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Formulation and Evaluation of Chitosan-Gellan Based Methotrexate Implants

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An implant controlled-release system for methotrexate delivery based on a polyion complex composed of chitosan and gellan was investigated. Multi-layered implant was prepared by using poly(vinyl alcohol), gellan and chitosan. Two chitosan layers sandwiched the poly(vinyl alcohol)-gellan layer, which acted as a methotrexate reservoir. The prepared implant was evaluated for swellability, *in vitro* and *in vivo* release and biodegradation studies. The equilibrium swelling and methotrexate release was found to depend on a concentration of calcium chloride, which was used as a crosslinking agent for gellan. Drug-loaded implants were subcutaneously implanted in the back of Wistar rats. The *in vivo* studies showed that methotrexate was released slowly for a period over 30 days and also there was no fibrous capsule formation around the implant indicating the biocompatibility of the implant.

Keywords: gellan; chitosan; biocompatibility; degradation; drug delivery

1 Introduction

Polymeric implants are one of the attractive devices for targeted drug delivery (1). The polymeric matrix can protect drugs from conditions that could degrade or render the drug in the body inactive. Therefore, various polymers have been investigated in order to obtain an ideal drug delivery system that would allow ease of incorporation of drugs without affecting their bioactivity, delivery to the target site at a desired rate and exhibit biocompatibility when in contact with the tissue. Gellan gum is a high molecular weight polysaccharide gum produced as a fermentation product by a pure culture of *Pseudomonas elodea* (2). The production organism is an aerobic, gram-negative bacterium, which has been very well characterized and demonstrated to be non-pathogenic. Chemical structure of the polysaccharide has been determined. It has a tetrasaccharide repeat unit consisting of two glucose residues, one glucuronic acid residue, and one rhamnose residue. It has been shown to produce three-dimensional networks in the presence of cations, either monovalent or divalent, due to the formation of coordinates by cross-linking with these cations. The apparent viscosity of the gellan gum dispersions can be markedly increased by an increase in both pH and cation concentration (3). Furthermore, its ingestion never produced adverse dietary, physiological or toxic effects in animals and humans. These properties make this polysaccharide suitable for several commercial applications, such as in the food industry and in drug delivery (4). Formation of polyionic complexation (PIC) between cationic chitosan and anionic gellan gum, which has carboxyl functional groups composed of tetrasaccharide repeat units comprising 1,3-β-D-glucose, 1,4-β-D-glucose and 1,4-α-L-rhamnose has been reported (5).

Methotrexate (MTX) is an anti-neoplastic agent, which acts as an anti-metabolite of folic acid. It also shows immunosuppressant properties, and may be given by mouth or injection as MTX sodium for treatment of various types of cancer for several months (6). Moreover, slow release of MTX is useful, because it's time effect (the sensitivity of cells to this drug increases with time) is greater than its dose effect. This slow release for several months can be achieved by providing the drug in the form of MTX implants. The objective of this study is to develop a gellan film device as an implant for MTX delivery. The procedure for the preparation of gellan film was optimized. The physical properties of the films were studied, and the *in vitro* and *in vivo* release characteristics and *in vitro* and *in vivo* degradation of the implants were investigated along with histopathological studies.

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2 Experimental

2.1 Materials

The Methotrexate was a kind gift from Biochem, Mumbai; Gellan, Polyvinyl alcohol (average molecular weight range 1,24,000–1,86,000 and degree of hydrolysis 99%), Chitosan (medium molecular weight, 75–85% deacetylated), Calcium chloride ($CaCl_2$), Potassium dihydrogen phosphate, sodium hydroxide and sodium chloride were obtained from SISCO Scientific Laboratories, Mumbai India.

2.2 Preparation of Chitosan-PVA-gellan Implant

The compositions of various formulations are given in Table 1. 10 ml of 0.5% w/v chitosan solution (in 0.2 M acetic acid) was poured into a glass mold (area 28.3 cm^2) and dried at 25°C under vacuum for 48 h. PVA was dissolved in water by heating to 90°C, to which gellan and glycerin (2% v/v) was added and stirred until a clear solution was obtained. To this solution, MTX dissolved in pH 7.4 phosphate buffered saline (PBS) was added at 25°C and stirred gently. The solution was then poured over the chitosan film in a glass mold and dried at 25°C under vacuum for 24 h. Over this layer, 10 ml of 0.5% w/v of chitosan was poured and was allowed to dry at 25°C under vacuum. The dry formulations were cut into 10 mm circular discs. The disc implants weighed 62 ± 0.47 mg and had a thickness of 1.036 ± 0.142 mm. The amount of MTX in each implant was 9.95 ± 0.17 mg (Figure 1).

2.3 Swelling Studies

Swelling studies were carried out for all formulations using phosphate buffered saline (PBS, pH 7.4). Implants were weighed to determine the initial dry weight and placed in 50 ml of PBS at 37 ± 0.5°C. At 15 min intervals, implants were removed, gently wiped with a tissue to remove surface water, weight was recorded, and then placed back into the vessel as early as possible. The mean weights were determined for each formulation and the degree of swelling (S) was calculated according to the relationship: $S = W_s - W_d/W_d$, where W_d and W_s are the dry and wet swollen implants weights, respectively at immersion time t in the buffer. The swollen degree was the mean value of three measurements (7).

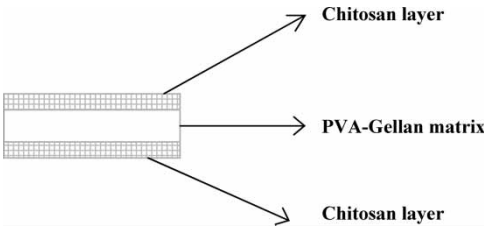

Fig. 1. Illustrative drawing of chitosan-gellan based methotrexate implant.

2.4 Scanning Electron Microscopy Studies

The surface morphology of dried implants was determined using a scanning electron microscope (JEOL 6320). The implant samples were mounted on the base plate and coated with gold using vapor deposition techniques. The surface was then scanned using a magnification of 500× and 1000×.

2.5 Differential Scanning Calorimetry

DSC analysis was performed on a DSC Dupont 9900, differential scanning calorimeter with a thermal analyzer. About 100 mg of the powdered sample was placed in a sealed aluminum pan, before heating under nitrogen flow (20 ml/min) at a scanning rate of 10°C min^{-1}, from 50°C to 400°C. An empty aluminum pan was used as reference.

2.6 In Vitro Drug Release Studies

The *in vitro* release of MTX from the implants containing 20 mg of the drug was carried out in screw-capped vials containing 50 ml phosphate buffered saline (PBS, pH 7.4). The vials were placed in an incubator shaker bath at 37 ± 1°C at a speed setting of 25 cycles per min (8). Samples were withdrawn at different time (24 h) intervals, filtered and analyzed for drug content spectrophotometrically at 303 nm.

Table 1. Formulation chart for methotrexate implants

Ingredients	F1	F2	F3	F4	F5	F6	F7
Methotrexate (g)	1	1	1	1	1	1	1
Gellan (%w/v)	2	1.5	1	2	2	2	2
Polyvinyl alcohol (%w/v)	4	4	4	4	2	4	4
Glycerin (%v/v)	15	15	15	15	15	15	15
$CaCl_2$ (%w/v)	0.1	0.1	0.1	0.05	0.1	0.15	0.2
Distilled water (ml)	20	20	20	20	20	20	20

2.7 Mathematical Analysis of Water Uptake and Drug Release

Analysis of the swelling behavior of implants in PBS of pH 7.4 was carried out using the equation:

$$M_t/M_\infty = 1 - 8/\pi^2 \exp(-\pi^2 Dt/4\delta^2) \text{ for } 0.4 < M_t/M_\infty < 1 \quad (1)$$

where D is the water diffusion coefficient, δ the half thickness of the implant, M_t the amount of water uptake at time t and M_∞ is the water uptake at equilibrium stage (9). Diffusion coefficient of MTX through the implants was calculated from Equations (1) and (2). Where D is the diffusion coefficient of MTX, δ the half thickness of the implant, M_t the drug release at time t and M_∞ is the amount of drug in the implant.

$$M_t/M_\infty = 4(Dt/\pi\delta^2)^{1/2} \quad \text{for} \quad 0 < M_t/M_\infty < 0.6 \quad (2)$$

2.8 Enzyme Mediated Biodegradation Studies

The implants were placed in 10 ml PBS (pH 7.4, 37°C) containing lysozyme enzyme (1 mg/ml). The PBS was changed for all the samples every day. Implants were taken out at 7, 14, 21 and 28 days washed with distilled water and air dried for 72 h. The resulting dry weights were recorded. The mass loss of the samples was determined by gravimetry (10).

2.9 In Vivo Drug Release Studies

Twelve Wister rats weighing around 300 ± 20 gm were obtained from the animal department of J.S.S. Medical College, Mysore which operates according to the requirements relating to the animal regulations. The rats were kept on a 12 h light/dark schedule, fed standard rat food and had free access to water. *In vivo* MTX release from the formulations F6 and F7 was studied. Implants were sterilized by gamma irradiation using a ^{60}Co source, at 32 kGy before implantation (11).

For each formulation, six rats divided into 2 groups were used. In the first group, rats were implanted with the formulation F6, and for the second group, with the formulation F7. The rats were anesthetized by an injection of Ketamine 80 mg/kg body weight. After anesthetizing, the back of the rats were shaved, and the surgery carried out for two groups with one group kept as control. A single incision, 1.5–2 cm long, was made on their backs; blunt-scissor dissection was then used to create a lateral implant site by tunneling immediately beneath the skin. The implant was then inserted a distance from the incision and sutured. The implants were inserted a distance from the incision, then the wound was closed in a standard surgical fashion (12). The *in vivo* drug release was estimated by excising the implant at predetermined time intervals, extracting the drug with PBS pH 7.4 and analyzing at 303 nm spectrophotometrically. The difference in the amount of the drug in the implants prior to the implantation and after *in vivo* release was calculated to determine percentage release of drug.

2.10 In Vivo Biodegradation Study

For *in vivo* biodegradation study, 16 Wistar rats weighing 260–290 gm were divided into three groups of 4 rats each, and were used to test biodegradability of the implant four times (7, 14, 21, 28 days). The implants were surgically placed subcutaneously as described in *in vivo* release studies. At regular intervals of time, rats were anesthetized and the implant was collected carefully, and then sonicated in 1% aqueous Triton X-100 detergent (sigma) for 10 min, rinsed twice in distilled water and in 70% ethanol solution to remove the cells. All implants were dried in vacuum to constant weight. The mass remaining was determined gravimetrically. Biodegradability of the implants was calculated using the equation:

$$\text{Degraded weight percent} = (W_1 - W_2)/W_1 \times 100\% \quad (3)$$

where, W_1 is the initial weight, W_2 is the final weight after degradation studies.

3 Results and Discussion

3.1 Determination of Swellability

Primarily three mechanisms could be responsible for the release of drugs from the hydrogels: swelling, diffusion and degradation. First, following exposure to an aqueous media, the polymer swells, due to uptake of the water. The rate of water uptake by the implant depends on the hydrophilicity of the polymer. Second, when the implant swells, the encapsulated drug is released by diffusion through the pores formed due to swelling. The third mechanism, which involves degradation of the polymer matrix, would occur under *in vivo* conditions as a result of enzyme activity.

The effect of crosslinking agents on the swellability of the polymer could be explained by the diffusion coefficient of water in the implant system. Moreover, the rate of drug delivery from an implant also depends on the rate of diffusion of water front into the device. It is known that the greater the

Table 2. Parameters A, k_2 and diffusion coefficient of water from swelling studies

Formulations	A	$k_2 \times 10^{-3}$ (min^{-1})	R^2	$D \times 10^{-6}$ (cm^2/min)
F1	0.25	3.1	0.992	7.34
F2	0.32	3.5	0.997	7.84
F3	0.41	5.3	0.986	8.65
F4	0.28	3.3	0.979	10.25
F5	0.21	2.8	0.985	6.92
F6	0.19	2.6	0.973	6.68
F7	0.28	6.5	0.991	5.74

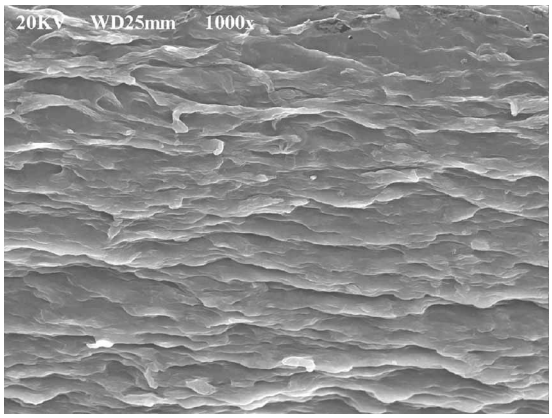

Fig. 2. Cross section of implant before swelling.

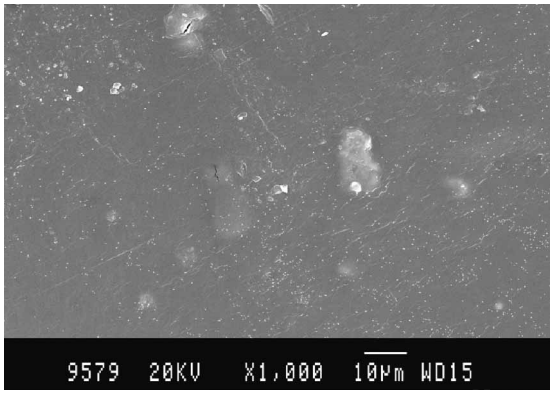

Fig. 4. Surface view of the implant before coating with chitosan.

molecular size/weight of the drug, the greater the sensitivity of the diffusion coefficient to changes in crosslink density. The swelling degree of the implants was found to be in the range of 1.24–1.62. The data obtained from the swellability assessment were fitted into the Berens–Hopfenberg differential equation $M_t/M_\infty = \{1 - A \exp(-k_2 t)\}$, to assess the overall kinetic mechanism governing the drug release. In this equation, M_t is the swelling at time t and M_∞ is the equilibrium swelling. The constants A and k_2 were calculated from the slopes and intercepts of the plot of $\log(1 - M_t/M_\infty)$ vs. time t at times later than those corresponding to $M_t/M_\infty = 0.6$. The calculated values of A and k_2 are listed in Table 2. The values of A were in the range of 0.19–0.41 indicating Fickian diffusion. Diffusion coefficient for the water transport studies was calculated by plotting $\log[\pi^2/8(1 - M_t/M_\infty)]$ v.s t for $0.4 < M_t/M_\infty < 1$ Equation (2). The slope of the plot was $\pi^2 D/2.303 \times 4\delta^2$ from which the diffusion coefficient D was calculated (Table 2). The diffusion coefficient of water through the implant was found to depend on $CaCl_2$ concentration. The maximum diffusion coefficient value was observed with implants prepared with $CaCl_2$ concentration of 0.05% w/v. When the crosslinker concentration was increased from 0.05% w/v (F4) to 0.1% w/v (F1) the diffusion coefficient of water through the implant decreased from 10.25×10^{-6} to $7.34 \times 10^{-6}\ cm^2\ min^{-1}$ and to $5.74 \times 10^{-6}\ cm^2\ min^{-1}$ when $CaCl_2$ concentration was increased to 0.2% w/v (F7). This may be explained by the fact that on increasing the crosslinker content there is a prominent decrease in the free volumes available between the chains of the macromolecular network and thus the swelling of implant decreases. The crosslink density of implant provides a restricted aqueous environment for diffusional migration of the MTX, by controlling both the degree of hydration and the permeability of implant to MTX (13).

3.2 Scanning Electron Microscopy Studies

The surface morphologies of the PVA/gellan implants were revealed by SEM (Figures 2 and 3). The SEM photographs clearly showed a difference in the microstructure of the swollen and dry state of the cross section of the implant. This provides structural proof for the hypotheses we made regarding release of MTX from the implant systems due to swelling of the polymer. Figures 4 and 5 show the surface morphology of the uncoated and chitosan coated gellan-PVA implant.

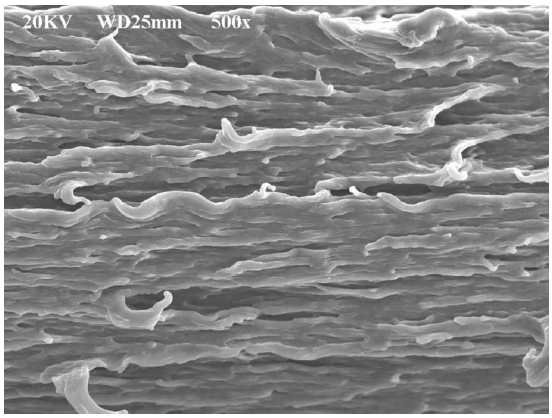

Fig. 3. Cross section of implant after swelling.

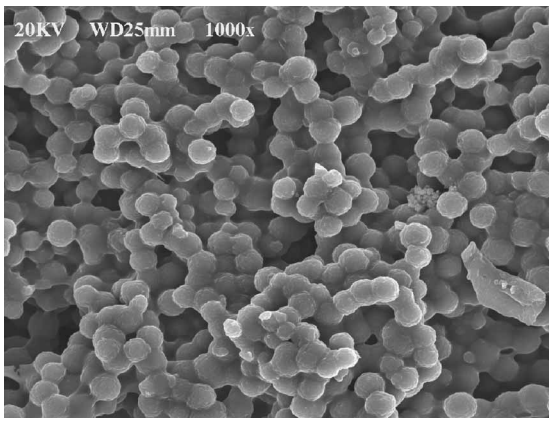

Fig. 5. Surface view of the implant after coating with chitosan.

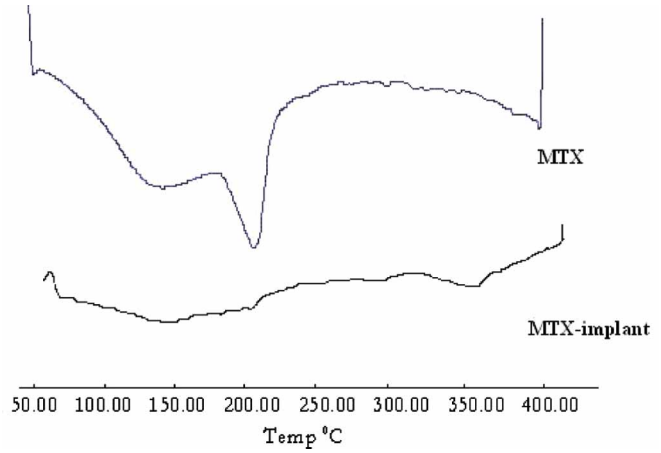

Fig. 6. Thermograms of pure drug methotrexate (MTX) and the formulation containing methotrexate (MTX-implant).

3.3 Differential Scanning Calorimetry

DSC thermogram of MTX showed three endotherms (Figure 6). The first two endotherms appearing at 95 and 120°C are associated with loss of free and bound water, respectively and the third endotherm at 212°C corresponds to the melting peak of MTX. These thermal characteristics of MTX implied that commercially obtained sample was a hydrate form (14). However, no characteristic peak of MTX was observed in DSC curves of the drug-loaded implant, suggesting that drug was molecularly dispersed in the polymer matrix.

3.4 In Vitro Drug Release Studies

The formulation F4 showed complete release of MTX in 15 days, whereas the formulation F1 showed complete release in 18 days whereas, F2, F3 and F5 showed complete release of MTX in 24 days. Formulations F6 and F7 showed release upto 28 days. Obviously, this infers that, the release depended on the $CaCl_2$ concentration in the formulation. Increase in $CaCl_2$ retarded the rate of MTX release. The *in vitro* release profiles of the various formulations are shown in the Figure 7. The fraction of MTX released less than 0.6 ($M_t/M_\infty < 0.6$) was fitted to the equation $M_t/M_\infty = kt^n$, where M_t is the MTX released at time t, M_∞ is the amount of MTX in the implant, k is the characteristic constant of the polymer, and 'n' is the characteristic exponent describing the penetration mechanism. For planar geometry, the value of $n < 0.5$ indicates a Fickian diffusion mechanism, for $0.5 < n < 1.0$ indicates non-Fickian or anomalous transport, and $n = 1$ implies case II (relaxation controlled) transport. The constant 'n' and 'k' was calculated from the slope and intercepts of the plots of $\log(M_t/M_\infty)$ vs. log t, respectively. The calculated value of n was in the range of 0.58–0.86, which indicated that the release of MTX followed non-Fickian or anomalous transport. This suggests that the release of MTX from the implant was controlled by a swelling of the matrix followed by water penetration into the implant.

Table 3. Diffusion coefficient data for in vitro drug release for different formulations

Formulations	Diffusion coefficient ($cm^2\ min^{-1}$)	
	Initial stage	Later stage
F1	5.92×10^{-6}	4.87×10^{-8}
F2	6.29×10^{-6}	4.96×10^{-8}
F3	6.42×10^{-6}	5.12×10^{-8}
F4	7.92×10^{-6}	5.63×10^{-8}
F5	5.65×10^{-6}	4.45×10^{-8}
F6	5.21×10^{-6}	3.85×10^{-8}
F7	4.45×10^{-6}	2.86×10^{-8}

The diffusion coefficient of MTX was calculated using Equations (2) and (3) and is shown in Table 3. Diffusion coefficient of MTX decreased when the concentration of $CaCl_2$ was increased due to the fact that with an increase in the cross-link density, the network becomes more compact. It is likely that the increase in the compactness restricts the mobility of

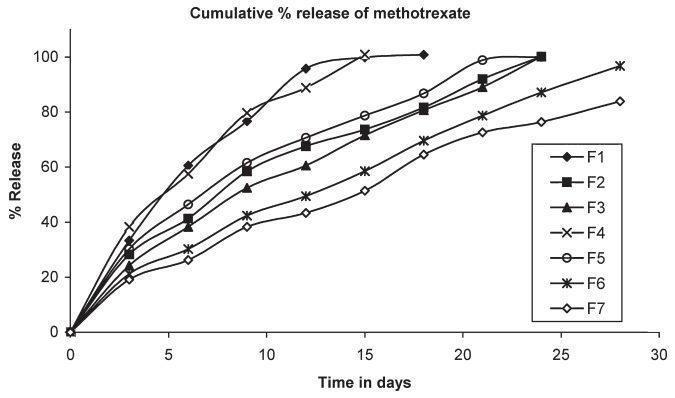

Fig. 7. *In vitro* release profile.

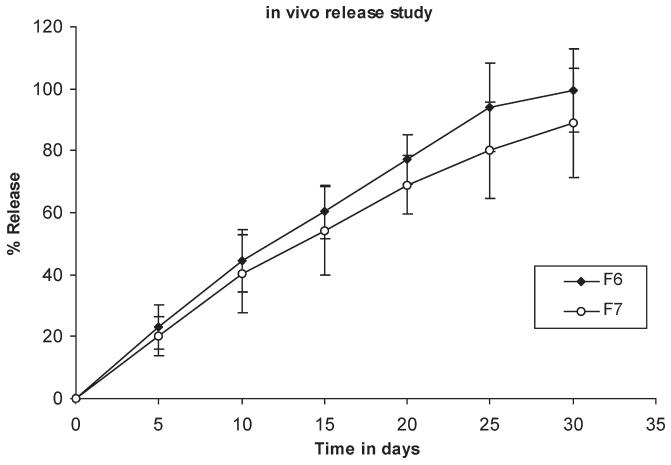

Fig. 8. *In vivo* release profiles of formulations F6 and F7.

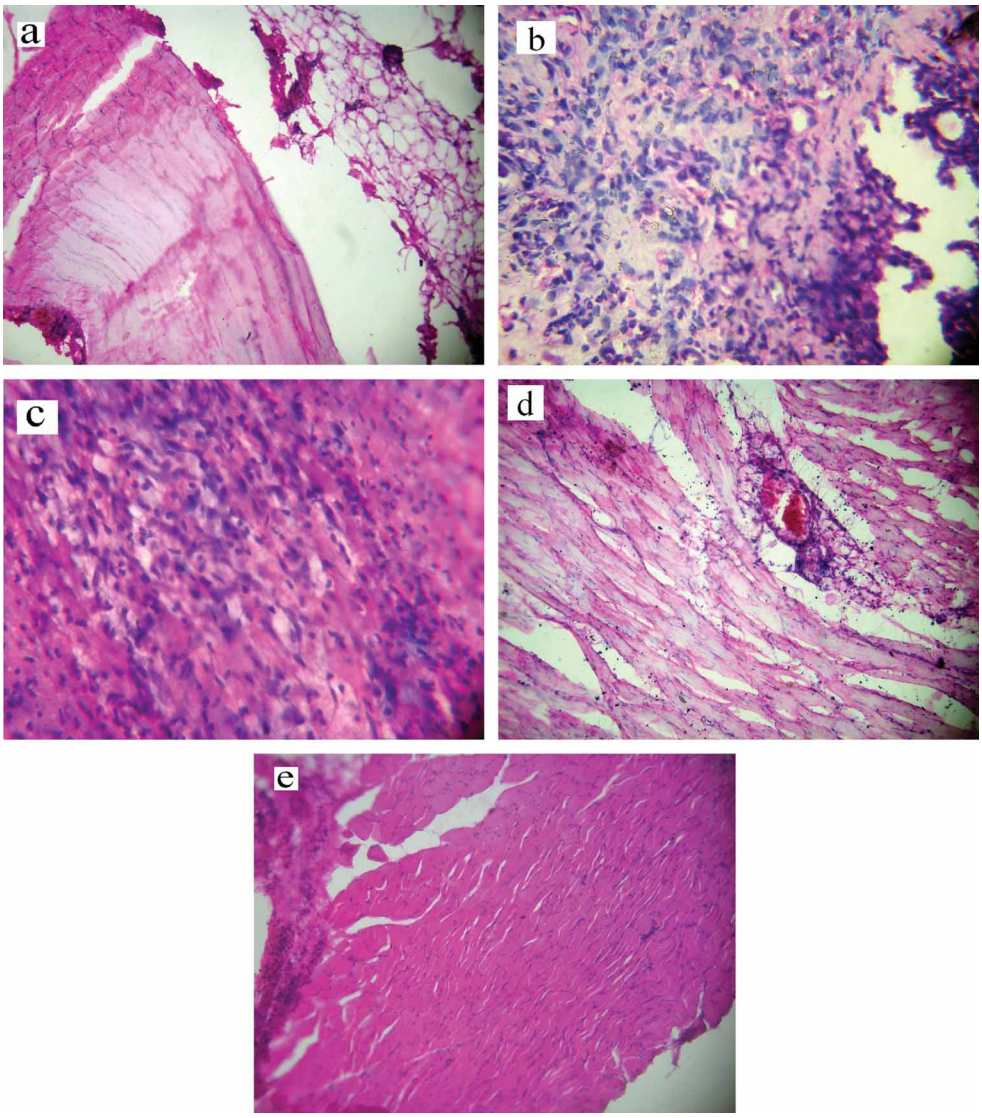

Fig. 9. Histopathology of the tissue; (a) control (b) formulation F6 after 1 week, (c) after 2 weeks, (d) after 3 weeks, (e) after 4 weeks of observations.

the network chains (chain relaxation) and the diffusion of the MTX from the implant into the release medium.

3.5 In Vivo Drug Release Studies

In vivo methotrexate release for two optimized formulation F6 and F7 were studied. Two groups, each containing six animals were used for the study. In the first group, rats were implanted with the formulation F6 and for the second group with the formulation F7. The *in vivo* drug release was estimated by excising the film at predetermined time intervals, extracting the drug with PBS pH 7.4 and analyzing at 303 nm spectrophotometrically. The difference in the amount of drug prior to the implantation and after the release was calculated. From the results of the *in vivo* drug release, it was observed that the drug release was slightly faster *in vivo* as compared to the *in vitro* release for the corresponding formulations. The reason may be due to the influence of enzymes or other *in vivo* factors. The formulation F6 showed complete release at the end of 30th day as compared to the formulation F7 which showed a 89% release. The results are shown in Figure 8. The 'n' value was of 0.94 for F6 formulation and 0.91 for F7 formulation indicating anomalous behavior (non-Fickian release, relaxation controlled). From the results of diffusion coefficient, it was observed that, the diffusion coefficient in the early stage was in the range of 8.16×10^{-6} $cm^2\,min^{-1}$, and at later stages, it was found to be 3.35×10^{-8} $cm^2\,min^{-1}$ for formulation F6 and for the formulation F7 it was 6.0×10^{-6} $cm^2\,min^{-1}$ at early stages and 1.63×10^{-8} $cm^2\,min^{-1}$ at later stages.

To evaluate the biocompatibility of the implants, the implantation site was observed, and the surrounding tissue was harvested for histopathological analysis. Macroscopic

evaluation of the implantation site revealed no inflammatory reaction (redness or swelling). After 30 days, the animals were sacrificed and the implants recovered to study the biocompatibility of the implant. The implant was surrounded by a capsule of collagen filaments. This capsule was thin and only one small area was characterized by a strengthened filament. The capsule has to be understood as a reaction to the mechanical irritation caused by the implant (15). The polymer itself can be considered very compatible with living tissues (Figure 9 (a, b, c, d, and e). The inflammatory cells like monocytes, lymphocytes, plasma cells, eosinophil and basophiles were in higher number during the first and second week, later, there was a decrease in the number of inflammatory cells during the third and fourth week.

3.6 In Vitro and In Vivo Degradation Study

It is known that chitosans with block structures and lower degrees of deacetylation (<75%) are more readily biodegraded due to the presence of blocks of glucosamine moieties containing acetyl groups that serve as a substrate for lysozyme. In the present study, we used chitosan (75–85% deacetylated) that has been shown to degrade *in vivo* in about 6 months (16).

The *in vitro* degradation studies showed that there was significant ($p < 0.001$) weight loss of the implants when placed in PBS pH 7.4 containing lysozyme enzyme indicating the degrading nature of the implants. The degradation studies were carried out for 4 weeks and the % wt. loss of the implants was found to be 20.83 ± 2.08. When compared to in vitro degradation study, in vivo degradation study showed much higher weight loss of the implant (28.32 ± 5.67), which can be attributed to the presence of other degrading enzymes at the site of implantation.

4 Conclusions

The studies provide validity for the potential utility of Chitosan coated PVA/Gellan multilayered implant system for the delivery of MTX. Both *in vitro* and *in vivo* results indicate that MTX release is slow from implants. The results of the in vitro release of MTX from the implants illustrated that percent release rate decreased proportionally to increase in $CaCl_2$ concentration. Chitosan coated PVA/Gellan multilayered implants can represent an effective delivery system for the sustained release of MTX. Moreover, the potential for toxicity is low because of the extended period over which the release occurs. Also the implants showed good biocompatibility, which is essential for implant based sustained delivery systems.

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