_2$ group, and the N–C bond changed into N=C bond. The FTIR spectrum of Ch/PVA film loaded 5-FU (Fig. 1c) showed the absorption peaks at about 3134 cm$^{-1}$ the O–H stretching from chitosan, the O–H stretching from PVA and the N–H stretching from 5-FU that superimposed with each other and at about 1664 cm$^{-1}$ for the C=O group of PVA which occurred in Ch/PVA film spectrum also revealed the presence of Ch, PVA and 5-FU that the lie over each other. Figure 1c also illustrated the effect of GA on the chemical structure of the Ch/PVA film. A decrease in the absorption peaks at OH and C–O groups in PVA found; whereas a strong absorption peak at 1090 cm$^{-1}$ for the –C–O–C group was shown. The overall results confirm the synthesis of the Ch/PVA film loaded 5-FU.

**The results of in vivo tests**

When studying influence of chitosan film, saturated with 5-FU, on eye structures, in terms from 7 to 28 days the macroscopic changes in both main groups wasn’t revealed. Distinctions were revealed at histological research of enucleated eyes. In both groups the lack of inflammatory reaction is noted, there was no formation of a connective tissue and macrophages. Toxicity neither in the first, nor in the second main groups morphologically wasn’t confirmed (there is no lysis of an internal boundary membrane). In the second main group the lysis of erythrocytes, which left the vascular course during operation, was quicker and without development of connective tissue and inflammation. In the first main group a small proliferative activity was noted, in the second was absent.

**Evaluation of in vitro FU release studies**

In vitro release kinetics of 5-FU from the crosslinked Ch/PVA film was investigated to determine the drug release properties. It was established that the release consisted of three main stages: water sorption by a film and its swelling, the drug diffusion in a film at the phase interface “polymer-system-environment” and the drug diffusion in the solvent volume. To determine the influence of drug loading on its release kinetics, polymer films were loaded with 250 mg/g and 500 mg/g of 5-FU. Compared with 250 mg/g to 500 mg/g of 5-FU, there was no significant effect on cumulative release of drug. Obtained results showed that the drug was diffused practically completely into Ringer-Locke solution within 60-70 h (Fig.4). The obtained release parameters are presented in Table 2. A novel Ch/PVA film treated with GA was prepared by direct blend process and solution casting method. This investigation studied the properties of the crosslinked Ch/PVA films loaded 5-FU in amounts of 250 mg/g (0.05 ml) and 500 mg/g (0.1 ml). The effects of stock Ch/PVA concentration, the treatment of GA on the structure and thermal property of Ch/PVA films were characterized with FTIR and TGA. The FTIR analysis confirmed the successful introduction of 5-FU in film. From the results it was concluded the thermal stability of the films increases with the treatment of GA. The evaluation of the permeation of 5-FU through the chitosan was conducted in this study. In vitro drug release could be resulted that the drug was diffused from the crosslinked Ch/PVA films after 70 h. According to results of morphological research, toxic action from intravitreal injection of chitosan film, saturated with 5-FU (0.05 and 0.1 ml) in ex-
experiment wasn’t observed. Some proliferative activity was revealed after implantation of the chitosan film saturated with 0.05 ml 5-FU.

**DISCLOSURE**

There are no potential conflicts of interest related to the contents of the article. No any pharmaceutical agency was involved in the study design, collection, management, analysis, interpretation of the data, writing of the manuscript, and decision to submit the manuscript for publication.

**ACKNOWLEDGEMENTS**

This work was supported by Institute of Chemical Sciences of the Republic of Kazakhstan and R. Cimdins Riga Centre for Biomaterial Innovation and Development, Riga Technical University. Biomedical tests of obtained films were carried out jointly with the staff of the National scientific surgery center of A. N. Syzganov of MHC RK, Almaty, Kazakhstan and Kazakh Science Research Institute of Eyes Disease, Almaty, Kazakhstan.

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