Self-cross-linking biopolymers as injectable in situ forming biodegradable scaffolds

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Abstract

The injectable polymer scaffolds which are biocompatible and biodegradable are important biomaterials for tissue engineering and drug delivery. Hydrogels derived from natural proteins and polysaccharides are ideal scaffolds for tissue engineering since they resemble the extracellular matrices of the tissue comprised of various amino acids and sugar-based macromolecules. Here, we report a new class of hydrogels derived from oxidized alginate and gelatin. We show that periodate-oxidized sodium alginate having appropriate molecular weight and degree of oxidation rapidly cross-links proteins such as gelatin in the presence of small concentrations of sodium tetraborate (borax) to give injectable systems for tissue engineering, drug delivery and other medical applications. The rapid gelation in the presence of borax is attributed to the slightly alkaline pH of the medium as well as the ability of borax to complex with hydroxyl groups of polysaccharides. The effect of degree of oxidation and concentration of alginate dialdehyde, gelatin and borax on the speed of gelation was examined. As a general rule, the gelling time decreased with increase in concentration of oxidized alginate, gelatin and borax and increase in the degree of oxidation of alginate. Cross-linking parameters of the gel matrix were studied by swelling measurements and trinitrobenzene sulphonic acid (TNBS) assay. In general, the degree of cross-linking was found to increase with increase in the degree of oxidation of alginate, whereas the swelling ratio and the degree of swelling decreased. The gel was found to be biocompatible and biodegradable. The potential of the system as an injectable drug delivery vehicle and as a tissue-engineering scaffold is demonstrated by using primaquine as a model drug and by encapsulation of hepatocytes inside the gel matrix, respectively.

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Keywords: Hydrogel; Alginate; Gelatin; Oxidation; Drug delivery; Cell encapsulation

1. Introduction

Biocompatible, biodegradable polymer scaffolds are important biomaterials for tissue engineering and drug delivery [1–3]. Molecular design of these materials has important role in determining their suitability in such applications. The simplest and the most convenient approach in these applications will be to inject the polymer-cell/drug entity into the body. Injectable systems offer specific advantages over preformed scaffolds, which include ease of application, confined delivery for a site-specific action and improved patient compliance and comfort [4]. Various methods have been employed for the preparation of injectable hydrogel systems. Water-soluble, thermosensitive and pH sensitive polymers exhibiting reversible sol–gel transition and photo-polymerizable hydrogels have been tailor-made as injectables [5–8].

Hydrogels derived from naturally occurring polymers mimic many features of extracellular matrix and thus have the potential to direct the migration, growth and organization of cells during tissue regeneration and wound healing and for stabilization of encapsulated and
transplanted cells. Many of them also demonstrate adequate biocompatibility and biodegradability. In situ gelling formulations from biopolymers are achieved by photo-polymerization of their custom-made monomers [9], enzymatic cross-linking [10,11] chemical cross-linking with metal ions [12] or by cross-linking agents such as glutaraldehyde, carbodiimide, adipic dihydrazide etc [13]. However, photo-polymerization often requires a photo-sensitizer and prolonged irradiation limiting their use. Cross-linking with metal ions is often reversible in the body and exerts cytotoxic effects [3]. Agents that are incorporated into the polymer matrix such as glutaraldehyde, polyeponides and isocyanates are highly toxic and are prone to leach out into the body on matrix biodegradation [14]. Agents that cross-link without incorporation by activating the carboxylic acid residues in biopolymers such as acyl azides and carbodiimides are considered less toxic. The toxicity of cross-linking agents is the major obstacle in the use of these polymers as injectable, in situ-forming polymer scaffolds, since their seepage into body fluids even at low concentrations can be catastrophic [15]. Potentially less toxic reagents such as adpic acid dihydrazide [16,17] and oxidized mono, di and polysaccharides have been investigated as cross-linking agents [18,19]. The gelation reaction leading to the three-dimensional network is rather slow with many of these reagents to be of practical use as injectable systems and modification of the biopolymer has been attempted to reduce the gelling time [20].

Alginates, derived from brown sea weeds are anionic linear polysaccharides composed of 1,4-linked β-D-mannuronate (M) and 1,4-linked α-L-guluronate (G) residues in varying proportions. An important feature of alginate is its gelation in the presence of divalent cations, such as calcium. The G-blocks are responsible for the “egg box” formation with calcium ions or other alkaline earth metal ions [21]. This gentle property has led to their wide use as cell transplanted vehicles to grow new tissues and as wound dressings. However, alginate hydrogels used in these applications have uncontrollable degradation kinetics and gels dissolve in an uncontrollable manner following the loss of divalent cations releasing high and low molecular weight alginate units [22]. There are reports showing degradability of low molecular weight (<80 KDa) alginates [23]. However, when used as wound dressings, there was no evidence of degradation or breakdown of the alginate fibres [24]. Also, high calcium concentration is reported to inhibit the growth of cells in culture [25]. Attempts have been made to covalently cross-link sodium alginate with gelatin and ethylenediamine using water-soluble carbodiimide [26,27]. Both ethylenediamine and carbodiimide are however, toxic reagents. Recently it is reported that although higher molecular weight alginate is non-biodegradable, its di-aldehyde derivative is biodegradable [28]. Here we show that an injectable, in situ-forming, non-toxic, biodegradable polymer scaffold can be prepared by self-cross-linking of oxidized alginate and gelatin, in the presence of small concentrations of borax without employing any extraneous cross-linking agents. Gelatin has a long history of medical use as a plasma expander as a wound dressing material and as adhesive and absorbent pads, etc. [14]. Borax also has a long history of medical use and the mean lethal dose in man exceeds 700 mg/kg [29]. Toxicity or discomfort has not been reported in humans receiving equivalent to 100 mg of boron intravenously [30]. Borax is reported to prevent osteoporosis at a dose between 3 and 6 mg/day and in persons with arthritis a dose of 3 mg of boron per day for 2–4 months is indicated [31].

The objective of the present study is to demonstrate an injectable system from oxidized alginate and gelatin for tissue engineering and drug delivery applications. By employing the commonly used double-syringe fibrin glue applicator wherein one syringe is filled with the gelatin solution containing the cell/drug entity and the other with the oxidized alginate in the presence of borax, injectable hydrogel matrices for cell encapsulation and drug delivery can be easily fabricated. The mixing of the polymer solutions inside the hypodermic needle of the applicator leads to gelation and cross-linking in a few seconds allowing the placement of an insoluble hydrogel-containing cell or drug entity inside the body. By varying the concentrations of the reactants, the gelation time can be varied from a few seconds to less than a minute allowing the system to be used in a number of applications.

2. Materials and methods

2.1. Materials

Sodium alginate (Medium Viscosity Grade, viscosity of 2% solution 3500 cps at 25°C), gelatin (Bloom 300, Type A, MW 100,000), sodium metaperiodate, borax (sodium tetraborate decahydrate), TNBS, primaquine phosphate, 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT), and Dulbecco’s Minimum Essential Medium were obtained from Sigma Chemical Co., St. Louis, MO, USA. Dialysis tubing (Spectra/ Por®, M.W.C.O 3500) was from Spectrum Laboratories Inc., CA, USA. Dextran (M_w/M_n < 1.2) standards of various molecular weights and glucose were from Polymer Laboratories (Amherst, MA, USA). Neutral red for measuring the viability of hepatocytes was procured from Hi Media Laboratories, Mumbai, India. Bromocresol-green reagent kit for albumin estimation was purchased from NPIL Reagents, Mumbai, India. Phosphate buffered saline (PBS, pH 7.4, 0.1 M) was prepared by dissolving 17.97 g of disodium hydrogen phosphate and 8.0 g of sodium chloride in 1 l of distilled water, adjusting the pH to 7.4 and diluting to 1 l.
phosphate, 5.73 g of monosodium hydrogen phosphate and 9 g of sodium chloride in 1 L distilled water. All other reagents were of analytical or equivalent grade. Double distilled water was employed throughout.

2.2. Methods

2.2.1. Periodate oxidation of sodium alginate

Into 20 g sodium alginate dispersed in 100 mL ethanol, different amounts of sodium metaperiodate in 100 mL distilled water was added and stirred in the dark magnetically at 25 °C for 6 h to obtain alginate dialdehyde (ADA) of different degree of oxidation. The degree of oxidation was followed by determining the concentration of periodate left unconsumed by iodometry after 6 h [32]. A 5 mL aliquot of the reaction mixture was neutralized with 10 mL of 10% sodium bicarbonate solution. Iodine was liberated by the addition of 20% potassium iodide solution (2 mL). This was kept under dark for 15 min and liberated iodine was then titrated with standardized sodium thiosulphate solution using starch as the indicator. Values reported for degree of oxidation are average of a minimum of three oxidation experiments. After reaction, solutions were dialysed against distilled water (2.5 L) for 48 h with several changes of water till the dialyzate was periodate free. The absence of periodate was checked by adding a 0.5 mL aliquot of the dialyzate to 0.5 mL of a 1% solution of silver nitrate and ensuring the absence of any precipitate. The dialyzate was then freeze dried. Typical yield of the oxidized products ranged from 50% to 60%.

2.2.2. Molecular weight (MW) measurements

Polymer MWs were determined by using HPLC-GEC [33]. A 30 cm × 0.75 cm TSK-G4000 PW column (Toyo Soda, Tokyo, Japan) preceded by a 2-µm filter (Rheodyne, CA) was equipped with a Hitachi pump (Model L-6000), a precision injection valve (Rheodyne, 50 µL sample loading) and a differential refractometer (R401 Waters Associates, France) connected to a computer for sample detection. Dextran standards and glucose were injected at a concentration of 1 mg/mL to establish the selectivity curve of the column. Injection volume was 50 µL for all analyses. The mobile phase was 100 mM NaNO₃ aqueous solution (pH 7) at a flow rate of 1 mL/min. A plot of log (Mw) versus Kd was used for the determination of the MW of the alginate derivatives.

2.2.3. Gelling time determination

One mL of ADA in 0.1 M borax (pH 9.4) and in PBS was reacted with 1 mL aqueous solution of gelatin in glass vials of 15 mL capacity (diameter 20 mm) under magnetic stirring using a teflon-coated stir bar (diameter 5 mm, length 10 mm) at 37 °C. Gelling time was noted as the time required for the stir bar to stop according to Mo et al. [20]. Values reported are average of 4–5 determinations ± S.D. Gelling time was studied by varying the concentration of ADA, gelatin and borax.

2.2.4. Swelling measurements

Swelling studies were conducted on hydrogels prepared from ADAs of different degree of oxidation and gelatin, in 0.1 M borax. Concisely, 0.5 mL each of a 20% solution of 87 and 57% oxidized ADA, or a 10% solution of 27% oxidized ADA dissolved in borax and 0.5 mL of a 15% aqueous solution of gelatin were mixed to obtain different gels. After 10 min, 5 mL PBS was added to the vial containing hydrogels and incubated at 37 °C for 24 h. The excess medium was then removed and the gels blotted gently with a filter paper and weighed. All experiments were done in triplicate. Degree of swelling (Q) was defined as the reciprocal of the volume fraction of the polymer in the hydrogel (vₒ).

\[ Q = \frac{1}{v_{o}} = \frac{1}{(1/\rho_p)((Q_m/\rho_s) + (1/\rho_p))^{-1}} \]

where \( \rho_p \) is the polymer density (0.825 g/cm³), \( \rho_s \) is the density of water (0.9971 g/cm³) at 25 ºC and \( Q_m \) is the swelling ratio, defined as the mass ratio of absorbed water and the dried gel [16].

2.2.5. Degree of cross-linking

Degree of cross-linking of the gels was determined by TNBS assay [16,34]. Briefly, gels were frozen within a minute after solutions of ADA and gelatin were mixed and lyophilized. About 5 mg of lyophilized gel was treated with a mixture of 1 mL of 0.5% solution of TNBS and 1 mL of 4% sodium bicarbonate at 60 ºC for 4 h. The unreacted gelatin in the hydrogel reacts with TNBS and forms a soluble complex. One milli liter of this solution was further treated with 3 mL of 6 N HCl at 40 ºC for 1.5 h and its absorbance was determined at 334 nm after dilution spectrophotometrically (Spectronic, Genesis 2, NY, USA). A standard curve was plotted for non-cross-linked gelatin by treating various concentrations of gelatin by TNBS in a similar manner. All the experiments were done in triplicate.

Degree of cross-linking (%)

\[ = \left(1 - \frac{\text{Absorbance of cross-linked gel}}{\text{Absorbance of non-cross-linked gel}} \right) \times 100. \]

2.2.6. Cytotoxicity studies

Cytotoxicity evaluation of ADA-cross-linked gelatin gel was carried out by the direct contact assay and test on extracts with a monolayer of L929 mouse fibroblast cells according to ISO standards [35]. Gels were prepared from a 15% solution of gelatin and 20% solution of 57% oxidized ADA in 0.1 M borax. Briefly, L929 cells were subcultured from stock culture (National Centre for Cell Sciences, Pune, India) by
trypsinization and seeded onto multi-well tissue culture plates (Nunc, Denmark). Cells were fed with Dulbecco’s minimum essential medium supplemented with bovine serum and incubated at 37 °C in 5% carbon dioxide atmosphere. When the cells attained a monolayer, the gel (0.75 cm²/mL) was kept in contact with the cells in triplicate for direct contact assay. After incubation at 37 ± 1 °C for 24 h, cell culture was examined microscopically using a phase contrast inverted microscope (Leica, WILD MPS32, Germany) for cellular response. The morphology of the cells was assessed in comparison with negative (high-density polyethylene) and positive control (copper wire). Cellular responses were scored as 0, 1, 2 and 3 according to non-cytotoxic, slightly cytotoxic, moderately cytotoxic and severely cytotoxic.

For test on extract, the extract was prepared by incubating the gel with media containing serum at an extraction ratio of 0.75 cm²/mL for 24 h at 37 ± 1 °C. One hundred micro liter of the extracts of the gel, negative control (high-density polyethylene), and positive control (diluted phenol) in triplicate were placed on subconfluent monolayer of cells. After incubation at 37 ± 1 °C for 24 h, cellular responses were microscopically examined and scored as before. Experiments were done in triplicate.

Cytotoxicity of gels were quantitatively assessed further by MTT assay [36] which measures the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to a coloured formazan by viable cells. Toxicity was evaluated on the extract of the material (0.75 cm²/mL) in medium containing serum as before. MTT dissolved at a concentration of 5 mg/mL in sterile PBS, filtered through a 0.22 μm filter to remove any formazan crystals and stored at 20 °C was used as working solution. Cells were cultured as before in multiwell tissue culture plates and when monolayer was attained, culture medium was removed, rinsed with PBS and 100 μL each of extracts of gel and negative control (high-density polyethylene) and 100 μL of diluted phenol (positive control) were added to different prelabelled wells containing cells. Cells with medium alone served as control. Culture medium (100 μL) was used as reagent blank. Plates were incubated for 24 h at 37 °C in 5% carbon dioxide atmosphere. After 24 h, the extracts/medium were removed and 200 μL of MTT working solution was introduced using a multi-channel pipette into each well. Plates were wrapped with aluminium foil and incubated at 95% humidified atmosphere at 37 °C for 8 h. After removing the reagent solution and rinsing with PBS, 200 μL of isopropanol was added to each well and incubated for 20 min at 37 °C in a shaker incubator (Labline Instruments, Melrose Park, USA). The absorbance of the resulting solution in each well was recorded immediately at 570 nm using automated micro plate reader (Bio–Tek Instruments, Vermont, USA). Results were expressed as O.D after blank (i.e; medium only) subtraction. Reported values are mean of three replicates.

2.2.7. Morphology

Morphology of ADA/gelatin gels was examined by scanning electron microscopy (SEM). Lyophilized gels were placed on double-sided tape, sputter coated with gold and examined in the microscope (Hitachi, Model S-2400, Japan). SEM images were analysed using an image analysis software (Optimas™ 6.1, West Ford, MA, USA).

2.2.8. In vitro drug release

In vitro release experiments were performed in a similar manner as reported by Jeong et al. [5] with minor modifications. One half milli liter of a 20% solution of ADA having a degree of oxidation of 87% and 57% or a 10% solution of ADA having degree of oxidation 27% in 0.1 m borax and an equal volume of a 15% solution of gelatin in water containing primaquine to give a drug payload of 5% by weight in the final gel were introduced into screw-capped test tubes of 10 mL capacity using a double syringe fibrin glue applicator fitted with a 20 G needle. Gelation occurred within seconds after the mixture was extruded out of the needle. After 10 min, 5 mL PBS was introduced and the tubes were incubated at 37 °C. At regular intervals, 1 mL aliquots were withdrawn and replenished with 1 mL fresh PBS. Absorbance of the released primaquine was read at 355 nm in a spectrophotometer (Spectronic, Genesys 2, NY, USA). All experiments were done in triplicate.

2.2.9. Hepatocytes encapsulation

Hepatocytes were isolated from adult, male Wistar rats, 7–8 weeks old, weighing 200–300 g as per modified Seglen’s method [37] and cultured in Iscove’s Modified Dulbecco’s Medium supplemented with 10% foetal calf serum and antibiotics. Gelatin and ADA were sterilized using ethylene oxide using standard protocols and solutions were prepared in sterile media. Fifty micro liter of a 20% solution of 57% oxidized ADA in 0.1 m borax mixed with 30 μL of single cell suspension (containing 2.65 × 10⁵ cells/mL) was introduced into each well of a 96 well tissue culture plate followed by 50 μL of 15% gelatin in distilled water. Transparent gels entrapped with the cells developed slight brown with time. Gels without cells were also prepared in a similar manner. Into each well, 150 μL of the medium containing serum was then introduced and the plates were incubated at 37 °C. Culture medium from the wells was collected at different time intervals for albumin estimation using bromocresol-green reagent at 578 nm using the kit according to manufacturer’s instructions. Values reported are average of eight experiments.

Viability of cells was assessed using neutral red assay [38]. Neutral red stock solution was made at a
concentration of 1 mg/mL in deionized water at room temperature. The stock solution was diluted (1:1) with 1.8% NaCl before use. Gels with cells and without cells (blank) were washed by adding 100 μL of PBS to each well and centrifuging at 1500 rpm for 3 min. PBS was then removed and 100 μL of working solution of neutral red was then added to each well and kept for 5 h at 37 °C. After the removal of the dye solution by inverting the plates, the gels were rapidly washed twice with PBS. The dye was then extracted from the cells by the addition of 100 μL of 0.05 M NaH2PO4 in 50% isopropyl alcohol. Optical density was recorded at 630 nm using a micro plate reader (Bio-Tek Instruments, Vermont, USA). The extract from gels without cells was used as blank. Values reported are average of eight experiments.

2.2.10. Degradation

Gels were prepared as in the case of in vitro release but without primaquine using 0.5 mL each of a 20% solution ADA with degree of oxidation 57% in 0.1 M borax and 15% solution of gelatin in distilled water and incubated with 5 mL PBS at 37 °C (n = 3). Degradation of gels was followed every week by aspirating the medium followed by freeze-drying the gel to dryness and normalising the weight obtained to their initial values [16].

2.2.11. Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA), assuming confidence level of 95% (p<0.05) for statistical significance. All data were expressed as mean ± standard deviation (S.D).

3. Results and discussion

Periodate oxidation specifically cleaves the vicinal glycols in polysaccharides to form their dialdehyde derivatives. This reaction is generally used for the elucidation of polysaccharide structure. Each α-β-glycol group consumes one molecule of periodate, and under given conditions, the rate of the reaction is dependent principally on the stereochemistry of the α-β-glycol group. Usually, the reaction is carried out in aqueous medium. Alginates form very viscous solutions even at low concentrations and are difficult to handle. Therefore, we examined the reaction in heterogeneous medium (1:1 ethanol: water) as a dispersion. By doing the reaction in this medium, solvent quantity needed was small even for preparing large quantity of the oxidized product, which made the reaction handier. The oxidation proceeded smoothly giving rise to alginates of different degrees of oxidation depending on the quantity of periodate employed (Table 1). Determination of the MW of the products obtained showed significant depolymerization of the alginate.

### Table 1

<table>
<thead>
<tr>
<th>Periodate equivalent (%)</th>
<th>Degree of oxidation (%)</th>
<th>MW (Da)</th>
<th>Gelling time (s)</th>
<th>Degree of crosslinking (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.0</td>
<td>27.4±0.4</td>
<td>28,790±1440</td>
<td>53±5</td>
<td>42.7±11</td>
</tr>
<tr>
<td>50.0</td>
<td>48.0±0.4</td>
<td>31,240±1560</td>
<td>32±4</td>
<td>44.6±4</td>
</tr>
<tr>
<td>65.0</td>
<td>57.5±0.2</td>
<td>30,245±1510</td>
<td>26±2</td>
<td>50.6±2</td>
</tr>
<tr>
<td>95.0</td>
<td>87.0±0.3</td>
<td>11,340±570</td>
<td>22±2</td>
<td>77.6±2</td>
</tr>
</tbody>
</table>

MW of the starting alginate was 4,89,000±50,000.

*Gelling time for 10% solutions of alginate dialdehyde with 15% solution of gelatin in 0.1 M borax.

*Gelling time for 20% solutions of alginate dialdehyde with 15% solution of gelatin in 0.1 M borax.

Depolymerization of alginates is a free radical-mediated reaction due to oxidation of phenolic impurities present, and aliphatic alcohols, usually isopropanol, act as a hydroxyl radical scavenger to prevent depolymerization giving rise to higher MW [39]. We expected the presence of ethanol would prevent depolymerization and give rise to oxidized alginate of higher MW. On the contrary, we observed extensive degradation, which we believe, was due to the generation of reactive 1-hydroxyethyl radicals during oxidation (as opposed to the more stable and less reactive 2-hydroxypropyl radicals) along with hydroxyl radicals cleaving the glycosidic bonds in alginate. Except for very high periodate equivalents, the MW of the product was found to remain the same due to the presence of excess ethanol in the reaction medium predominantly influencing the depolymerization of the alginate. At very high periodate equivalent, the effect is believed to be synergic, both alcohol and the periodate influencing the cleavage of the alginic acid chains (Table 1).

Hydrogels were prepared by cross-linking gelatin with ADA (Scheme 1). Cross-linking is predominantly due to Schiff’s base formation between the ε-amino groups of lysine or hydroxylsine side groups of gelatin and the available aldehyde. The solubility of oxidized alginate was found to be increasing with increase in degree of oxidation. While we were able to prepare a 20% solution from ADAs having degree of oxidation 57% and 87%, solubility was limited to 10% for ADA having a degree of oxidation of 27%. It was found that the more oxidized the alginate is, the faster the gelation reaction. This was evident from the gelling time obtained by using sodium alginate of different degree of oxidation (Table 1). Degree of cross-linking of the gels analysed by measuring the amount of unreacted gelatin present by TNBS assay showed that the higher the degree of oxidation of the alginate, the higher the degree of cross-linking as the presence of a large number of aldehyde groups facilitates the gelation formation.
(Table 1). Statistical analysis of the data showed a significant effect of degree of oxidation on gelling time as well as the degree of crosslinking (p < 0.05).

Swelling ratio ($Q_m$) and degree of swelling ($Q$) of these gels were calculated by swelling the gels until equilibrium was attained. Degree of swelling ($Q$) is the reciprocal of volume fraction of the polymer ($\nu_2$) in the hydrogel, which is a measure of interaction between polymer chains. Cross-linking density ($\nu_c$, mol/cm$^3$) of the hydrogels was subsequently calculated from the Flory–Rehner equation,

$$
\nu_c = \frac{-\ln (1 - \nu_2) + \nu_2 + \chi_1 \nu_2^2}{\nu_2 [V_1 (\nu_2^{1/3} - 2\nu_2/f)]}^{-1},
$$

where $\chi_1$ is the interaction parameter, $f$ is the cross-linking functionality, $V_1$ is the molar volume of water (18.062 cm$^3$/mol) and $\nu_2$ is the volume fraction of polymer in the hydrogel when it reaches the equilibrium swelling state. The interaction parameter, $\chi_1$ was assumed to be 0.35 as it has been previously reported for similar interaction [16]. It has been reported that the reactive functional groups present per 100 g of high quality gelatin are primarily, hydroxyl, carboxyl and amino at an amount of approximately 100, 75 and 50 meq of each of these groups, respectively [40]. On this basis, the functionality of gelatin in terms of reactive amino groups was assumed to be 50.

The values of $Q$ and $Q_m$ for all the gels decreased with increase in degree of oxidation (Table 2). Statistical analysis revealed that there was significant difference ($p < 0.05$) in the values of $Q$ and $Q_m$ on varying the degree of oxidation of ADA. Hence, cross-linking density calculated from the swelling measurements (see Table 2) increased with increase in the degree of oxidation which can also be correlated to stronger mechanical properties.

The effect of concentration of ADA, gelatin and borax on gelling time was systematically examined further. Fig. 1 shows the gelling time at different concentrations of gelatin, ADA and borax. As a general rule, the gelling time decreased with increase in concentration of ADA, gelatin and borax as well as with increase in the degree of oxidation of alginate. Particularly striking is the influence of borax concentration on the gelling time. This we believe is due to the alkaline pH of the medium which facilitates the formation of the Schiff’s base as well as the ability of borax to complex with hydroxyl groups of polysaccharides [41] (Scheme 1). Since the gelling time decreased rapidly with increase in the concentration of borax, it supports the fact that not only the alkaline pH of the medium, but the ability of borax to complex with hydroxyl groups is also responsible for the rapid gelation. When we employed ADA having a low degree of oxidation (10%), gelation did not take place at all even after hours, demonstrating that a minimum aldehyde content is essential for rapid gelation. Gelation of ADA with gelatin also occurred in PBS, although reaction was less rapid in this medium (Fig. 1(a)).

To reconfirm the non-toxic nature of the gelatin-ADA hydrogel preparations in borax, a preliminary cytotoxicity evaluation using mouse fibroblast cells was carried out. Neither the gel nor its extract induced any morphological changes to the cells confirming its non-toxic nature. The morphology of the cells growing on the surface (scored as zero) is depicted in Fig. 2(a). Quantitative assessment of the cytotoxicity by MTT assay of cells after contact with the material extract showed 93% metabolically active cells compared to cells without the material for 24 h of contact. Statistical analysis of absorbance values obtained for control and gel samples showed that there was no statistically significant difference ($p > 0.07$). The MTT reduction for 24 h contact for the gel is shown in Fig. 2(b) along

![Scheme 1. Gelatin cross-linking with ADA in the presence of borax.](image-url)
SEM showed interconnecting pores in the gelatin-ADA gel matrix (Fig. 3) and pore size analysis gave an average pore diameter of 100 μm, demonstrating the suitability of the matrix for cell encapsulation. It was found that pore size distribution changed with change in composition of the gel (data not shown).

We examined the potential of the system as an injectable drug delivery vehicle. Primaquine was chosen as the model drug as its amino functions can enter into with the reduction observed for positive and negative controls.

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Schiff’s reaction with the aldehyde groups in ADA and give rise to both diffusion and degradation controlled release (Scheme 2). While release was faster from matrices cross-linked with ADA having lower degree of oxidation which can be attributed to poor drug conjugation, the release from ~87% oxidized sample was slowed down due to the highly cross-linked nature of the matrix as well as better drug conjugation due to the availability of more aldehyde functions (Fig. 4). The system will be well suited for the controlled release of therapeutic peptides and proteins also, since at lower aldehyde contents, these molecules will be less chemically conjugated to the matrix enabling release in their most native form [19].

For examining the potential of the system as a scaffold for tissue engineering, we encapsulated rat hepatocytes in the hydrogel matrix. Since 50 μL each of gelatin and ADA was mixed with 30 μL of cell suspension, we characterized this particular gel for its physical properties. The degree of crosslinking of the gel was found to be 54.48 ± 3%. The swelling ratio \( Q_m \) and the degree of swelling \( Q \) were found to be 9.68 ± 0.5 and 9.52 ± 0.5, respectively. The crosslinking density of the gel \( n \) was determined to be \( 10^{-5} \times 23.5 ± 7 \text{ mol/cm}^3 \).

Hepatocytes are anchorage-dependent cells with the matrix playing an important role in cell shape, division, differentiation and function and are difficult to culture and propagate in vitro for extended periods [2]. In 2-D cultures, cell matrix interaction is minimal resulting in

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**Fig. 3.** SEM image of lyophilized ADA cross-linked gelatin gel prepared from 20% solution of 57% oxidized ADA and 15% solution of gelatin.

**Fig. 4.** Cumulative release of primaquine from gels made from 87% (▲), 57% (▼), and 27% (□) oxidized ADA and gelatin alone (●).

**Scheme 2.** Interaction between primaquine and the gel matrix.
limited cell survival and proliferation. Fig. 5 shows the characteristic morphology of hepatocytes 4 weeks after encapsulation. The viability of hepatocytes was examined by neutral red assay. Absorbance increase for 4 weeks can be attributed to the presence of more active cells within the matrix (Fig. 6). Estimation of albumin secretion up to 2 weeks also showed that cells were maintaining their protein producing ability (one of the most liver-specific functions) (Fig. 7) demonstrating the suitability of the scaffold for tissue engineering.

The degradability of the hydrogels was studied by examining the weight loss of gels with time in PBS at 37°C. A linear decrease in weight with time was seen up to 4 weeks with complete dissolution of the gel at the end of 5 weeks demonstrating the degradability of the matrix (Fig. 8). It has been recently reported that oxidized alginates are rapidly degraded at physiological

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Fig. 5. Optical photomicrograph (320 x) of hepatocytes encapsulated in the gel derived from 15% solution of gelatin and 20% solution of 57% oxidized ADA in 0.1 M borax.

Fig. 6. Viability of hepatocytes encapsulated in the gel with respect to time as shown by increase in absorbance.

Fig. 7. Albumin secretion of encapsulated hepatocytes with respect to time.

Fig. 8. Degradation profile of gel prepared from 20% solution of 57% oxidized ADA and 15% solution of gelatin.
pH unlike alginates [28], which are not broken down in mammals and have a very slow clearance from the body [42]. Therefore, the hydrolytic susceptibility of Schiff's linkage between gelatin and oxidized alginate and the degradability of both gelatin and ADA would make the scaffold fully biodegradable with time in the body.

4. Conclusion

We have shown here that an injectable, in situ-forming, non-toxic, biodegradable polymer scaffold can be constructed from biopolymers of well-known biocompatibility and bioresorbability without employing any extraneous cross-linking agents. By employing oxidized alginate having appropriate MW and degree of oxidation, we have demonstrated that rapid cross-linking and gelation with gelatin is possible in the presence of borax to fabricate a truly injectable system. The use of low concentrations of borax has the beneficial effect of increasing the solubility and compatibility of biopolymers due to complexation, and enhancing the rate of Schiff's reaction giving rise to injectable hydrogel systems. Cytotoxicity screening using mouse fibroblasts have shown the non-toxic nature of these hydrogels. Potential applications such as tissue engineering, drug delivery, in situ-forming wound and burn dressings, tissue adhesives etc. are envisaged for these polymer scaffolds.

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