JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Preparation And Evaluation Of Injectable Scaffold Loaded With Raloxifene Hydrochloride For Bone Regeneration

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Abstract : Bone injury is very serious in elder people or osteoporotic patients. This study intended to design an injectable scaffold oxidized sodium alginate/gelatin-based 3D scaffolds loaded with raloxifene hydrochloride as a selective estrogen receptor modulator was essential to make use of its anti-resorptive properties for bone regeneration. The prepared formulae were tested for their *in-vitro* gelation time, drug release, injectability, viscosity properties, and morphological properties. Results presented that the prepared scaffolds were highly porous, additionally, the scaffold containing drug/OSA-gelatin kneaded complex showed the most controlled drug release pattern with the least burst effect and reached almost complete release at 120 h.

IndexTerms **- Raloxifene hydrochloride, gelatin, oxidized alginate, osteoporosis, injectable scaffold, bone regeneration, bone injury.**

I. INTRODUCTION

Bone repair of large bone defects due to severe trauma, resection of bone tumors, reduced normal bone regeneration in cancer, vascular necrosis, and osteoporosis remains a great challenge in the field of orthopaedic surgery. Osteoporosis is a condition in which bones become fragile due to reduced bone density and decreased bone strength, which increases the overall risk of bone fractures. Bone injuries can be divided into two types according to the size of the injury: critical sized and non-critical sized injuries. A critical-sized bone defect is known as a non-union defect. A non-union defect is an osseous size defect that does not heal spontaneously or form non-union healing filled with fibrous connective tissues unless there is a surgical intervention and injecting osteoinductive or osteoconductive materials [1].

However, non-critical defects may heal spontaneously without surgery. Surgical treatment is the first line of treatment for bone injuries; however, the post-surgical healing process can be delayed due to other health issues or age. Therefore, a great attention has been given to the development of non-surgical injectable drug delivery systems, which have a simple application, local drug delivery for a site-specific action, sustained delivery periods, and enhanced patient compliance [2].

Recently, bone tissue engineering (BTE) has gained increasing attention from researchers across the globe owing to its excellent compatibility and access. The BTE avoids several disadvantages of traditional treatments such as chronic inflammation, complicated surgical processes and immune rejection [3]. The principle of BTE lies in the combination of knowledge of cells, engineering, and biochemical factors to effectively regenerate the damaged bone tissues. A tissue engineering scaffold is the one which supports the extracellular matrix (ECM) and serves as a place for the proliferation and differentiation of bone cells and tissues [4].

An ideal scaffold for BTE would possess similar chemical composition of natural bone with additional features such as surface chemistry, good mechanical strength, biocompatibility and bioactivity. In these conditions, the new cells could perform the biological functions of bone. Therefore, materials intended for scaffold preparation should be biocompatible, biodegradable and non-toxic in nature which could degrade into non-toxic entity. In general, materials originated from biomacromolecules and

inorganic constituents could mimic natural ECM and could provide sufficient mechanical strength which will allow the favourable environment for the cell proliferation and tissue regeneration [5].

Using a natural polymeric biomaterial as starting materials for biomedical applications can be a field of great interest from the economic as well as ecologic point of views [1] . Sodium alginate is a cell wall component of marine brown algae, and contains approximately 30 to 60% alginic acid. The conversion of alginic acid to sodium alginate allows its solubility in water, which assists its extraction [6].

Alginate is one of the most preferred materials for BTE owing to its chemical similarity with the natural bone and offers excellent biodegradability and biocompatibility. To further increase the osteogenic properties. Additionally. Alginate has been reported as one of the most commonly used scaffolding materials due to its excellent in vivo properties such as non-toxicity, biocompatibility, biodegradability, cell adhesion, and cell proliferation characteristics [7]. Moreover, alginate is structurally similar to that of glycosaminoglycan's (GAG) which forms the ECM of body tissues. Many scaffolds have employed sodium alginate as one of the important materials for tissue regeneration [8].

Researchers have produced oxidized sodium alginate using different chemical and mechanical treatment methods. Oxidized alginate has possible medicinal applications due to its benign properties like safety, versatility, biodegradability and good mechanical properties with high surface area and low density.[1] The exploration of oxidized alginate in regenerative medicine is well-reported due to its proliferative activity [9,10. Also, due to the robust fiber network and high functionality; sodium oxidized alginate can control drug delivery.

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II. MATERIALS AND METHODS

2.1. Materials

Apotex Pharmachem India Pvt Ltd provided Raloxifene Hydrochloride as a gratis sample. Sodium alginate was obtained from Sigma Aldrich, sodium metaperiodate and sodium tetraborate (borax) were obtained from Merck. Ethanol from Loba Chemie PVT. LTD. Throughout the practical work, distilled water from within the institution was used.

2.2. Preparation Of Oxidized Sodium Alginate

Oxidized sodium alginate (OA) was prepared according to a previously reported method with some modification [11]. Briefly, 100 ml solution of 1% (w/v) sodium alginate in ethanol solution was prepared. The solution was mixed with aqueous solution containing various specified concentrations of sodium periodate at room temperature. The concentrations were calculated according to molar ratio of sodium periodate/urinate to have OA with a different oxidation degree (OD). The mixing was continued for 6 h in the dark to avoid any undesirable reactions. In order to stop oxidation reaction, ethylene glycol was added to the system and stirring was continued for 30 min. Sodium chloride and ethanol were added while the system was under stirring. After 15 min, the precipitates were collected by a centrifuge and re-dissolved in distilled water (100 ml). The prescribed amount of sodium chloride and ethanol was added again to the system and after 15 min stirring, the precipitates were isolated by a centrifuge, dried by vacuum, and kept in a refrigerator prior to use.

Table 1: Selection of sodium alginate to sodium metaperiodate ratio for the synthesis of oxidized sodium alginate

Batch	Sodium alginate	Sodium metaperiodate
OSA1	8	2
OSA2	7	3
OSA3	6	4
OSA4	5	5

2.3. Preparation Of Injectable Scaffold Scaffolds

The sol-gel method is a popular technique for preparing hydrogels with controlled porosity, biocompatibility, and drug delivery properties. Following steps were used to prepare the formulation.

Oxidized sodium alginate (OSA) was dissolved in borax and stirred for at least 2 hours until fully dissolution. The solution was filtered through a 0.22 µm filter to remove any impurities. Gelatin was dissolved in distilled water by stirring it overnight at room temperature then heated to 37°C to completely dissolve the gelatin. The OSA and gelatin solution were mixing and stirred for 30 minutes to form a homogeneous mixture. Finally, raloxifene hydrochloride was added to the mixture and stirred til fully dissolved.

The mixture was heated to 40-50°C to initiate gelation. At this temperature, the solution should be in a sol state and able to flow freely. The final solution ws kept in the fridge till further use [12].

2.4. STATISTICAL METHOD

For, optimization, CCD has been used to study the effect of independent variables on dependent variables. All the batches were prepared according to the design and analysed using design expert 13 software. Following the application of statistical design and optimization, the batch was chosen that exhibit a shorter gelling time, 100% drug content and optimum swelling ratio.

2.5. EVALUATION OF OSA/GELATIN INJECTABLE SCAFFOLDS LOADED WITH RLX HCL

2.5.1. DETERMINATION OF OXIDATION DEGRE

Using UV-Vis absorption spectroscopy, the oxidation degree (OD) of oxidized sodium alginate could be calculated based on the difference between the initial and final concentrations of $NaIO_4$ throughout the oxidation reaction. The reaction solution was transferred and diluted with distilled water from one millilitre to 250 ml. The indicator solution was made by mixing equal volumes of 20% (w/v) KI and 1% (w/v) soluble starch solutions, and 3.5 mL of this diluted solution was then added to 1.50 mL of pH 7.4 PBS, which served as the solvent for the experiment. The absorbance of a diluted using a UV-visible spectrophotometer set to 290 nm. A calibration curve, which was created by standardising the concentration of $NaIO_4$ in PBS, was used to determine the concentration of $NaIO_4$ in the solution [12,13]. This calibration curve's linear regression equation was fitted as y = 0.0938x + 0.0815 (R^2 = 0.9921). As a result, the amount of $NaIO_4$ was used to calculate the actual OD of OSA using the following equation:

$$OD = \frac{\text{Ni} - \text{Nr}}{\text{No}} \text{ x } 100\%$$

where N_i, N_r, and N_o, respectively, represent the initial moles of $NaIO_4$, the residual moles of $NaIO_4$, and initial moles of alginate .

2.5.2. DEGREE OF CROSS-LINKING

Using the TNBS (Trinitrobenzene sulphonic acid) assay, the degree of cross-linking of the gels was evaluated. The prepared scaffold was lyophilized. A mixture of 1 mL of 0.5% TNBS solution and 1 mL of 4% sodium bicarbonate was applied to around 5 mg of the lyophilized gel at 60° C for 4 hours. A soluble complex is created when the hydrogel's unreacted gelatin reacts with TNBS. After dilution, the absorbance of 1 ml of this solution was measured spectrophotometrically at 286 nm after being further treated with 3 mL of 6 N HCl at 40ºC for 1.5 h. By using TNBS to similarly process different concentrations of gelatin, a standard curve was created for non-cross-linked gelatin [14]. There were three duplicates of each experiment and results are shown in table 3.

$$Degree\ of\ cross-linking = \{1 - \frac{\text{Absorbance of cross link gel}}{\text{Absorbance of non cross link gel}}\}\ X\ 100$$

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2.5.3. FTIR OF SODIUM ALGINATE AND DIFFERENT BATCHES OF OSA

To verify the oxidised sodium alginate peak of the functional group present in sodium alginate, IR spectra of the compound were obtained using an FTIR spectrophotometer and KBr pellets [73]. The FTIR spectra of different batches of oxidised alginate are displayed in figures 1 and 2.

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2.5.4. GELLING TIME

The prepared PBS 7.4 was filled in a glass beaker and 1 ml of the prepared formulation was poured in the PBS 7.4 under magnetic stirring at 37ºC. Gelling time was noted as the time required for the stir bar to stop [13]. All experiments were done in triplicate. Results are shown in able 3.

2.5.5. SWELLING DETERMINATION

Fluid absorption studies are of paramount importance for preliminary analysis of hydrogels. For fluid-uptake measurements, the hydrogel was dried in a vacuum oven until a constant weight was reached (Wi), and then it was immersed into PBS solution at 37 ºC for 48 h to reach its equilibrium state. The surface water of the scaffold was removed gently with blotting paper and then the

scaffold was weighed again (Wf) [15]. The swelling ratio was defined as the weight ratio of absorbed water to the dried scaffold and was calculated using the following formula:

$$Swelling\ Ratio = \frac{Wi - Wf}{Wi}$$

Where, Wi = initial weight of scaffold

Wf = final weight of scaffold

2.5.6. VISCOSITY STUDY

The injectability test was carried out by applying manual shear using a syringe. About 1 ml of the optimized batch F5 hydrogel was loaded into a 5 ml syringe and a slow and controlled shear was applied [80]. The flow was observed visually [16]. Results are shown in table 3.

2.5.7. DRUG CONTENT

1 ml of gel was diluted up to 100 ml in PBS pH 7.4 as part of the procedure. Following a 45-minute bath sonication of the resulting solution, the drug concentration was ascertained by measuring the filtrate's absorbance at a UV spectrometer's maximum value of 286 nm. Additional dilutions were made if necessary [17]. Results are shown in table 3.

2.5.8. *IN-VITRO* DRUG RELEASE

The in-vitro release of raloxifene hydrochloride from the formulations were conducted using the dialysis bag technique. Accurately measured 1 mL of the investigated formulations containing 10 mg raloxifene hydrochloride was added in a dialysis bag, molecular weight cut-off which was pre-soaked in phosphate buffer solution pH 7.4 for 12 h. Each dialysis bag was secured using two clamps at both ends and for the in vitro release research, a USP dissolving equipment type II was employed. PBS pH 7.4 was used to perform in vitro release profiles on optimized batch F5 (to mimic IV conditions). A 10 mg of raloxifene hydrochloride formulation was obtained and packaged in a dialysis membrane sac; the sac was connected to the paddle of the shaft in such a way that the membrane just touched the dissolving medium surface. At 50 rpm, the dissolving media was agitated. The release study lasted 120 hours, and at pre-determined time intervals, an aliquot of 3 mL of the sample was removed from the cylinder and refilled with new medium. Filtration was performed on the aliquots using 0.45 mm membrane filters. A UV spectrophotometer was used to determine the drug concentration at 286 nm. To determine the release of raloxifene hydrochloride from the scaffold, the cumulative percent drug release was plotted as a function of time [18]. All tests were carried out in triplicate.

2.5.9. SCANNING ELECTRON MICROSCOPE

SEM was used to test the morphological characteristics of the optimized batch F5. 1 mL of the samples of batch F5 was added to 2 mL of PBS solution (pH = 7.4), which was then kept at 37°C for 24 hours. On filter paper inside a desiccator, the generated gel was allowed to dry in the air overnight. Double-sided tape was used to mount the dried samples on metal stubs. After that, samples were sputter-coated with a thin layer of gold so they could be imaged by a SEM with a 20 kV excitation voltage [19].

2.5.10. STERILIATION STUDY AND TERILITY STUDY

All prepared formulations were sterilized by membrane sterilization using 0.22 m membranes individually [20].
By using the direct inoculation of the culture media technique, injectable scaffold sterility was tested using the pharmacopoeia method. Aseptically, samples of gelatin in distilled water solution and alginate dialdehyde in 0.1M borax solution were transferred to fluid thioglycolate medium and soy bean casein digest media, respectively, using sterile pipettes or syringes. These samples were incubated for 14 days at 30 to 35 °C for the fluid thioglycolate medium and 20 to 25 °C for the soy bean casein digest medium in order to monitor for turbidity on a daily basis, which served as a gauge for microbial development [21].

2.5.11. STABILITY STUDY

At accelerated temperatures of 25°C and 60°C relative humidity, a stability analysis for an optimized batch formulation was conducted. At 0-, 15-, and 30-day intervals, the sample was analyzed. By measuring the gelling time, swelling, drug content, degree

of cross-linking, and in-vitro drug study, it was possible to monitor the physical stability of injectable scaffold on a regular basis [22].

III. RESULTS AND DISCUSSION

3.1.determination of oxidation degree

In this section, the effect of amount of sodium periodate on OD was investigated. Furthermore, the corresponded OD were calculated by using equations (1), and the results are summarized in Table 2. As can be seen, as expected, OD has increased by increasing the amount of periodate. Balakrishnan et al. also investigated the effect of periodate amount on OD and molecular weight of prepared alginate. They found that the OD is low at low amount of periodate and it can reach up to 85% at high amount of periodate. Our reported OD was 99%, which is in good agreement with our results. The synthesized OA were used to prepare the injectable scaffold [23].

Table 2: Results of optimized oxidized alginate

Batch	Sodium alginate: sodium metaperiodate Ratio	Colour	% Degree of oxidation Mean ± SD
OSA1	8:2	Yellowish White	53±0.45%
OSA2	7:3	White	66.66±0.22%
OSA3	6:4	White	86.51±0.5%
OSA4	5:5	White	90.52±0.22%

(Where n=3, Mean ± SD)

3.2. FTIR of alginate and optimized batch of prepared oxidized sodium alginate

Prepared oxidized alginate was characterized by FTIR. FTIR spectra of oxidized sodium alginate were taken using a FTIR spectrophotometer with KBR pellets to confirm oxidized sodium alginate peak of functional group present in sodium alginate. FTIR spectra of various batches of oxidized alginate shown in figure 1 and 2. As shown in figure, both SA and OSA revealed the broad hydroxyl stretching vibration absorption peaks in the range of 4000~3000 cm^{-1}. In detail, SA exhibited main characteristic peaks at 3276, 2890, and 1606 cm^{-1} owing to the C-H stretching vibration of the polysaccharide structure and the asymmetric and symmetric stretching vibration of -COO. Additionally, the absorption peaks at 1033 cm^{-1} were attributed to the C-O stretching vibration on the polysaccharide skeleton. In comparison with SA, OSA4 had a new characteristic peak at 1413.83 cm^{-1} which were assigned to the vibration absorption peak of the C=O bond on the aldehyde group. In addition, the hydroxyl stretching vibration absorption peak at 2890 cm^{-1} in the spectrum of SA became blue-shifted in the spectrum of OSA, implying a decline in the amount of -OH groups of alginates. These results indicated that the adjacent hydroxyl groups at the C-2 and C-3 positions on alginate uronic acid were oxidized to aldehyde groups by $NaIO_4$. Moreover, OSA4 displayed similar FT-IR spectroscopy results to the SA, which demonstrated the periodate ion only cleaved the C2–C3 linkage by the oxidation reaction, leading to the formation of a dialdehyde [24].

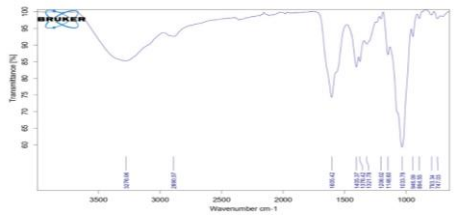

Figure 1 FTIR spectra of SA

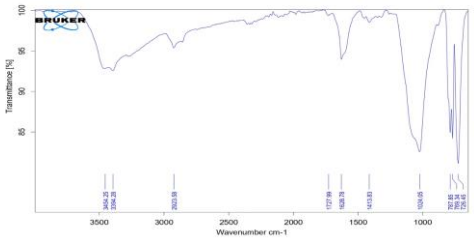

Figure 2: FTIR spectra of OSA4

3.3. Preparation Method of Injectable Scaffold

A central composite design was selected for optimization of oxidized sodium alginate and gelatin concentration. Different ratio of oxidized alginate in borax solution and gelatin in distilled water were optimized, which shows in table 3. Required quantities of polymers were taken from various batches for preparing injectable scaffold and optimization of various batches. Prepared scaffold was evaluated for gelling time, swelling ratio, drug content and degree of cross-linking. Based on this characterization, injectable scaffold batch was optimized. The results of the gelling time, swelling ratio, drug content and degree of cross-linking of optimization of drug to polymer weight ratio are shown in table 3.

Table 3:CCD design for optimization of oxidized alginate in 0.1 M borax and gelation in distilled water

Batch	**Oxidized sodium alginate %W/V**	**Gelatin in distilled water %W/V**	**Gelling time (sec)**	**Drug content (%)**	**Swelling ratio**	**%Degree of cross-linking Mean ± SD**
F1	10	10	183 ± 1.32	79.63 ± 0.5	7.29 ± 0.5	43.6 ± 0.21
F2	30	10	194 ±1	73 ± 1.32	8.83 ± 1.05	39.05 ± 0.07
F3	10	30	34 ± 1.05	75 ± 0.5	2.38 ± 1.63	51.2 ± 0.42
F4	30	30	50 ±1	80 ± 1.16	3.45 ± 1.32	35 ± 0.49
F5	10	20	87 ± 0.57	85 ± 0.32	3.84 ± 0.23	77 ±0.56
F6	30	20	56 ± 0.5	80 ± 0.6	5.12 ±0.86	55.35 ± 0.14
F7	20	10	198 ± 1.34	81.3 ± 0.65	8.33 ± 1.40	42 ± 0.3
F8	20	30	43 ± 0.6	76 ± 1.06	2.94 ± 0.12	36.15 ± 0.56
F9	20	20	88 ±1.2	81.28 ± 0.2	5.55 ± 0.86	75 ± 0.3
F10	20	20	88 ± 1.2	81.28 ± 0.2	5.55 ± 0.86	75 ± 0.3
F11	20	20	88 ± 1.2	81.28 ± 0.2	5.55 ± 0.86	75 ± 0.3
F12	20	20	88 ± 1.2	81.28 ± 0.2	5.55 ± 0.86	75 ± 0.3
F13	20	20	88 ± 1.2	81.28 ± 0.2	5.55 0.86	75± 0.3

Above results shows gelling time, drug content, swelling ratio and degree of cross-linking of different batches. Whenever volume of gelatin in distilled water was increased and oxidized alginate in borax was deceased, gelling time and swelling index were

decreased, drug content and degree of cross-linking were increased. It can be related to the higher amount of free amine groups or the rapid physical crosslinking of gelatin polymers in higher concentrations (Balakrishnan et al., 2005 a; Sarkar et al., 2014 a) [25]. So here batch F5 and batch F9 shows good gelling time, high amount of drug content, best swelling ratio and good cross-linking behaviour compare to the other batches. So, it had been concluded that these two bathes show optimum gelling time, drug content, swelling index and degree of cross-linking than other batches. Thus, further optimization of gelling time, drug content and swelling index of these two batches were performed by taking higher volume ratio of oxidized alginate in 0.1 M Borax solution and gelatine in distilled water. Batches containing oxidized alginate in 0.1 M Borax solution and gelation in distilled water solution based in injectable scaffold were further statically analysed by CCD design.

3.4. Statistical Analysis

For optimisation CCD design was employed to study the effect of independent variables on dependant variables (Y1) gelatine (second). All the batches were prepared according to the design and analysed using the design expert. The results of ANOVA along with response surface and contour plot generated for each response were given in table 4, 5, 6, 7, 8 and figure 1, 2, 3, 4.

Response 1: Gelling time

Polynomial equation for gelling time was found to be as follows;

Gelling time=349.563 + 4.11092 * A + -22.8891 * B + 0.0125 * AB + -0.11069 * A^2 + 0.37931 * B^2

Table 4: Result for ANOVA for gelling time

Source	Sum of Squares	Degree of Freedom	Mean square	F value	p-value	Decision
Model	37470.24	5	7494.05	68.00	<0.0001	significant
A-Oxidized alginate in borax solution(%w/v)	2.67	1	2.67	0.0242	0.8808	
B-Gelatin in water solution(%w/v)	3345.067	1	33450.67	303.52	<0.0001	
R^2	0.9798					

. From the ANOVA results the **Model F-value was found to be** 68.00 which implies the model was significant**. P-value was** less than 0.0500 indicate model terms are significant, it was concluded that all the factors had the prominent effect gelling time.

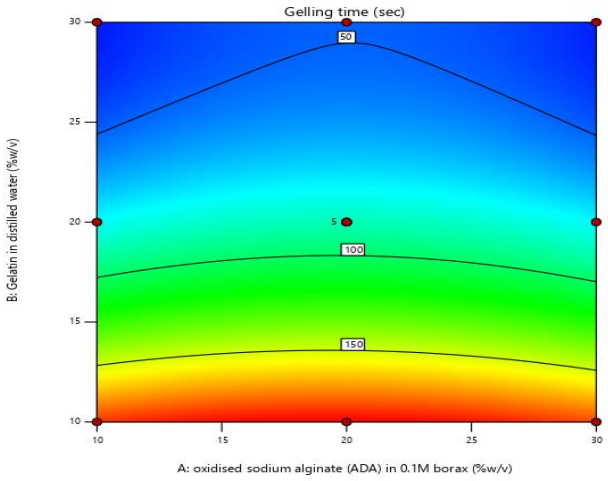

Figure 1: Response surface plot of effects of oxidized sodium alginate in 0.1 M borax (A) and gelatin in distilled water (B) on gelling time.

Response 2: Drug content

Polynomial equation for drug content was found to be as follows;

Drug content= 88.7436 + -0.5 * A + 0.261667 * B

Table 5: Result for ANOVA for drug content

Source	**Sum of Squares**	**Degree of Freedom**	**Mean square**	**F value**	**p-value**	**Decision**
Model	191.08	2	95.54	20.59	0.0003	significant
A- Oxidized alginate in borax solution(%w/v)	150.00	1	150.00	32.33	0.0002	
B-Gelatin in water solution(%w/v)	41.08	1	41.08	8.86	0.0139	
R^2	0.7046					

From the ANOVA results the **Model F-value was found to be** 20.59 which implies the model was significant. **P-value was** less than 0.0500 indicate model terms are significant, it was concluded that all the factors had the prominent effect drug content.

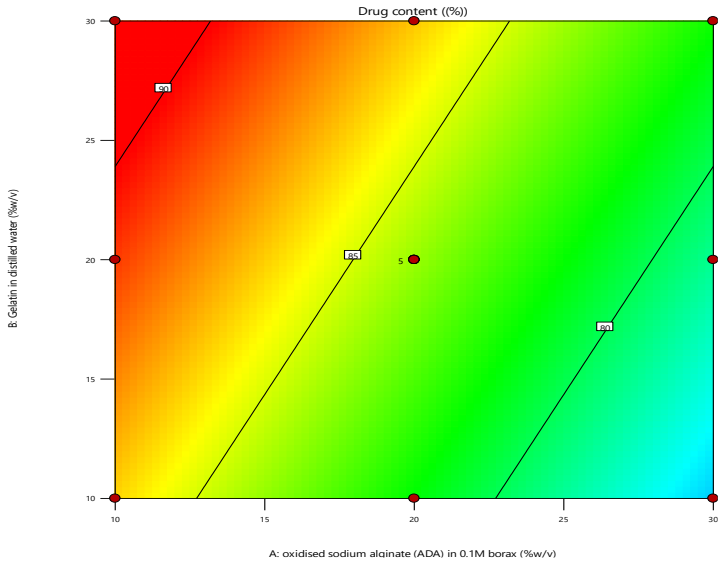

Figure 2: Contour plot of effects of oxidized alginate in 0.1 M borax (A) and gelatin in distilled water (B) on drug content.

Response 3: Swelling ratio

Polynomial equation for drug content was found to be as follows;

Swelling ratio: 8.50552 + 0.338195 * A + -0.449971 * B + -0.001175 * AB + -0.00624655 * A^2 + 0.00530345 * B^2

Table 6: Result for ANOVA for swelling ratio

Source	Sum of Squares	Degree of Freedom	Mean square	F value	p-value	Decision
Model	44.91	5	8.98	103.56	<0.0001	significant
A-Oxidized alginate in 0. M borax solution(%w/v)	2.52	1	2.52	29.08	0.0010	

B-Gelatin in water solution(%w/v)	40.98	1	40.98	472.46	<0.0001	
R^2	0.9867					

From the ANOVA results the **Model F-value was found to be** 103.56 which implies the model was significant. **P-value was** less than 0.0500 indicate model terms are significant. In this case A, B, A^2, B^2 are significant model terms, it was concluded that all the factors had the prominent effect drug content.

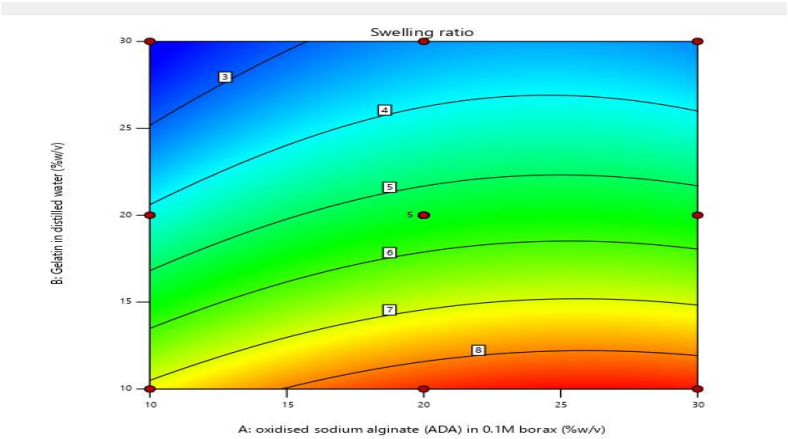

Figure 3: Contour plot of effects of oxidized alginate in 0.1 M borax (A) and gelatin in distilled water (B) on swelling ratio

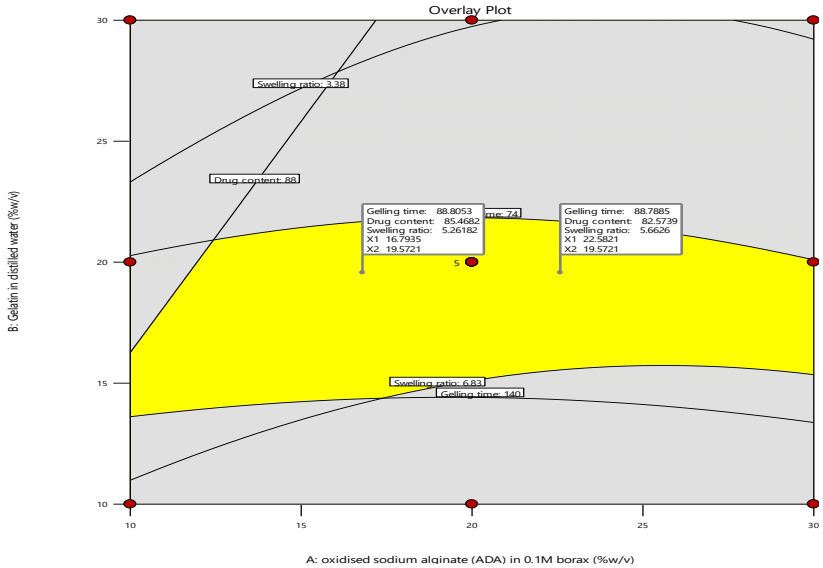

Figure 4: Overlay plot of oxidized alginate in 0.1 M borax and gelatin in distilled water

The overlay plot reflected that the "Yellow region" of the area shown in the Figure is the area of interest (experimental region). The three sets of independent variables values in the yellow region of the area were found. Near to the yellow region Formulation (F5) having OSA in borax (10%) (X1) and gelatin in distilled water (20%) (X2) was found best in the region of overlay plot and having higher desirability than other check point batches. So, it was selected as optimized batch.

Table 7: Check point batch F5

Check Point Batch CP1	
Concentration of oxidised sodium alginate in 0.1 M borax	10
Concentration of gelatin in distilled water	20

Responses	Predicted	Actual	% Error
Gelling	88.80	87 ± 0.57	0.020
Drug content	87.4	85 ± 0.35	0.022
Swelling	5.01	3.45 ± 0.84	0.3

Table 8: Check point batch F9

Check Point Batch CP2	
Concentration of oxidized sodium alginate in borax	20
Concentration of gelatin in distilled water	20

Responses	Predicted	Actual	% Error
Gelling	88.78	88 ± 0.627	0.87
Drug content	82.57	81.28 ± 0.029	0.032
Swelling	5.66	5.55	0.019

Here, we found that relative error was less than 2% for response like gelling time for the levels of batches, so equation obtain from selected responses are validated in selected ranges of variables. On the basis of contour plot and surface response study, optimization of formulation parameters was done and F5 was selected as suitable batch for preparation of injectable scaffold.

3.5. Scanning Electron Microscope sem

Here the morphology of hydrogels after freeze-drying was characterized by SEM is shown in figure 5. It was found that the hydrogels had the interconnecting porous structure. This structure made the hydrogels show largespecific surface area and allow the water molecules to enter the cross-linked network to keep highwater content. This structure is closely related to the crosslinking density. So, the pore size distribution changed with thevariation of hydrogel network. The higher the crosslinking density of hydrogels, the more compact of the network structure, the smaller the pore size, and also the higher the mechanical strength. In addition, the interconnecting pores in the hydrogel also providing the suitability of thematrix for cell growth [36].

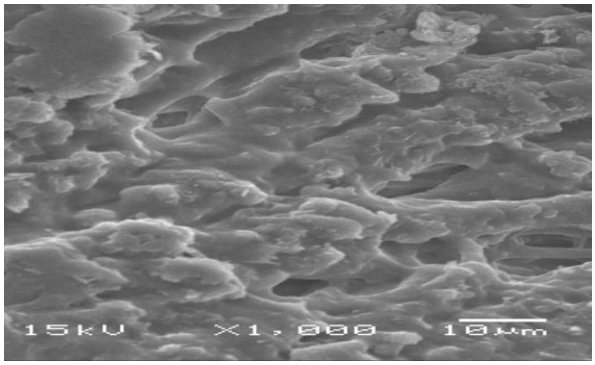

Figure 5: SEM images of dry oxidized sodium alginate/gelatin hydrogel scaffold

3.6. Injectability Test and Viscosity Measurement

The injectability of the hydrogels was tested by loading them into a 5 ml syringe and subjecting them to manual shear. Figure shows the good injectabilityof the developed hydrogels. Also indicatesa shear-thinning behavior, in which the gel is capable of forming a thick gel in situ when the shear force generated by injection is removed. Enhanced injectability was observed for thedeveloped hydrogels [38]. After running the sample in the viscosimeter, the viscosity was found to be 86412 ± 2.58 cP (centipoise).

Figure 6: injectability test of optimized batch

3.7. *In-vitro* Drug Release Study

In-vitro drug release study of optimized batch formulation was performed by using acetate buffer saline pH 7.4. Drug concentration was analyzed using UV spectrophotometer at 286 n at regular time interval [39]. Results of *in-vitro* drug release of injectable scaffold are shown in figure 7 and table 9. Drug release study is a crucial step for monitoring the developed formulation giving a clue about the drug behaviour after administration [40]. The release of RLA from its suspension was more than 85% in the first 3 h, which confirmed the suitability of the method and the dialysis membrane used. The combination of oxidized alginate and gelatin is expected to influence drug release; this could be elicited by the possible chemical interactions between them. After 120 hours, the in-vitro rug release for the injectable scaffold containing raloxifene hydrochloride was determined to be 89.5 ± 0.42%, indicating a slow and sustained release of the drug from the dosage form. The release rate of the drug from the scaffold was thus found to be lower if we included it because the drug was mixed with an oxidized sodium alginate cross-linked gelatin preparation for better drug conjugation. This burst effect after the first 24 h was previously discussed in literature and accredited to the presence of some drug on the surface of the formed scaffolds that permitted a rapid drug escape out from the matrix [41, 42]. As well, the presence of the porous matrix might assist in the ease of the penetration of the release medium inside the scaffold and the extraction of the drug [42, 44]. Moreover, it was observed that the type of the gelatin used possessed a significant impact on the burst drug effect within 24 h.

Table 9: Data for Drug release study at pH 7.4

Time (hrs)	**%Cumulative drug release**
0	0
0.5	1.59
1	3.18
1.5	5.6
2	9.32
3	9.37
4	9.7
5	10.02
24	22.95

48	37.97
96	70.9
120	89.5

(Where n=3, Mean ± SD)

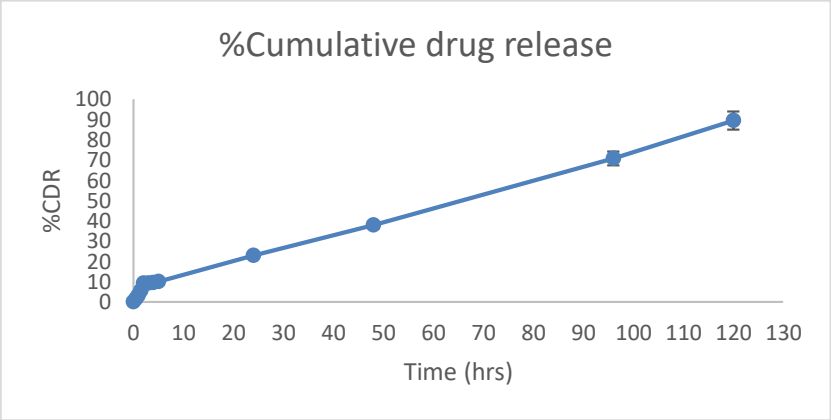

Figure 5.16: *In-vitro* drug release profile of raloxifene hydrochloride in PBS 7.4

3.8. Sterilization And Sterility Testing

Sterilization of two different solutions oxidized alginate in borax and gelatin in distilled water of optimized batch were performed by membrane filtration method using 0.22 ug/ml membrane filter paper under aseptic condition. After performing sterilization, sterility testing was performed for aerobic and anaerobic bacteria and fungi by using fluid thioglycolate and soya bean casein digest medium respectively [45,46]. The samples were incubated for 14 days at 30 to 35ºC in case of fluid thioglycolate medium and 20 to 25ºC in case of soya bean casein digest medium. There was no microbial growth (turbidity) observed in both mediums. The sterility results is shown in figure 10 and 11. We can infer from the results above that membrane sterilization was successful in sterilizing the final preparation. Both the fluid thioglycolate medium and the soya bean casein digest medium were found to be sterile because there was no growth in the negative control. The positive control's presence of turbidity proved that the medium can induce growth with a small number of bacteria [47].

Figure 5.17: Sterility testing for negative control of oxidized alginate in 0.1M borax and gelatin in distilled water in soya bean casein and fluid thioglycolate medium

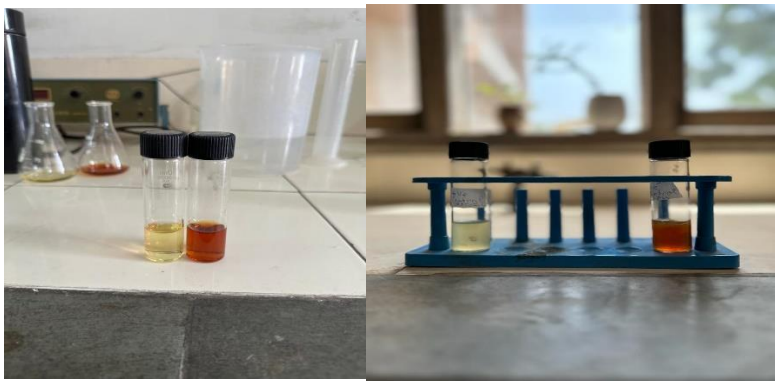

Figure 5.18: sterility testing for positive control of oxidized alginate in 0.1M solution and gelatin in distilled water for aerobic bacteria

1.1. Stability Study

Stability study was carried out for optimized batch at accelerated condition at 40°C ± 2°C temperature and 75% ± 5% relative humidity condition evaluated at different interval for gelling time, swelling ratio, degree of cross-linking, drug content *in-vitro* release study for 1 month. The results of sterility study of optimum batch are shown in table 10. From the stability studies of the optimized injectable scaffold for one month, it was found that there was not significant change in gelling time, degree of cross-linking, degree of swelling, drug content and drug release behavior. No significant change in any of above parameter during storage at 40°C ± 2°C temperature and 75% ± 5% relative humidity which indicated that the developed injectable scaffold was stable after one month [48].

Table 10: Stability study data for optimized batch

Sr No.	Parameters	Storage periods (Day) at 40°C ± 2°C temperature and 75% ± 5% relative humidity		
		Before storage	After 15 days	After 30 days
1	Gelling time (sec)	10	12	11
2	Degree of swelling measurement	2.37 ± 0.3	2.46 ± 0.26	2.41 ± 0.35
3	Degree of cross-linking (%)	71.05 ± 0.14	72.45 ± 0.55	71.15 ± 49
4	Drug content	94.65 ± 0.31	94.06 ± 0.23	95.01 ± 0.35
5	*In-vitro* drug release (%)	86.42 ±0.41	87.30 ± 0.80	86.29 ± 0.80

(Where n=3, Mean ± SD)

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