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Gelatin hydrogels: enhanced biocompatibility, drug release and cell viability

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Abstract Biodegradability and enhanced biocompatibility with pH-sensitivity of hydrogels are becoming very important issues for biomaterials applications so as to minimize the host-body reactions such as, inflammatory, antigenic, and immunogenic problems. This study involves development of hydrogel matrices of gelatin conjugated/modified with highly hydrophilic, pH-sensitive and biocompatible polymer, poly (2-ethyl-2-oxazoline) and glyoxylic acid respectively. Various compositions of gelatin conjugated/modified with poly (2-ethyl-2-oxazoline) (gp) and glyoxylic acid (gg) were synthesized. The swelling kinetics, cell viability and drug release capability from the gels at pH 4.5 and 7.4 were investigated. The results of swelling kinetics showed that, both the degree of swelling (DS) and the maximum degree of swelling (MDS) increased as function of modification (increase in modification) and pH with an increase of time, which is due to increase in ionic groups. The drug-release (1% chlorhexidine) studies at pH 7.4 and 4.5 confirmed a proportional drug release with an increase in degree of swelling. The results of in-vitro cytotoxicity tests using mouse embryonic 3T3 fibroblast cells indicated, an improved cell viability for gelatin gels conjugated/modified with poly (2-ethyl-2-oxazoline) (gp) and glyoxylic acid (gg) gels, when compared with 1% glutaraldehyde cross-linked gelatin gels (gx). Hence, cross-linked gelatin gels can be replaced with gp/gg for potential use in biomedical applications as a matrix for drug delivery.

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1 Introduction

Hydrogels with improved hydrophilicity are attracting increased attention lately because of their potential in biomaterials and biomedical applications [1–5]. Amongst the various polymers, poly (ethylene glycol) (PEG) has been extensively used due to its biocompatible, hydrophilic, non-toxic, non-antigenic, and non-immunogenic properties [2, 6–8]. From the literature survey, it was observed that a degradable, pH sensitive polymer, poly (2-ethyl-2-oxazoline) (PeOz), is least explored for biomedical applications [9–13]. The hydrophilicity and biocompatibility of gelatin hydrogels has been improved with the conjugation of poly(2-ethyl-2-oxazoline) for the applications in drug delivery.

Similarly, the excellent properties of glyoxylic acid [14] have motivated me to conduct an investigation on the hydrogels of gelatin modified with glyoxilic acid. The glyoxilic acid is a natural product present in fruits, leaves, and sweet beet. The present article reports the synthesis, characterization, swelling behavior and cell viability for conjugated/modified gelatin gels with dialdehyde derivatized PeOz/glyoxylic acid. The release of antibacterial drug, chlorhexidine from the above mentioned hydrogels has been investigated.

The properties, such as swelling (DS and MDS), cell viability and drug release were recorded and compared with the control gel i.e., cross-linked gelatin loaded with (Dgx) and without drug (gx).

2 Materials, characterization and methods

2.1 Materials

2-ethyl-2-oxazoline (eOz), glyoxylic acid, glutaraldehyde, and sodium cynoborohydride ($NaCNBH_3$) were obtained from ACROS Organics, Belgium. Gelatin (300 bloom), trinitrobenzenesulfonic acid (TNBS), 20% chlorhexidine digluconate and trypan blue were procured from Sigma Chemical Co., USA and 1,4 dibromo butene from Aldrich. Mouse embryonic 3T3 fibroblast cells (Cell No. CCRC 60063) purchased from FIRDI, Taiwan. (Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, trypsin-EDTA, penicillin, and streptomycin, were obtained from GIBCO, Grand Island, NY. All other reagents were of analytical grade.

2.2 Characterization

The infrared spectrum for PeOz and PeOzDA were recorded from 4,000–1,000 cm^{-1} using Bio-Rad FTS 155 spectrophotometer (USA). The KBr discs were made using 1−2 mg of the sample/100 mg of KBr. ^1H and ^{13}C NMR spectra were recorded on a Bruker AM-500 NMR spectrometer. Chloroform-d ($CDCl_3$) was used as a solvent for recording ^1H and ^{13}C NMR spectra. For ^1H NMR spectra, 30–50 mg of the polymer was dissolved in 1 mL of chloroform-d. And for ^{13}C NMR spectra, 100–150 mg of the polymer was dissolved in 1 mL of chloroform-d.

Molecular weight of PeOz was determined using Gel Permeation Chromatography (GPC). Measurements on GPC were performed using a Macherey-Nagel NUCLEOGEL with a refractive index detector using columns 2 × 300/7.7 NUCLEOGEL® GPC 100–5 (300 mm length, and 7.7 mm i.d). HPLC grade *N*, *N*-Dimethyl formamide was used as a mobile phase at a flow rate of 1.0 mL/min. For analysis, 25 µL at a concentration of 0.5–0.7% w/v of PeOz was dissolved in acetonitrle and injected using 220-µL syringe. The column was calibrated with poly (methyl methacrylate) standards. The percent of conjugation/modification of lysyl residues of gelatin with dialdehyde derivatized PeOz and glyoxylic acid was determined using trinitrobenzenesulfonic acid (TNBS) method [15, 16]. Estimation of unmodified amino groups in gelatin (protein) and the amount of the drug (chlorhexidine) released were determined using Perkins–Elmer UV/Vis Spectrometer Lambda 2S at 415 and 285 nm respectively.

2.3 Methods

2.3.1 Synthesis of poly (2-ethyl-2-oxazoline) (PeOz)

Synthesis and characterization of PeOz was performed according to the reported procedure [17, 18]. To 1–4-dibromo-2-butene (1.75 g), under dry nitrogen atmosphere, acetonitrile (75 mL), and 2-ethyl-2-oxazoline (24.5 g) were added and refluxed with stirring for 14 h. The reaction was terminated by cooling the reaction mixture to room temperature and with the subsequent addition of 0.1N aqueous potassium hydroxide. Acetonitrile was removed using rota-vapor, and the crude precipitate was dissolved in minimum amount of acetone. This concentrated solution was precipitated in diethyl ether and vacuum dried. The obtained pure product was analyzed using GPC, NMR and FTIR.

2.3.2 Synthesis of poly (2-ethyl-2-oxazoline)-dialdehyde (PeOzDA)

The PeOzDA was synthesized using the procedure reported earlier [19]. To 1.9 mL (20 mM) of acetic anhydride, 2.0 g (1 mM) of PeOz dissolved in 10 mL of dry DMSO was added and stirred at room temperature. After 2 h of stirring, the reaction mixture was precipitated in 100 mL of diethyl ether. The obtained crude product was re-dissolved in minimum amount of methylene chloride and re-precipitated in 100 mL diethyl ether and vacuum dried. The obtained pure product was analyzed using FTIR and NMR spectroscopy.

2.3.3 Conjugation of gelatin with poly (2-ethyl-2-oxazoline)-dialdehyde (gp)

Conjugation of gelatin with PeOzDA was done according to the reported procedure [20]. To 1 mM gelatin solution at 60–70 °C, aqueous solutions of 1 mM of PeOzDA (10 mL) and 7 mM of $NaCNBH_3$ (10 mL) were added simultaneously with two dropping funnels. After 24 h of stirring at 70 °C, the reaction mixture was dialyzed and lyophilized to obtain the dry product. Various percent of PeOzDA conjugation with gelatin was achieved by increasing mole ratio of PeOzDA. The percent of PeOzDA conjugation with gelatin was estimated by trinitrobenzenesulfonic acid (TNBS) method [15, 16].

2.3.4 Modification of gelatin with glyoxylic acid (gg)

Modification of gelatin with glyoxylic acid was performed according to reported procedure [20]. About 5 g of gelatin was dissolved in 150 mL of double distilled water. To this solution, 0.78 g of glyoxylic acid was added and stirred for half an hour. While stirring, 0.56 g of sodium cyanoborohydride was added drop wise and after the addition, the stirring was continued for 24 h. The reaction was terminated and the reaction mixture was dialyzed and

lyophilized to obtain the dry product. The percent of lysyl (amino groups) modification was estimated by TNBS method [15, 16].

2.3.5 *Quantification of conjugated/modified lysyl residues using TNBS method*

The extent of modification of gelatin (protein) with PeOzDA/glyoxylic acid was determined by quantification of free lysyl groups (free ε-amino groups) using TNBS method in triplicates as described earlier [15, 16]. This method of analysis is restricted for proteins only. To 0.5 mL of 1% gelatin solution (with and without conjugated/modified), 4% $NaHCO_3$ and 0.2 mL of TNBS (12.5 mg/mL) solutions were added. After incubation of the solution mixture at 40 °C for 2 h, 3.5 mL of concentrated hydrochloric acid was added and left the solution mixture at 110 °C for 3 h. The solution mixture was cooled and made up to the volume of 10 mL; it was extracted twice with equal amount of ether. The aqueous solution was separated and left at 40 °C to remove the traces of ether. The absorbency of the resulting yellow solution was measured at $\lambda = 415$ against blank. The amount of reactive lysyl residues for with and without conjugated/modified gelatin (protein) was determined using an equation:

$$\text{Optical density} \times \text{number of dilutions}/1.5 \times 10^7/\text{one mole of protein} = \text{amountofmodifiedlysylresidues}$$

2.4 Preparation of gels with drug (D) or without loaded drug

Ten percent hydrogels of cross-linked gelatin (gx), conjugated or modified gelatin with PeOzDA (gp), and glyoxylic acid (gg) were prepared by dissolving the respective amount of the polymers in de-ionized water at 100 °C. The dissolved homogeneous polymer solutions were poured onto individual petri dishes and allowed to set overnight. After setting the gels, small circular discs were punched and cross-linked with 1% glutaraldehye solution. After 6 h of cross-linking, the discs were washed thoroughly with de-ionized water to eliminate surface glutaraldehyde and dried them to constant weight. Gels loaded with drug such as Dgx, Dgp, and Dgg were prepared by allowing the respective dry gels to swell in 1% aqueous solution of chlorhexidine for 24 h. Later the drug-loaded gels were washed with de-ionized water to remove the surface drug and dried to constant weight. The various gel compositions of with or without loaded drug are listed in Table 1.

Table 1 Gel formulations with or without loaded 1% chlorhexidine. D is drug loaded gx is cross-linked gelatin; gp is gelatin conjugated with PeOzDA; gp is modified gelatin with glyoxylic acid

Sample no	Sample code	Conjugation/modification of gelatin (%)
1	gx/D gx	0
2	25 gp/D 25 gp	25
3	35 gp	35
4	48 gg/D 48 gg	48

2.4.1 *Estimation of total amount of the drug loaded in the disc*

The total amount of the drug loaded in the disc was determined as follows. A drug-loaded disc was weighed and crushed into fine powder. The crushed powder was subjected to soxhlet extraction with water at 100 °C for 48 h. The extractions were collected and made to standard volume and measured the optical density of the drug at λ 285 using UV/Visible spectrophotometer. The total amount (M_∞) of the drug present was calculated using a standard curve of optical density vs. concentration of the drug (mg/mL).

2.4.2 *Swelling kinetics*

Swelling studies for all the gels were carried out in de-ionized water, and in phosphate buffers of pH 9.4, 7.4 or 4.5, respectively, at 37 °C. The dried circular discs were weighed and transferred into the swelling medium; the swollen weights were recorded at regular intervals of time until the gels reached an equilibrium swelling. During weighing, the excess solvent on the disc surface was removed gently with Kim Wipes [21]. For all gels, swelling studies were done in duplicates.

The degree of swelling (DS) at time 't' and the maximum degree of swelling at equilibrium (MDS) were calculated according to the equations given below:

$$\text{DS} = W_t - W_i/W_i \text{ and } \text{MDS} = W_{\max} - W_i/W_i$$

where, W_i, the dry weight of a gel and W_t, the weight of a swollen gel at time t respectively. $W_{\max}$, the maximum weight of a swollen gel.

2.4.3 *In-vitro drug release studies*

Drug release studies for all the gels were performed in duplicates in phosphate buffers of pH 7.4 or 4.5 at 37 °C. Exact amount of chlorhexidine released into the buffer at intervals of time was monitored using UV/Visible spectrometer by measuring optical density of the drug at λ 285.

Dried drug loaded samples were transferred into 5 mL buffer solution; at intervals of time the swollen gel was pressed gently with Kim Wipes, weighed and transferred into fresh buffer. This process was repeated until the gels achieved zero amount of release. The concentration of drug in the buffer solution was obtained from the calibration curve, and the fraction of drug release was calculated using an equation $M_t/M_\infty = Kt^n$, where M_t/M_∞ is the fraction of the drug released at time 't' and at infinity, '∞' (the total amount of the drug loaded prior to drug release), k is the constant and n is the index. Gels without drug were used as controls to observe the interference of the optical density read out as a result of any unreacted glutaraldehyde and/or soluble gelatin.

2.4.4 *Extraction of solvent fraction and sterilization of hydrogels*

Before conducting the cytotoxicity studies, the respective hydrogels were subjected to soxhlet extraction. The extraction was done for 48 h using water as a solvent. This was done to remove the uncross-linked homopolymer and unreacted glutaraldehyde. The extracted hydrogels were dried at room temperature, autoclaved and used for further studies.

2.4.5 *In-vitro cytotoxicity studies*

The biological property such as, cytotoxicity for the various gel compositions were determined using mouse embryonic fibroblast 3T3 cells. Per well 2.5×10^5 cells were cultured in 6-well cultured plates and incubated at 37 °C in the 5% CO_2 incubator for 8 h, removed the medium (Dulbecco's Modified Eagle Medium (DMEM) with 10% calf serum, 1% L-glutamine, and 1% penicillin/streptomycin) and washed the attached cells twice with PBS (phosphate buffer solution) and calculated the cell number to be 3×10^5 per well. The hydrogels (25gp, 48gg and gx) of (0.01 g/10 mL) were allowed to swell separately in aqueous medium for 3 days. From the respective pre-swollen hydrogel medium, 1.5 mL of the medium was added to each well of 6 well-cultured plate containing 3×10^5 cells. After addition the 6 well-cultured plate was incubated for 3 days in 5% CO_2 incubator at 37 °C. The cell viability per day was estimated using cell counting assay method [22]. One of the well without gel medium was used as a control. All the studies were done in triplicates.

2.4.6 *Statistical analysis*

Statistical analysis was performed for data using a program of student t-test. Each experiment was repeated twice in duplicates. Statistical significance was determined at $p \leq 0.05$ for all data analysis.

3 Results and discussion

3.1 Synthesis of poly (2-ethyl-2-oxazoline) (PeOz)

Monomer, 2-ethyl-2-oxazoline was polymerized without any catalyst in the presence of an initiator 1,2 di-bromo-butene (Fig. 1 Scheme-a). The monomer to initiator ratio was set to 50 and the molecular weight obtained was 4,600 Daltons, which was confirmed from GPC. The ^{1}H NMR spectral analysis indicated two peaks for $(-CH_2)_2$ at $\delta = 3.4$, and $\delta = 3.455$ for 2-ethyl-2-oxazoline, which merged to a single peak ($\delta = 3.43$) after polymerization.

3.2 Synthesis of poly (2-ethyl-2-oxazoline)-dialdehyde (PeOzDA)

Figure 1 (scheme-b), shows the derivatization of PeOz to PeOzDA. The terminal hydroxyl groups of PeOz are derivatized to aldehyde in a view to conjugate it with the lysyl ($-NH_2$) residues of gelatin as shown in scheme-c (Fig. 1). The formation of PeOzDA was confirmed by FTIR and NMR spectroscopy. From the FTIR spectrum for PeOzDA, the carbonyl peak of –CHO was detected at 1,742 $cm^{-1.}$ The ^{1}H and ^{13}C NMR spectrum for PeOzDA of –CHO functional group indicated characteristic peaks at 9.2 ppm and 201 ppm respectively.

3.3 Synthesis and estimation of conjugated/modified gelatin with PeOzDA/glyoxylic acid

Trinitrobenzenesulfonic acid (TNBS) assay indicates the content of lysyl residues present in medical grade gelatin of 300 blooms. The results indicate the presence 3.1 lysyl residues per 10,000 molecular weight of gelatin. Scheme-c, in Fig. 1, shows the conjugation of lysyl residues ($-NH_2$) of gelatin with the terminal aldehyde (–CHO) groups of PeOzDA in the presence of sodium cynoborohydride. As shown in Table 1, different mole ratios were used to achieve 25 and 35% conjugation of gelatin with PeOzDA (25gp and 35gp). Scheme-d in Fig. 1, shows the synthesis for 48% modification of gelatin with glyoxylic acid (48gg). Table 1, shows various gel formulations with or without loaded drug of conjugated gelatin with PeOz/glyoxylic acid. As described earlier, TNBS method is used for estimation of exact percent of modification of gelatin.

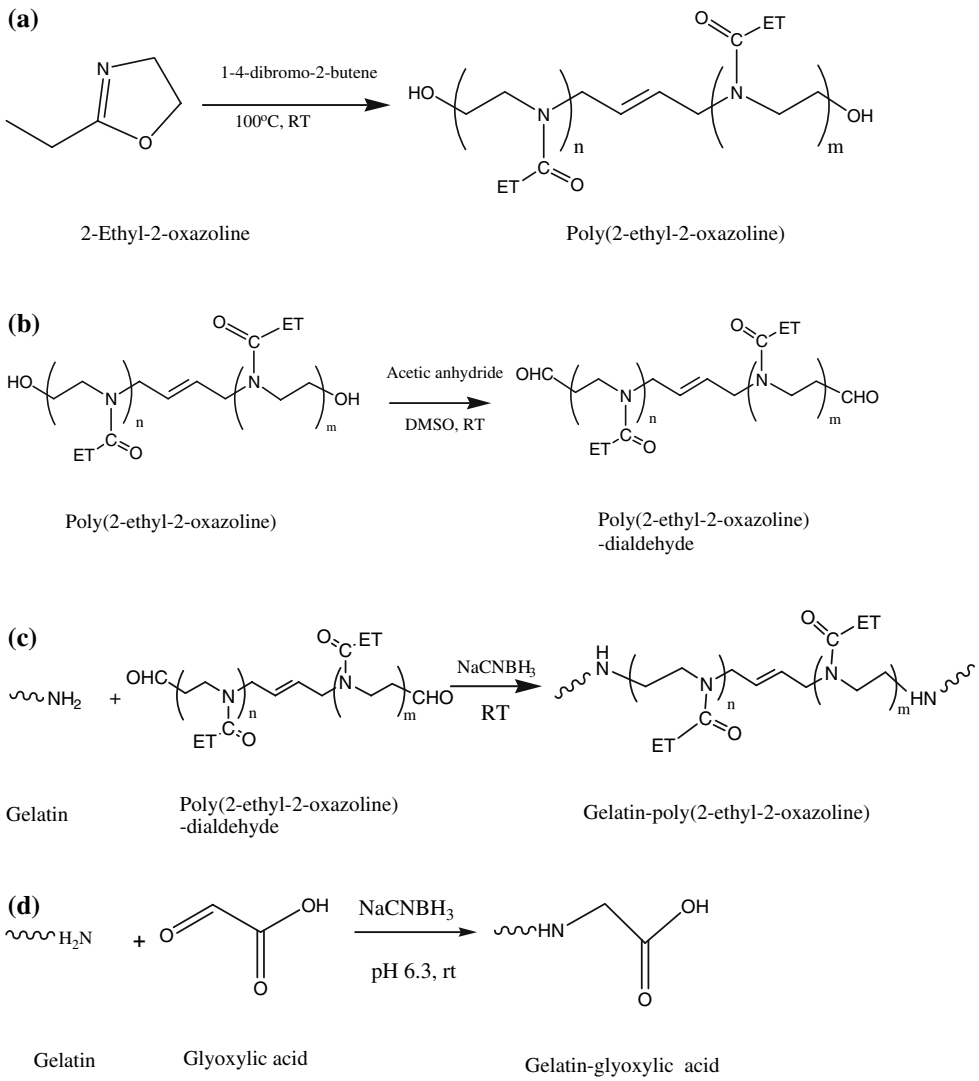

Fig. 1 Schematic representation of reaction methods (**a**) Synthesis of PeOz (**b**) Derivatization of PeOz to PeOzDA (**c**) Conjugation of PeOzDA with gelatin (**d**) Modification of gelatin with glyoxylic acid

3.4 Swelling studies and in-vitro drug release

Figure 2 shows, the degree of swelling for various gels with or without the drug loaded in the buffer of pH 4.5. In this buffer, gels with and without the drug show a gradual increase in degree of swelling with increase in time. The swelling also, increased with an increase in percent of modification of gelatin with PeOzDA/glyoxylic acid (gp/gg). This can be attributed to the increase in hydrophilicity. Besides, in pH 4.5, it is observed that the affect of the drug on the swelling profiles is insignificant for gels since the drug is de-ionized in acidic pH due to the presence of ionizable groups and hence no affect of drug on swelling for the drug loaded gels.

Figure 3 shows, the degree of swelling for various gels in the buffer of pH 7.4. In this buffer too, it is observed that the degree of swelling increased with increase in time. Also the figure shows increased swelling with increase in

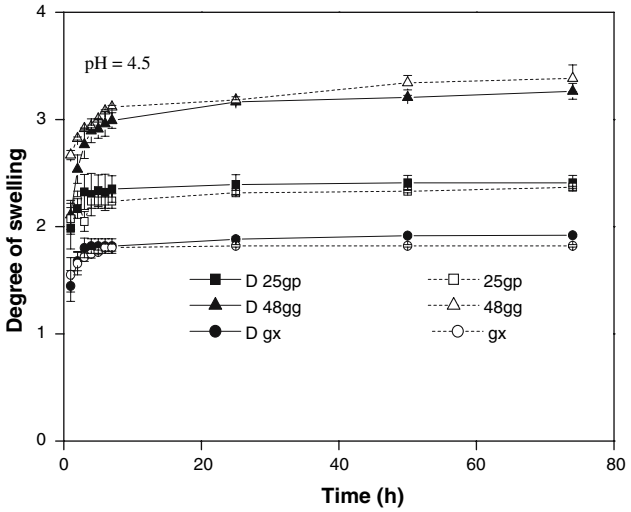

Fig. 2 Degree of swelling for gels of with or without loaded drug at 37 °C in buffer of pH 4.5

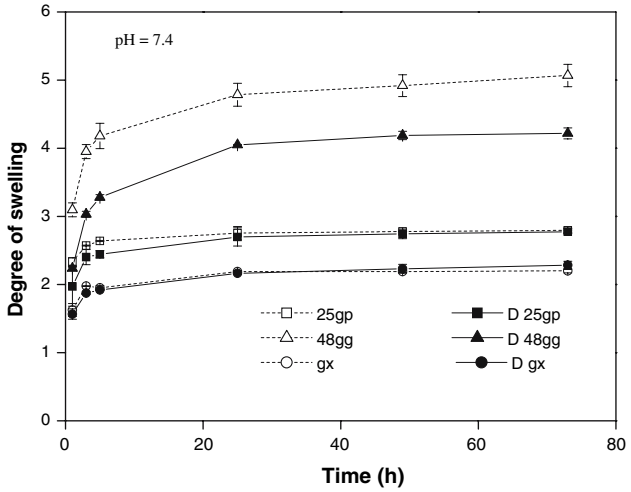

Fig. 3 Degree of swelling for gels of with or without loaded drug at 37 °C in buffer of pH 7.4

percent of modification of the gels, which is due to the ionizable groups, favoring hydrophilicity. However, it is observed that there is a significant decrease in degree of swelling for the drug loaded gelatin gels conjugated with glyoxylic acid (Dgg) in comparison to the gels without drug. This behavior is in contrast to the behavior in buffer of pH 4.5. The abnormal behavior of swelling at pH 7.4 is due to the ionization of carboxylic groups of glyoxylic acid. The negative charges of carboxylic groups interact with the positive charge of amine groups of the drug leading to reduction of swelling.

Figure 4 depicts, the maximum degree of swelling (MDS) as a function of pH for all the gel formulations without drug loaded. The results indicate that with the rise in pH there is an increase in MDS for the all the gels. Maximum MDS is observed for gels modified with

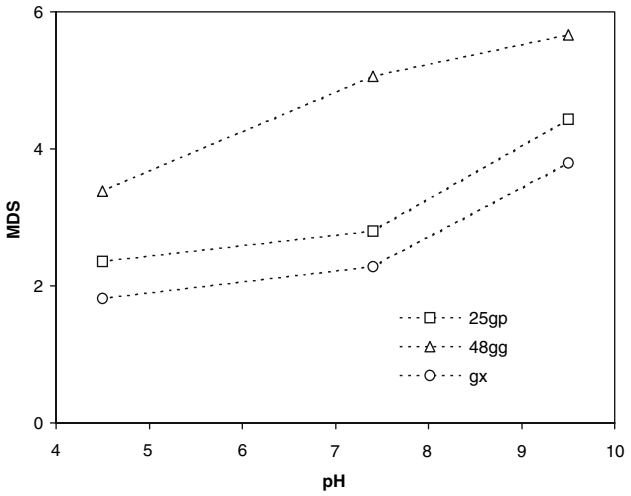

Fig. 4 Maximum degree of swelling for gels without loaded drug at 37 °C in buffers of pH 4.5, pH 7.4 and pH 9.4

glyoxylic acid with increase in pH. Based on earlier investigations [21] it is understood that weakly acidic gels swell gradually with the rise in pH due to the ionization of acidic groups and gradually shrink with decrease in pH. Hence a similar trend is observed for the weakly acidic gels of gelatin modified with glyoxylic acids without drug loaded (gg). Gelatin being non-ionic, the cross-linked gelatin gels (gx) should not respond to the rise in pH. However, an increase in swelling with an increase in pH is observed because the gelatin used in the experiments is basic derived.

Figure 4, also shows that with an increase in pH there is a gradual increase in maximum degree of swelling for gelatin gels conjugated with PeOzDA though they are non-ionic. As reported [18], at lower pH (below 5.2), the amide groups present in PeOz tends to increase the ionization and interacts with the hydrogen atoms thereby restricting the swelling of gels. A similar effect is observed for gelatin gels (without drug loaded) conjugated with PeOzDA in buffer of pH 4.5, and hence the degree of swelling is significantly lowered at low pH and gradual increase of swelling with an increase in pH is observed.

In our earlier drug release experiments, the loading of the drug was done, by adding the drug into the gel solution during gel preparation; hence it was easy to determine the exact amount of the drug loaded in the gels as reported earlier [23]. In the present study, the drug is loaded by the swelling method. The exact amount of the drug loaded is calculated depending on the amount of buffer absorbed. However, from the drug release studies, an excess drug release is observed than the calculated amount due to absorption of buffer. This is due to the interaction of drug with the possible sites present on the surface of the gels. Hence to estimate the precise amount of drug (M_∞) in the matrix, the respective gel is dried to constant weight, and ground to powder. The powder is subjected to refluxing for 48 h and the extractions are made to standard volume and optical density is measured using UV/Visible spectrophotometer at λ 285. From calibration curve the amount is determined. The profiles of Figs. 5 and 6 shows cumulative fractional drug release from the gels of 1% loaded drug in buffers of pH 4.5 and 7.4 respectively. In the corresponding figures, M_t/M_∞ indicates the cumulative fractional drug release, where M_t is the mass of drug released at time, 't' and M_∞ is the total mass of drug loaded. In individual buffer the profiles show a sustained type of release for 124 h. However, initially the drug release is higher, which is attributed to the drug release from the surface as well as due to faster initial swelling of gels [23]. About 98% of the drug is released from gels, gx and 25gp, where as, 85% of drug release is observed from gels, 48gg. The reduction in the percent of drug release is attributed towards an interaction of drug (chlorhexidine) with that of the network.

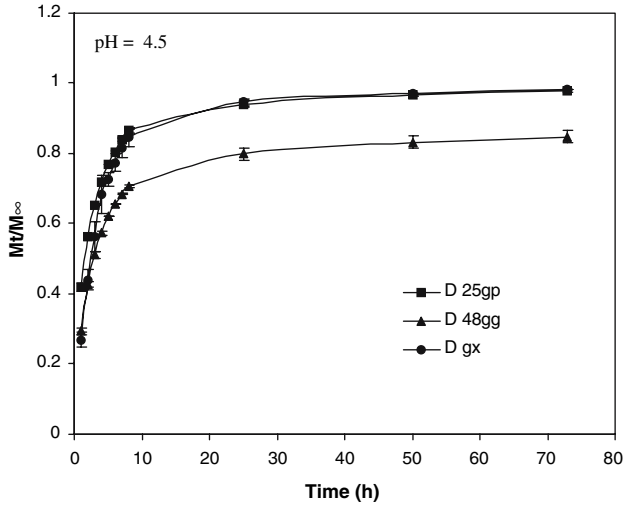

Fig. 5 Cumulative fractional drug release from gels with loaded drug in buffer of pH 4.5 at 37 °C

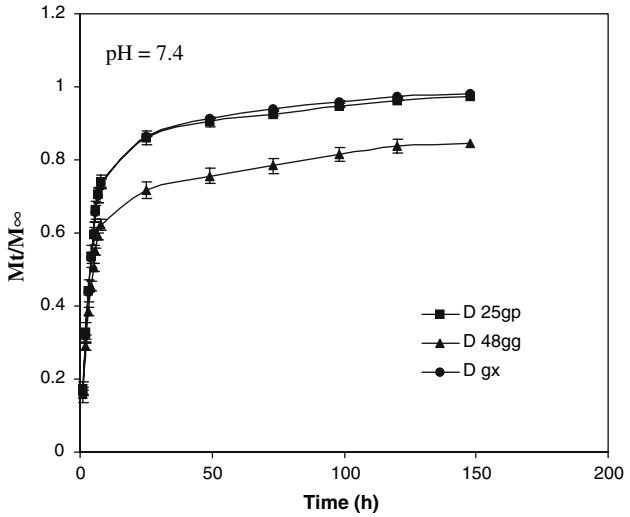

Fig. 6 Cumulative fractional drug release from gels with loaded drug in buffer of pH 7.4 at 37 °C

The greater the interaction of the drug with gel matrix, lesser is the drug release.

3.5 In-vitro cytotoxicity studies

The response of cell viability for the developed hydrogels is evaluated by in vitro cytotoxicity test using mouse embryonic fibroblast 3T3 cells. The number of surviving cells after incubation is counted at different incubation times by cell counting assay method. The results shown in Fig. 7 indicate that cytotoxicity studies are comparable to control. There is an insignificant increase in cell number for all the gel formulations including the control for first and second day of incubation. But the studies on third day

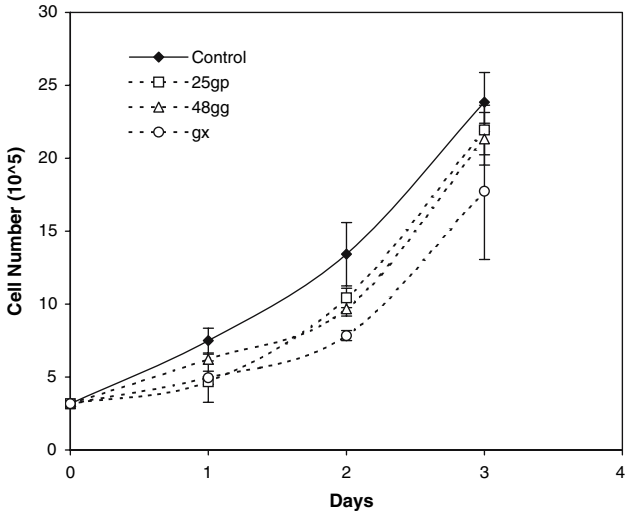

Fig. 7 Cytotoxicity studies for gels without loaded drug after 1, 2, and 3 days of incubation at 37 °C

indicate a significant increase in cell number which follows an increasing order of control >25gp > 48gg > gx. Thus, cell viability is more for 25gp gels than 48gg and gx gels. Although FDA suggested 1% glutaraldehyde solution for cross-linking the gels for biomedical applications, the results from the studies indicate that with an increase in incubation time, a significant number of living cells tend to decrease. In contrast, the gelatin gels conjugated with PeOzDA and gelatin gels modified with glyoxylic acid show significant increase in number of cells with an increase in incubation time, indicating that these gels are most viable to cells than gx.

4 Conclusion

Poly (2-ethyl-2-oxazoline) was synthesized by cationic ring opening polymerization of the monomer 2-ethyl 2-oxazoline and derivatized it to PeOzDA. Gelatin was conjugated with PeOzDA/glyoxylic acid and was loaded with 1% chlorhexidine. The swelling studies of gels showed an increase in swel1ing with an increase in modification due to an increased hydrophilicity. The effect of drug on swelling behavior was insignificant in the buffer of pH 4.5 for both conjugated/modified gelatin gels. However, at physiological pH, the gels modified with glyoxylic acid (gg) had restricted to swelling, due to a stronger interaction of the functional groups of the gel matrix with the ionized drug. The in vitro cytotoxicity studies indicate that, the gels conjugated with PeOz were more viable to cells because of its hydrophilicity and biocompatibility. Based on our studies, we confirm that the gelatin gels cross-linked with 1% glutaraldehyde are more toxic. The toxicity due to

glutaraldehyde could be minimized or even nullified with an increase in percent of conjugation/modification of PeOz/glyoxylic acid with gelatin and hence can be safely used in biomedical applications.

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