JOURNAL OF Applied Polymer SCIENCE

Fabrication and Characterization of Natural Origin Chitosan-Gelatin-Alginate Composite Scaffold by Foaming Method Without Using Surfactant

Chhavi Sharma,[1] Amit Kumar Dinda,[2] Narayan Chandra Mishra[1]

[1]Department of Paper Technology, Indian Institute of Technology Roorkee, Saharanpur Campus, Saharanpur, Uttar Pradesh 247001, India

[2]Department of Pathology, All India Institute of Medical Sciences, New Delhi 110029, India

Correspondence to: N. C. Mishra (E-mail: mishrawise@googlemail.com.)

ABSTRACT: A natural origin tripolymer scaffold from chitosan, gelatin, and alginate was fabricated by applying foaming method without adding any foam stabilizing surfactant. Previously, in foaming method of scaffold fabrication, toxic surfactants were used to stabilize the foam, but in this work, the use of surfactant has been avoided strictly, which can provide better environment for cellular response and viability. In foaming method, stable foam is produced simply by agitating the polymer (alginate-gelatin) solution, and the foam is crosslinked with $CaCl_2$, glutaraldehyde, and chitosan to produce tripolymer alginate-gelatin-chitosan composite scaffold. Microscopic images of the composite scaffold revealed the presence of interconnected pores, mostly spread over the entire surface of the scaffold. The scaffold has a porosity of 90% with a mean pore size of 57 μm. Swelling and degradation studies of the scaffold showed that the scaffold possesses excellent properties of hydrophilicity and biodegradability. *In vitro* cell culture studies by seeding L929 mouse fibroblast cells on scaffold revealed excellent cell viability, proliferation rate and adhesion as indicated by MTT assay, DNA quantification, and phase contrast microscopy of cell-scaffold construct. The natural origin composite scaffold fabricated by the simplest method i.e., foaming method, but without adding any surfactant, is cheap, biocompatible, and it might find potential applications in the field of tissue engineering. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: foam; polymer; composite; tissue engineering; scaffold; surfactant

Received 22 August 2011; accepted 19 March 2012; published online
DOI: 10.1002/app.37755

INTRODUCTION

The loss or failure of an organ or tissue is a frequent, devastating and costly problem in health care, occurring in millions of patients every year and to overcome this problem current clinical therapy available, comprises "autografting," "allogenic implants," and "prosthetics."[1,2]

Autografting can cause morbidity at harvested site with problems such as pain, infection and blood loss. Allogenic implants are attributed to immunogenic reactions in the patient's body while prosthetics are often subjected to fatigue, toxicity, and do not remodel with time. For all these reasons, there is an intense need of finding an alternative solution to these problems, and tissue engineering serves as a promising approach to address this need. Tissue engineering is an interdisciplinary field, applying a set of tools at the interface of biomedical and engineering sciences that uses living cells or attract endogenous cells to aid tissue formation or regeneration, to restore or maintain tissue function.[3,4] Tissue engineering involves three key elements: cells, scaffold, and growth factors (cell signaling molecules). Scaffold is a 3-D (3 dimensional) polymeric matrix, which plays a critical role in tissue engineering by acting as a temporary tissue-construct or building block for cell accommodation, proliferation, and differentiation.[5] It has been proved that the limiting step in trying to generate a tissue/organ is not the number of cells seeded, but the complexity of creating a 3-D polymeric network, i.e., scaffold.[6] There are many techniques available for the fabrication of tissue engineering scaffolds, and these include "solvent casting and particulate leaching,"[7] "phase separation,"[8] "emulsion freeze drying,"[9] "foaming,"[10–12] "electrospinning,"[13] "solid free form fabrication technique"[14] etc. All these scaffold fabrication techniques have several advantages as well as disadvantages. Thus, there is a need to find new methods of scaffold fabrication or improving and exploring the existing methods, so

that one can fabricate scaffold easily and economically for its wide-spread applications in tissue engineering. Here, we aim to fabricate a natural origin scaffold by foaming method where foam will be produced by agitation.

The idea behind this work of scaffold fabrication was inspired by a video clipping from the National Geographic Channel, which shows how frogs, during mating, make nest of foam wherein the fertilization takes place to spawn tadpoles. In the clip, it was shown that during mating, the female frog secretes mucous containing eggs, and the male and female frogs, with their hind legs, whip up foam which hardens with time and looks like crust. Male frog adds sperm to the foam nest where fertilization takes place. The foam nest provides protection to the fertilized eggs, and also aids in keeping the eggs moist and supply oxygen to eggs, which facilitate them to survive. After 5 days, the tiny tadpoles slip out from the foam nest and drop into the pond where they complete their development. By seeing this video clip, it came across in mind that whether a similar foaming system can be generated *in vitro* by using a system of polymer or polymer combinations, which might facilitate in accommodating living cells, exactly in the same way as the foam nest accommodating frog eggs.

Therefore, our first step is to hunt for a system of polymer or polymer combinations that can generate efficient foam upon shearing without any toxic foam stabilizing surfactant and be stable for a long time.

We started the work with natural polymers, agarose, alginate, gelatin, and chitosan, and found a polymer combination of alginate and gelatin, which can produce foam without any surfactant and the foam is highly stable. Alginate was also used previously to fabricate scaffold by foaming method, but to stabilize the foam, toxic surfactant was added to the alginate solution.[10]

For applying a scaffold in tissue regeneration, the scaffold should have some desired properties: it should be biocompatible, biodegradable, it must aid in cell adhesion, proliferation and differentiation. By using a single polymer system, one cannot impart all the properties to the scaffold, and that is why scientists are using a composite system where more than one polymer is used to fabricate the scaffold. Two or three polymers in combination, should impart properties of the individual polymers to the scaffold, which in turn might facilitate cell adhesion, proliferation, and differentiation. And, in this work, we studied on the fabrication of a composite scaffold based on natural origin polymer system alginate-gelatin-chitosan. The use of natural origin polymers is one of the present trends in tissue engineering applications, because natural origin materials are biocompatible and biodegradable, and have been demonstrated to promote healing at a faster rate and more importantly, they are expected to exhibit greater compatibility with human tissues.[11] Thus, the inclusion of chitosan in scaffold is beneficial as chitosan is often associated with the structural similarities with glycosaminoglycans—an important component of extra cellular membrane. Chitosan is biodegradable to normal body constituents and thus, it is highly useful in wide range of applications in tissue engineering.[15,16,17] It binds to mammalian and microbial cells aggressively (antibacterial). It accelerates the formation of osteoblast which is responsible for bone formation.[18] It is to mention that the foam producing capacity of chitosan is very low as confirmed from our experiments. The scaffold with an informational function, e.g., material containing the arginine-glycine–aspartate (Arg–Gly–Asp) sequence which facilitates cell attachment, should be better than non-informational synthetic polymers.[7,8] Gelatin used here, is a hydrolysed form of collagen and it is well known that the interactions of glycosaminoglycans with collagens and other glycoproteins in extracellular matrix play important roles in cell adhesion and extracellular matrix assembly. Since gelatin is basically denatured collagen, it presumably retains informational signals, such as the Arg–Gly–Asp sequence.[8] It has excellent cell adhesion property and its aqueous solution produces sufficient foam as observed by us. Alginate, a naturally occurring polysaccharide, can produce foam vigorously and is biocompatible, but it lacks specific cellular interactions, which limits its potential for wider applications.[10] On the other hand, alginate, in presence of multivalent cations, produces mechanically strong scaffold which can be handled easily in comparison with chitosan and gelatin scaffold which are difficult to handle because they are not so strong mechanically.[19,20] Thus by using alginate as one of the component of the scaffold, the mechanical strength of the scaffold is improved for its application in tissue engineering.[21] Besides providing mechanical strength, alginate also plays a significant role in transmitting preliminary mechanical signals to the cells and developing tissue.[22] The composite scaffold, if formed from these three polymers, should provide various functional groups like $–NH_2$, –COOH, –OH, etc., which are responsible for cell adhesion, and might provide proper chemical cues to cells for proliferation and differentiation.

Therefore, a composite scaffold, if prepared with a combination of chitosan, alginate and gelatin, should exhibit the characteristics of cell adhesion, proliferation and differentiation, which are important for tissue regeneration. Moreover, the scaffold can be handled easily. To the best of our knowledge, till date, there is no study on fabricating composite scaffold using the natural origin polymer combination (tripolymer) of chitosan-gelatin-alginate, and we aim to fabricate this composite scaffold.

Foaming method—generating foams upon shearing, will be used here for fabricating the scaffold. This method is simple and economic, and previously it was used by Eiselt et al.[10] where they used surfactants to stabilize the foam. Even though the foam scaffold fabricated by foaming method, is washed several times, but a trace amount of surfactant molecules always remain within the scaffold, which reduce cell viability.[23]

In this study, we restrict the use of any surfactant to stabilize the foam while using foaming method. To the best of our knowledge, till date, there is no work on "foaming method without using any surfactant."

MATERIALS

Chitosan (MW 100,000-300,000), was obtained from Sigma, St. Louis, MO. Alginate (300 kDa) were purchased from Acros Organics, New Jersey. Sodium bicarbonate, calcium chloride, and glacial acetic acid were obtained from Qualigens Fine

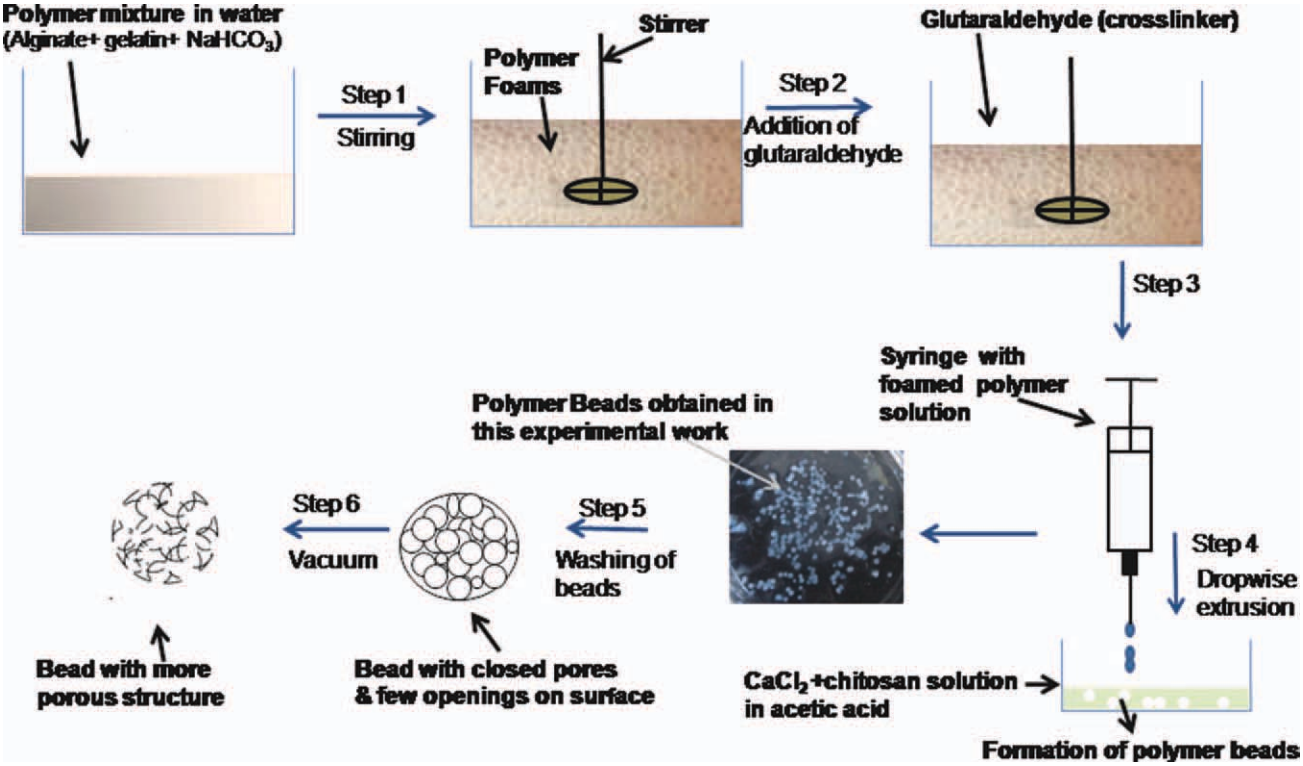

Figure 1. A schematic showing the whole process used in this study, to fabricate tripolymer composite scaffold from chitosan, alginate, and gelatin (the beaded scaffold in a glass petri plate shown here, were prepared in our laboratory). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Chemicals, Mumbai, India. Gelatin was obtained from Merck Specialities, Mumbai and glutaraldehyde was purchased from SD Fine-Chem 3-(4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide) (MTT), Dulbecco's modified eagle medium (DMEM), and phosphate buffer saline (PBS) were obtained from Sigma. Fetal bovine serum was received from Hyclone and L929 fibroblast cell lines were received from NCCS, Pune, India. F127, a polyoxypropylene-polyoxyethylene copolymer type non-ionic surfactant (trade name: Pluronic® F127 and generic name: poloxamer 407) was received from Sigma. Double distilled water was prepared in our lab and the same was used for preparing the polymer solution as well as for washing the scaffold.

METHODS

Analysis of Foam Stability of Polymer Mixtures

In this study, polymer mixture in water produces foam on agitation, which in turn produces 3-D foam scaffold followed by crosslinking. The important consideration in foaming method, is to check the stability of the foam of the polymer or polymer mixtures which will used for scaffold fabrication. To check the foam stability, 50 ml of polymer solution was taken in a graduated beaker. The solution was stirred continuously for 10 h at an agitator-speed of 500 rpm (Remi, India), while foam was produced vigorously: the final volume of foam solution in the beaker was noted. Then the beaker was left standstill and the decrease in foam volume was measured at different time intervals, which gives a measure of the stability of the foam.

In this work, the foam stability has been measured for different polymer systems: system-1 consists of mixture of alginate + sodium bicarbonate ($NaHCO_3$) + glutaraldehyde; system-2 consists of mixture of alginate + gelatin + $NaHCO_3$ + glutaraldehyde; system-3 consists of chitosan solution in acetic acid + sodium bicarbonate + glutaraldehyde; and system-4, alginate + gelatin + chitosan + $NaHCO_3$ + gluteraldehyde.

Foam stability of all the four polymer systems, was determined by repeating the experiment three times independently.

Scaffold Fabrication by "Foaming-Technique"

Figure 1 explains the scaffold fabrication by foaming method without using any surfactant. Solutions of alginate (2 wt %) and gelatin (5 wt %) was prepared in double distilled water, and mixed in the ratio of 1 : 1. Thereafter, 0.9% $NaHCO_3$, a gas generating agent, was added to this mixture and was continuously stirred for 2 h. Next, 0.025% glutaraldehyde solution (crosslinker for chitosan and gelatin) was added to the alginate-gelatin-$NaHCO_3$ mixture (volume ratio 1 : 40), and allowed to react/crosslink with the mixture for 10 h under continuous agitation. Because of the continuous agitation/stirring, foam was generated extensively. Scaffold in bead form, was formed by drop-wise extrusion of the foam into a solution containing chitosan, acetic acid and $CaCl_2$—a crosslinking agent for alginate. During the bead formation, acetic acid reacts with $NaHCO_3$ to evolve CO_2 from inside the bead—promoting high porosity in the bead scaffold. The beads were allowed to remain in solution for 12 h, to facilitate efficient crosslinking among alginate, gelatin and chitosan and washed with double distilled water several times to remove excess of glutaraldehyde. Finally, the beads were exposed to vacuum for 12 h to create more porous structures inside. The sizes of 50 beads were measured individually by using microscopy imaging and calibration, and average

size of the beads was determined. Here, the solution containing chitosan, acetic acid and $CaCl_2$, was prepared by mixing a "solution of 2 wt % chitosan in 1 wt % acetic acid" with a "solution of 0.1M $CaCl_2$", in the volume ratio of 10: 1.

In parallel to experiment as mentioned above, beads were also prepared by the same (foaming) method, but by adding F127 (0.0025 wt %) surfactant in the alginate-gelatin-$NaHCO_3$ mixture to study the effect of surfactant on cell viability.

SCAFFOLD CHARACTERIZATION

Scaffold Morphology

Scaffold morphology was studied by using environmental scanning electron microscope (ESEM) (Quanta 200, FEI, Netherland). Since the ESEM technique does not require dehydration of samples, the scaffold in their hydrated state was directly observed by ESEM. To study the surface morphology of the scaffold, 30 beads were examined through ESEM at the saturation pressure of water vapor (1 torr) and an accelerating voltage of 15 KV. Each time, five beads prepared from independent experiments, were examined. Cross-section of the scaffold was also examined after sectioning or cutting the beads with a sharp razor blade. The size of the pores was determined for the 30 beads, and the average pore size was calculated.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is an important tool to carry out semi-quantitative functional analyses and to investigate intermolecular interaction between different compounds. Infrared (IR) spectra were recorded with a Nexus Thermo FTIR spectrophotometer (Nicolet Co.). The samples were prepared by processing compressed potassium bromide disks. The ratio of sample to KBr used for performing FTIR analysis was 1 mg sample/900 mg KBr.

Porosity

Porosity is defined as the percentage of void space in a solid and it is a morphological property independent of the material.[24] The volume of pores within beads was determined by liquid displacement method by measuring the total volume of porous beads and the amount of solvent required to fill the porous component.[10] In brief, individual beads were placed in a graduated cylinder filled with a known volume of ethanol (V_1). The total volume following bead immersion was recorded (V_2). The beads were removed with the volume V_T whereby, solvent is entrapped in the pores, and the remaining volume of ethanol in the graduated cylinder was denoted by (V_3). The total volume (V_T) of the beads was calculated according to equation (**1**)

$$V_T = V_2 - V_3 \tag{1}$$

The porosity χ was determined using the following equation:

$$\chi = \frac{V_1 - V_3}{V_T} \times 100 \tag{2}$$

The porosity-determination experiment was repeated for six times, and the mean porosity was calculated.

Swelling Property

The swelling gives a measure of hydrophilicity and it is defined by the following equation:

$$S = (w_s - w_d)/w_d) \times 100\% \tag{3}$$

where, S = percentage swelling, w_s = wet weight of the bead-scaffold after swelling, and w_d = weight of the bead-scaffold after drying.

Briefly, the beaded scaffolds were immersed in PBS (pH = 7.4) at room temperature for an hour. After 1 hour immersion, in every 10 minutes, a known quantity of the bead-scaffolds was retrieved and excess water was removed using filter paper. The wet weight of the scaffold (w_s) was determined using an electronic balance, after which the swollen scaffold was dried in an oven at 50°C for half an hour, and the dry weight (w_d) was measured. Each time, the percentage swelling (S) was calculated from the values of w_s and w_d. The experiment was carried out until the time point, where no further swelling of beads was observed and the equilibrium point of swelling of beads was determined. The experiment was repeated for six times individually.

In Vitro Biodegradability

A known quantity (w_i = 0.5 g) of freshly prepared beads was taken in a tissue culture plate in triplicate. The beads were immersed in phosphate-buffered saline PBS (pH = 7.4), and kept at 37°C in a water bath. To determine the degradation profile, the beads were removed after 1st, 3rd, 5th, 7th, 14th and 21st day and weighed (w_f). The percentage weight loss was calculated by the following equation.

$$\%\text{weight loss} = \frac{w_i - w_f}{w_i} \times 100 \tag{4}$$

The pH value of the resultant PBS solution was also measured using pH meter at different time intervals.

IN VITRO CELL CULTURE STUDIES

Cytotoxicity Assay: MTT Assay

L929 mouse fibroblast cell line was maintained in DMEM with 10% fetal bovine serum at 37°C in a CO_2 incubator (BINDER, Germany). L929 cell suspension in complete DMEM media (200 μl) with cell density of 5×10^4 cells/ml was plated in 96 well plate in triplicate such as the cells per bead is 10,000. Scaffold bead of size 1 $\pm$ 0.3 mm was placed in the wells with 1 bead/well and incubated for three time point viz. 24 h, 48 h, and 72 h at 37°C in CO_2 incubator in order to test the toxicity of leachable from the scaffold towards L929 fibroblast cells. A control, with cells grown in the presence of complete culture medium, was also included. After the incubation points, the beads were removed from wells and the media was discarded. 90 μL of fresh complete media was added to the wells and 10 μL of MTT solution (5 mg/ml stock in PBS) was added to the media for final volume of 100 μL. The plate was incubated at 37°C for 4 h until purple formazan crystals were formed due to reduction of MTT by viable cells. The media was discarded and 200 μL of dimethyl sulphoxide was added to the wells to

Table I. Comparison of Foam Stability of Different Polymer System to Determine Their Potential Use in Scaffold Fabrication by Foaming Method

System	Foam producing capacity	Remarks
System-1: Chitosan (2 wt %)+ sodium bicarbonate(0.9%) + glutaraldehyde (0.025 wt %)	Only scarce amount of foam was produced that started to collapse within 10 min.	Not suitable for fabricating foam scaffold
System-2: Alginate (1 wt %) + gelatin (5 wt %)+ chitosan (1 wt %)+$NaHCO_3$(0.9%)+ gluteraldehyde (0.025 wt %)	Insoluble precipitate formed	Not suitable for fabricating foam scaffold
System-3: Alginate (1 wt %) + sodium bicarbonate (0.9%) + glutaraldehyde (0.025 wt %) in solution in water	Sufficient quantity of foam produced Foam volume started reducing sharply within 10 min, and disappeared almost completely in 30 min	Not so suitable to fabricate a foam scaffold
System-4: Alginate (1 wt %) + gelatin (5 wt %) + sodium bicarbonate (0.9 %)+ glutaraldehyde (0.025 wt %) in solution in water	Large volume of foam produced Foam volume remained almost constant for about 30 min	Suitable for fabricating a foam scaffold

dissolve the formazan crystals. Absorbance was taken in a Bio-rad plate reader at 490 nm with the substraction for plate absorbance at 650 nm. Cell viability was expressed by the following equation:

$$\%\text{cell viability} = \frac{I_{\text{sample}}}{I_{\text{control}}} \times 100 \quad (5)$$

where, I_{sample} = absorbance of the wells incubated with sample (bead-scaffold) taken in triplicate and I_{control} = Mean absorbance of control wells (wells incubated with cells but without bead).

The results were compared to the control wells incubated with cells in which no sample beads were present.

Cell Morphology Studies

Morphology of the scaffold after cell culture was studied in their wet form using ESEM (Quanta 200, FEI, Netherland). For this purpose, cells over the scaffold were fixed with glutaraldehyde (2.5%) at 4°C for 6 h and then rinsed with PBS and then observed under ESEM.

Phase-contrast microscopy for the acquisition of cell images were carried out with cultured L929 fibroblasts on composite scaffold. After the incubation of cells, the cell-scaffold constructs in the culture medium were viewed under an inverted phase-contrast microscope (Olympus, Tokyo).

Visualization of Cells on 3-D Scaffolds by Calcein AM Staining

Scaffold beads of size 1 ± 0.3 mm were taken in 12 wells tissue culture plate in triplicate with 10 beads/well. 1 ml of L929 cell suspension in complete DMEM media with cell concentration of 1×10^5 cells/ml, were added to the beads in wells. Thus the number of cells per bead is 10,000. The cells were incubated in a 5% CO_2 incubator for 10 h so as to allow cells to adhere to the beads. After the incubation time, the media was discarded. Calcein green AM at a concentration of 10 μM was prepared by dissolving it in dimethyl sulphoxide (less than 0.01%) and adding it to fresh DMEM media. The cells were then incubated with calcein AM containing media for 1 h to load calcein AM into the cells. Inside the cells, calcein-AM is hydrolyzed by endogenous esterases into the highly negatively charged green fluorescent calcein, which is retained inside the cytoplasm. After the incubation time point, the media was discarded and the cells were thoroughly washed with PBS (pH = 7.2). Around 8-10 beads were then taken on a clean microscopic slide and mounted with glycerol: PBS (1: 1 ratio), and observed under an upright fluorescence microscope (Nikon eclipse E_{600}, Wavelength = 450 nm) using a green filter (wavelength = 490 nm). The cell-scaffold constructs were sectioned and the cell adhesion and viability were also observed in the inner regions of the scaffolds. The images were captured using an Olympus DP71 CCD camera attached to the microscope.

Cell Proliferation by DNA Quantification

L929 mouse fibroblast proliferation on composite scaffold was determined by DNA quantification using a NanoDrop ND-1000 spectrophotometer (Thermofisher). For this purpose, beads of size 1 ± 0.3 mm were plated in each well in six well plate with 6 beads/well. The 3 ml of L929 cell suspension in complete DMEM media with cell density of 2×10^4 cells/ml was plated in each well, and thus the concentration of the cells/bead were maintained at 10,000. The plates were incubated at 24 h, 48 h, and 72 h, in a CO_2 incubator at 37°C and 5% CO_2. Total cellular DNA from cell-scaffold construct was isolated by using alkaline lysis method.[25] The DNA content extracted was measured by nanodrop ND- 1000 spectrophotometer at the absorbance of 260 nm.

RESULTS AND DISCUSSION

Analysis of Foam Stability of Polymer Mixtures

Foam stability of the polymer mixture is very important because the foam made of polymer, itself produces scaffold upon cross-linking among the polymer molecules. Therefore, if the foam itself is unstable, it cannot produce foam scaffold. The foam stability of different polymer systems was studied and their foam

Table II. Foam Stability with Time (50 ml Polymer Solution (Without any Foam) Stirred Continuously for 10 h to get Foams)

Time (min)	System-3: Volume of foamed solution of alginate + sodium bicarbonate + glutaraldehyde (ml)	System-4: Volume of foamed solution of alginate + gelatin+ sodium bicarbonate + glutaraldehyde (ml)
0	100 ± 0.5	100 ± 0.5
5	100 ± 0.5	100 ± 0.6
10	82 ± 1.4	100 ± 0.7
15	78 ± 1.8	100 ± 0.5
20	70 ± 1.6	100 ± 0.6
25	64 ± 1.5	100 ± 0.9
30	54 ± 1.3	100 ± 1.3
60	50 ± 1.2	96 ± 1.5
180	50 ± 1.1	92 ± 1.8
300	50 ± 0.7	86 ± 2.4
420	50 ± 1.1	70 ± 2.5
720	50 ± 0.8	52 ± 2.7

Results are expressed as mean ± S.D. ($n = 3$)

producing capacity is briefed in Table I. Different concentrations of alginate (1 wt % to 3 wt %), gelatin (1 wt % to 5 wt %) and chitosan (1 wt % to 3 wt %) was used to get the concentrations that produces most stable foam. The result of only the most suitable concentrations of polymers that gives foam was shown in Table II.

Upon stirring the system-1 (chitosan+ sodium bicarbonate + glutaraldehyde), only scarce amount of foam was produced that started to collapse within 10 min. Therefore, if we use this system for making foam scaffold, we need to finish crosslinking reaction among the polymer molecules present in foam, within a very short time after foam formation, which would be very difficult because of slow diffusion of crosslinking molecules into the interior of the foam. Thus, the system-1 is not so suitable to fabricate a foam scaffold.

Upon stirring the polymer systm-2 (alginate + gelatin+ chitosan + $NaHCO_3$ + gluteraldehyde), an insoluble precipitate resulted. Thus, polymer system-2 was also not suitable for scaffold fabrication by foaming method. The precipitate is possibly the result of crosslinking among the polymer and glutaraldehyde molecules.

Upon stirring the system-3 (consisting of alginate + sodium bicarbonate + glutaraldehyde in solution in water), sufficient quantity of foam was produced, but the foam volume started reducing sharply within 10 min, and disappeared almost completely in 30 min (Table II, Figure 2). In this case also, there is not enough time for the crossliking molecules to diffuse into the scaffold and therefore, this system is also not so suitable to fabricate a foam scaffold.

Upon stirring the polymer-system-4 (consisting of alginate+gelatin+sodium-bicarbonate+ glutaraldehyde in solution in water), again a large volume of foam were produced but in this case the foam volume remained almost constant for about 30 min, and then the volume was being reduced gradually and slowly (Table II, Figure 2). Therefore, for system-4, if we perform the crosslinking reaction immediately after foam formation, then there will be sufficient time available for the crosslinking-molecules to diffuse into the scaffold and there will good crosslinking—a high possibility of obtaining foam scaffold without losing the structural integrity of the foam. Gelatin used in the polymer system 4, might be responsible for stabilizing the foam for a long time. The use of gelatin as foam stabilizer was also reported by Schrieber and Gareis in 2007.[26]

Therefore, out of these four polymer system, polymer-system-4 has the potential to form stable foam and thus polymer system-4 has been considered for fabricating scaffold, in this study.

Scaffold Fabrication by "Foaming-Technique"

Here, we have fabricated a tissue engineering scaffold in bead form from the natural origin polymer combination of chitosan-gelatin-alginate by applying foaming method, without using surfactant. The size of beads is in the range of 0.5 mm to 2 mm, and the average bead-size was determined to be 1.19 ± 0.46 mm.

Upon stirring the alginate-gelatin-$NaHCO_3$ mixture (Step 1 in Figure 1), alginate was supposed to interact weakly with gelatin due to oppositely charged amino and carboxylate groups of gelatin and alginate, respectively. On addition of glutaraldehyde to this polymer mixture (Step 2 in Figure 1), aldehyde groups present in glutaraldehyde, was believed to crosslink with the amino groups of gelatin present in the mixture. The proposed mechanism of crosslinking was shown in Figure 3.

Scaffold-beads were formed, upon dripping the foamed-mixture (alginate-gelatin-$NaHCO_3$-glutaraldehyde) into the solution containing chitosan in acetic acid and $CaCl_2$ (Step 3 in Figure 1). Here, the free aldehyde groups of glutaraldehyde molecules, were supposed to crosslink with amino groups of chitosan by

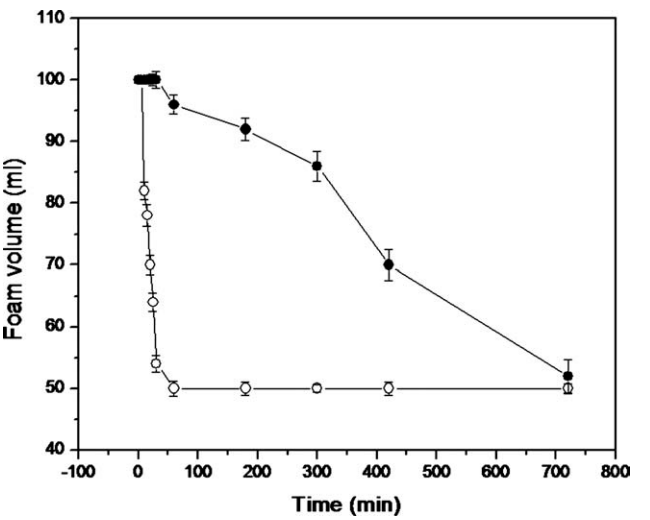

Figure 2. Change in foam volume with time to characterize foam stability of two different polymer systems—System 3: mixture of alginate + sodium bicarbonate + glutaraldehyde in water (-O-) and System-4: mixture of alginate + gelatin + sodium bicarbonate + glutaraldehyde in water (-●-).

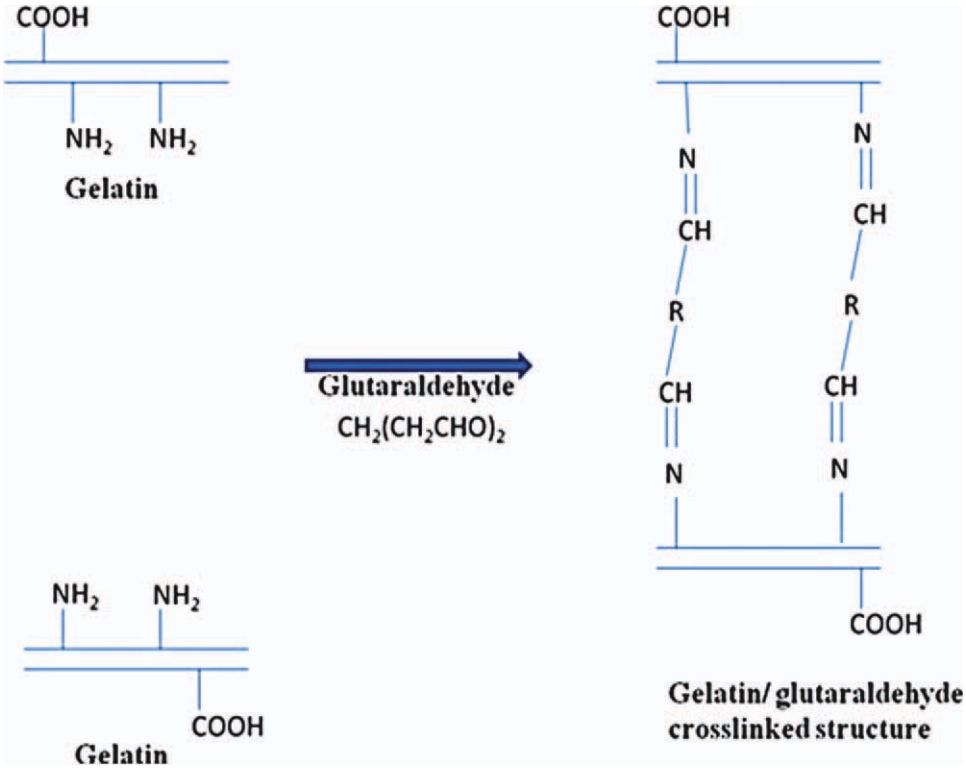

Figure 3. Schematic showing possible reaction between gelatin and glutaraldehyde. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

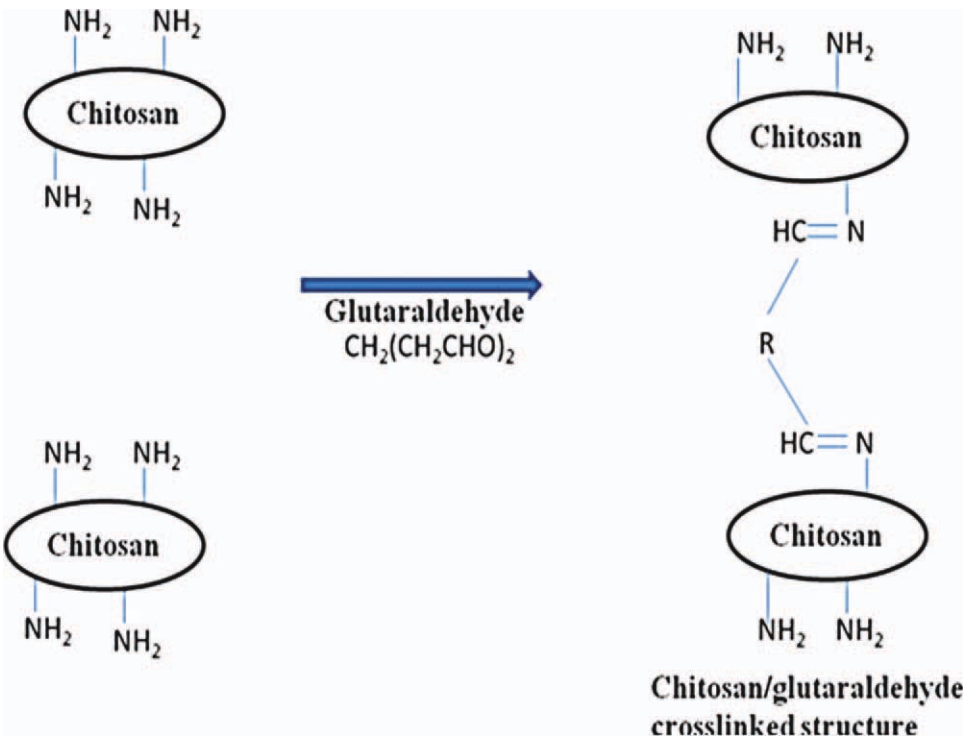

Figure 4. Schematic showing possible reaction between chitosan and glutaraldehyde. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

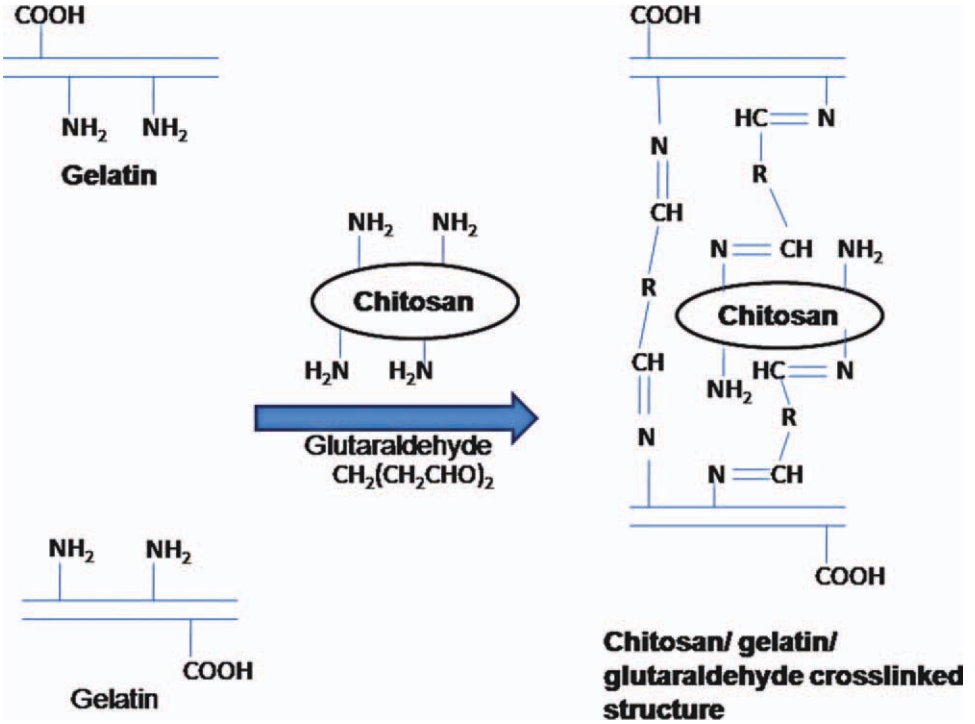

Figure 5. Schematic showing possible reaction of gelatin cross-linked with glutaraldehyde in the presence of chitosan. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

forming imine bond, following the crosslinking reaction as proposed in Figure 4. The formation of imine bonds between aldehyde groups of glutaraldehyde and amine groups of chitosan by covalent linkage, was also reported earlier.[27]

Glutaraldehyde crosslinks both chitosan and gelatin in different stages of scaffold formation, and the proposed crosslinking reactions among chitosan, gelatin and glutaraldehyde, was depicted in Figure 5. A similar type of crosslinking was also proposed by Ma et al., 2003 between collagen (hydrolysed form of gelatin) and glutaraldehyde in presence of chitosan.[28]

In the 4th step of scaffold fabrication (Step 4 in Figure 1), alginate interacts with both Ca^{2+} ions and chitosan, and beads are formed. Ca^{2+} ions present in the solution of acetic acid containing chitosan and $CaCl_2$ (Step 4 in Figure 1), served to ionically crosslink alginate, while the acetic acid reacted with the sodium bicarbonate to release CO_2, according to the reaction given below (equation 6).

Some of the gas is released immediately and some is entrapped inside the scaffold. The exposure of the beads to vacuum (Step 6 in Figure 1) drew entrapped gas bubbles out and created a highly interconnected porous network with openings on the surface of the matrix.

$$NaHCO_3 + CH_3COOH \rightarrow CH_3COONa + H_2O + CO_2 \uparrow \quad (6)$$

Some of the chitosan molecules present in gelling solution (Step 4 in Figure 1), might also bond to alginate weakly, forming polyelectrolyte complex due to electrostatic interactions between oppositely charged groups (—COO^- of alginate and —NH_3^+ of chitosan). The polyelectrolyte complex formation between chitosan and alginate, was also reported previously.[29]

Ultimately, a complex 3D network of alginate, gelatin and chitosan is developed, because of the crosslinking among these polymer molecules. Thus, successfully, a stable natural composite-polymeric-scaffold was fabricated by foaming technique and subsequent crosslinking reactions.

It is well known that free glutaraldehyde molecules are toxic to the cells, but if somehow, the aldehyde groups are blocked by reacting with some other molecules then it will not show toxicity.[30,31] Here, we have used very low concentration of glutaraldehyde (0.025 wt %), so that all the aldehyde groups participate in crosslinking reaction with gelatin and chitosan, and there are no free aldehydes available to show any toxic effects. The scaffold was rinsed with double distilled water 20 times, to remove any extra glutaraldehyde, if remained within the scaffold.

Morphology of Scaffold Surface and Cross-Section

The Figure 6(A-E), ESEM images of the surface of the scaffold, show that there are pores spread over the entire surface of the scaffold.

The ESEM images, at higher magnification, indicate the presence of open and interconnected pores of different sizes on the surface of the scaffold [Figure 6(C-E), arrows indicates interconnectivity between pores]. The pore sizes of each of the scaffold (beads) are in the range of 10-100 μm and the mean pore size as determined is to be 56.1 $\pm$ 34.1 μm. This pore size range was reported to be suitable for fibroblast growth over chitosan based scaffold.[31] Cross section of the scaffold was also examined

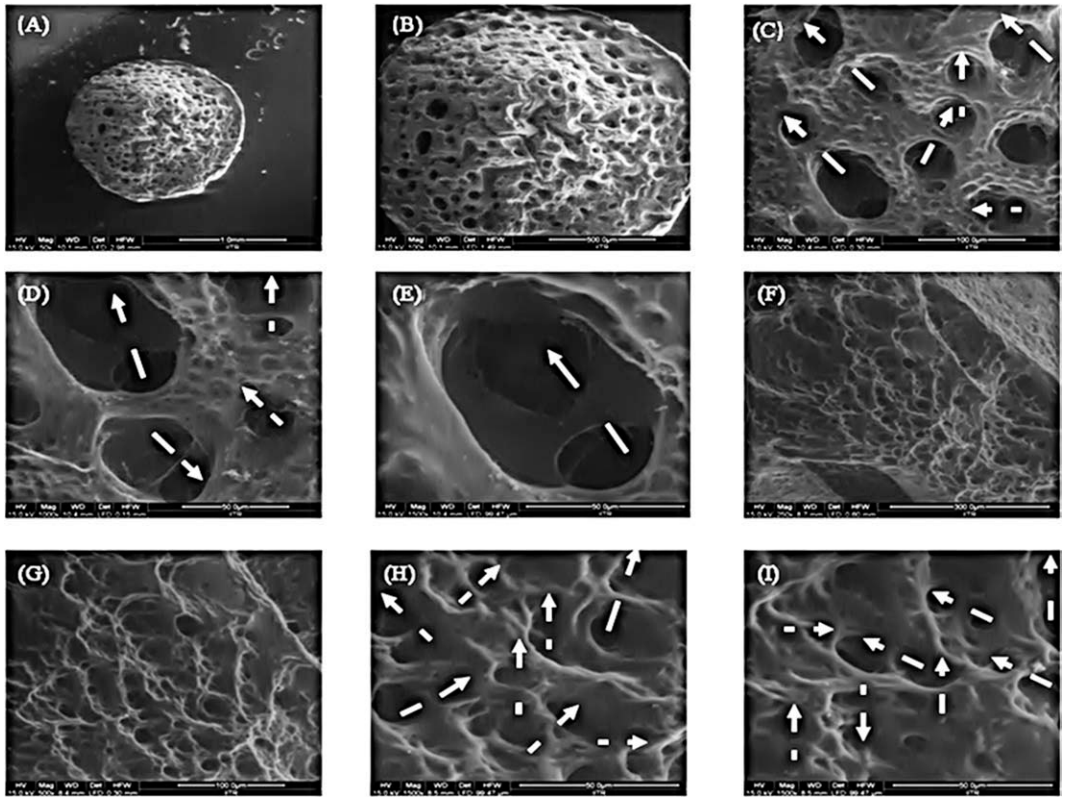

Figure 6. Scaffold morphology by ESEM analysis. (A, B) ESEM images of whole scaffold at 50× and 100×; (C-E) ESEM images of a portion of surface of the scaffold at 500×, 1000×, and 1500×, respectively. (F-I) Cross-section of the scaffold at 250×, 500×, and 1500×, respectively. The arrows indicate the interconnectivity between the nearby pores in the scaffold.

and the ESEM images of the cross-section are shown in Figure 6(F-I). Sectioned beads clearly show porous structures with interconnectivity as indicated by the arrows in the figure. Thus, the 3-D architecture of the scaffold possesses sufficiently porous morphology and has similar structure like a sponge.

FTIR

FTIR spectra of: (a) chitosan; (b) gelatin; (c) alginate/gelatin/chitosan composite beads; (d) alginate, were shown in Figure 7. The IR spectra of chitosan confirms the presence of –OH and N-H stretching vibration at 3442 cm^{-1}, in which the –OH stretching vibration are overlapped by N-H stretching. The absorption of C-H stretching of methyl or methylene group of chitosan is at 2921 cm^{-1}. The peak at 1641 cm^{-1} corresponds to N-H bending vibrations of secondary amide. The C–O–C, C–O and C–OH bending was visible at 1156 cm^{-1}. The C–H bending was seen at 1378 cm^{-1}.

IR spectra of gelatin showed peak at 3443 cm^{-1} due to N–H stretching of secondary amide, C=O stretching at 1640 cm^{-1}, N–H bending at 1543 cm^{-1} and N–H out-of-plane wagging at 665 cm^{-1}. The C–N stretching peaks were between 1078 and 1240 cm^{-1}. The $–CH_2$ stretching vibration at 1456 cm^{-1} was seen. The C–H bending was seen at 1395 cm^{-1}.

The IR spectra of alginate showed characteristic peaks for its glucoronic (G) and manuronic (M) acid units at 1031 cm^{-1} and 1091 cm^{-1}, respectively. The –OH stretching peak was observed 3407 cm^{-1}. The H–C–H and O–C–H stretching vibration was seen at 1416 cm^{-1}. The $–COO^-$ stretch was visible at 1609 cm^{-1}. The peaks at 886 cm^{-1} and 818 cm^{-1} indicate β-glycosidic linkages between G and M units of alginate. The IR spectra of polymeric composite beads showed –NH stretching vibration shifted to 3436 cm^{-1}, while in gelatin and chitosan FTIR, the –NH stretching vibration was observed at 3443 cm^{-1} and 3442 cm^{-1} respectively. This indicates some possible interactions, which may occur between gelatin and chitosan during scaffold formation. The sharp intensity vibration at 1648 cm^{-1} corresponds to imine bond (C=N) which might be formed between gelatin and gluteraldehyde as well as chitosan and gluteraldehyde present in the polymer mixture. This peak was not observed in case of pure gelatin and chitosan. This implies the possibility of crosslinking reactions among chitosan, gelatin and gluteraldehyde molecules during scaffold formation. The possible crosslinking reaction between chitosan and gelatin in presence of glutaraldehyde, is also mentioned earlier in Figure 5. Moreover, the C–H bending vibration was shifted to 1385 cm^{-1}, which was between the C–H bending-vibration peaks seen in chitosan (1378 cm^{-1}) and gelatin (1395 cm^{-1}). This shifted peak also indicates that some interaction might have occurred among the polymers during scaffold fabrication.

Porosity

A high porosity is necessary to facilitate cell seeding and diffusion throughout the whole structure of both cells and

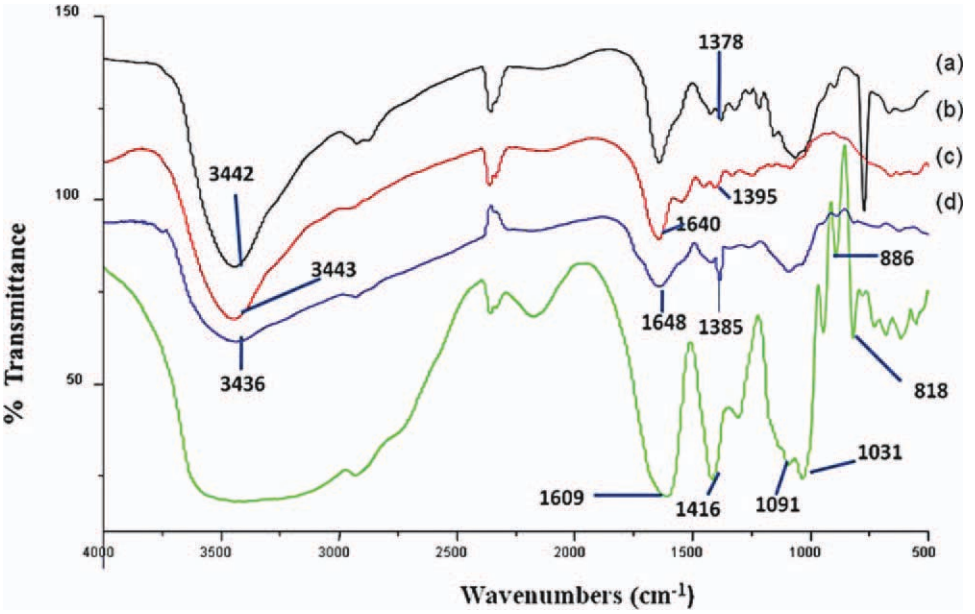

Figure 7. FTIR spectra of (a) chitosan; (b) gelatin; (c) alginate/gelatin/chitosan composite scaffold, and (d) alginate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

nutrients.[1] The porosity of the beads was assessed utilizing the liquid displacement method. The scaffold shows porosity of about 90% (mean porosity 88.56 ± 2.03%), which is excellent for cellular invasion. In previous study, for the scaffold prepared by gas foaming method, it was found the porosity ranges from 87 to 97%.[32] In another study, the porosity of chitosan-gelatin scaffold was in range of 90–95%.[33]

Liquid displacement method used here gives only the rough estimation of the porosity. More accurate estimation of porosity can be obtained by using mercury intrusion porosimetry.[34]

Swelling Property

The hydrophilicity of the scaffold serves as one of the crucial factor in the evaluation of biomaterials for tissue engineering as it is essential for the absorption of body fluid and for a transfer of cell nutrients and metabolites. As shown in Figure 8, the swelling ratio of the beads is above 621% after immersion in PBS for 10 min and increases with time, which reflects the excellent hydrophilicity of the scaffold. After 60 min, no further swelling of beads (negligible swelling effects) was observed, which indicates that the equilibrium point of swelling was reached. This data is consistent with another study which showed the swelling ratio of chitosan-gelatin scaffold above 600%, after immersion in PBS, and its increasing trend with time.[35]

In Vitro Biodegradability

Figure 9 shows the percentage biodegradation of scaffold in PBS solution. Biodegradability was assessed by determining the percentage weight loss of beads which comes out to be 36% on day 5. It is observed that the degradation rate proceeds to 63 and 69% on day 7th and 21st, respectively. The chitosan-gelatin-alginate composite scaffold showed good biodegradability

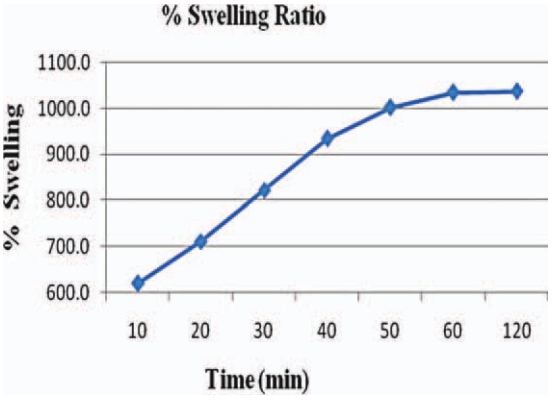

Figure 8. Swelling ratio (%) of composite scaffold. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

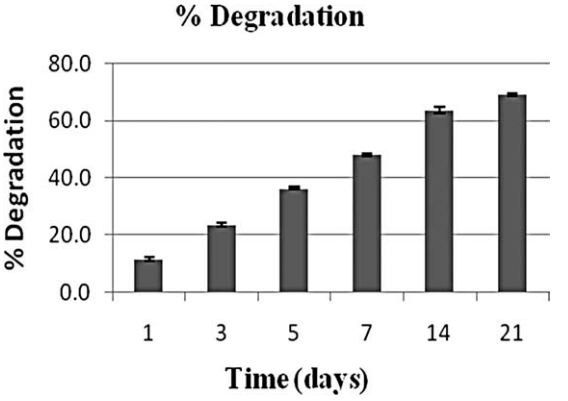

Figure 9. Percentage degradation of chitosan-alginate-gelatin scaffold in PBS solution.

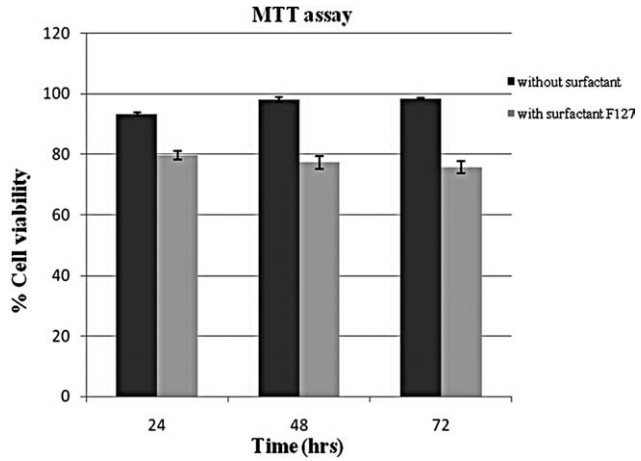

Figure 10. Viability level of L929 mouse fibroblast cells on composite scaffold up to 72 h.

when kept in PBS solution (pH 7.4), as indicated by weight loss at various time points. Previous literature revealed that the degradability of chitosan-gelatin porous scaffold involves chitosan degradation and gelatin dissolution, and nearly 35% weight loss occurred by day 6 in PBS solution containing lysozyme, which is consistent with our finding.[16] The degradation byproducts have little effect on the pH value of the resultant PBS solution which was found to be 7.18 at 21st day of degradation experiment. This value was close to the resultant pH value of 7.22 for chitosan-gelatin scaffold degradation in previous study by Jiankang et al., 2009.[33]

In Vitro Cell Culture Studies

Cytotoxicity Studies: MTT Assay. In order to evaluate the cytotoxicity of the substances that leach out of the developed scaffold, a viability assay (the MTT test) was performed. This test is based on mitochondrial viability, as only functional mitochondria can oxidize the MTT solution, giving a typical blue–violet end product. Cytotoxicity studies were performed for the scaffold prepared with and without surfactant F127 in order to determine the effect of surfactant on cell viability that was often reported to decrease the cell viability (Figure 10). The percentage of viable cells in surfactant-free scaffold was around 99% up to 72 h, and thus comparable to the control which is considered to have 100% viability. On the other hand, the percentage of viable L929 cells in contact with scaffold fabricated using surfactant F127 went on decreasing from 79% to 75 in 24 h and 72 h, respectively. This result indicates that the substances leached out from the surfactant-free scaffold during incubation is non-toxic to the cells while the substances leached out from the scaffold with surfactant showed toxicity toward L929 cells as indicated by reduced cell viability (75%).

Cell Morphology Studies. The absence of cytotoxicity does not confer any information about the biocompatibility of a biomaterial.[11] In order to authenticate whether the developed scaffold support the functions shared by many cell types, such as membrane integrity, adhesion to surfaces, and replication, adhesion studies with the cell line L929 were performed. At the end of 24 h, the scaffold was seen to be covered with a monolayer of cells (Figure 11). After 48 hrs of culture, the fibroblasts had taken a typical spindle-shaped morphology and exhibited cytoplasmic projections strongly attached to the scaffold which is suggestive of cell activation [Figure 11(b,c)]. A similar type of morphology of L929 on chitosan-based scaffold was also reported in previous studies.[11] Phase contrast microscopy of cell-scaffold construct also showed viable and healthy cell that has increased in density with incubation time. The L929 cells cultured on composite scaffold showed its characteristic spindle shaped morphology throughout the culture period (Figure 12).

In our opinion, these results are quite promising since it demonstrates that the composite scaffold in bead form possess an adequate pore size and pore distribution to effectively allow the cells to adhere and maintain their functions. Therefore, the results of cell morphology studies using fibroblast L929 cells showed that the composite scaffold in bead form can be used as a porous scaffold for tissue engineering applications.

Visualization of Cells on 3-D Scaffolds by Calcein AM Staining. Cell viability of the composite scaffold formed without surfactant was further confirmed by fluorescence

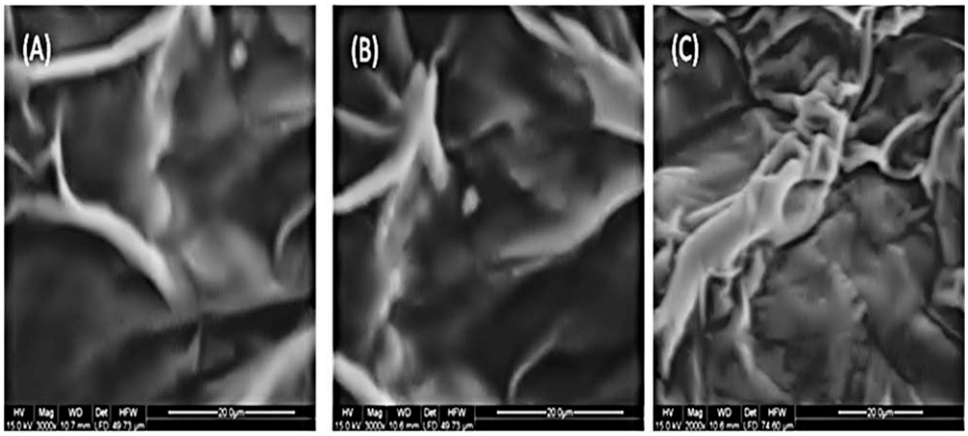

Figure 11. ESEM images of L929 cultured composite scaffold (A-C-at 24h, 48h, and 72h, respectively). The cell morphology over the scaffold showed change from round to characteristic spindle-shaped.

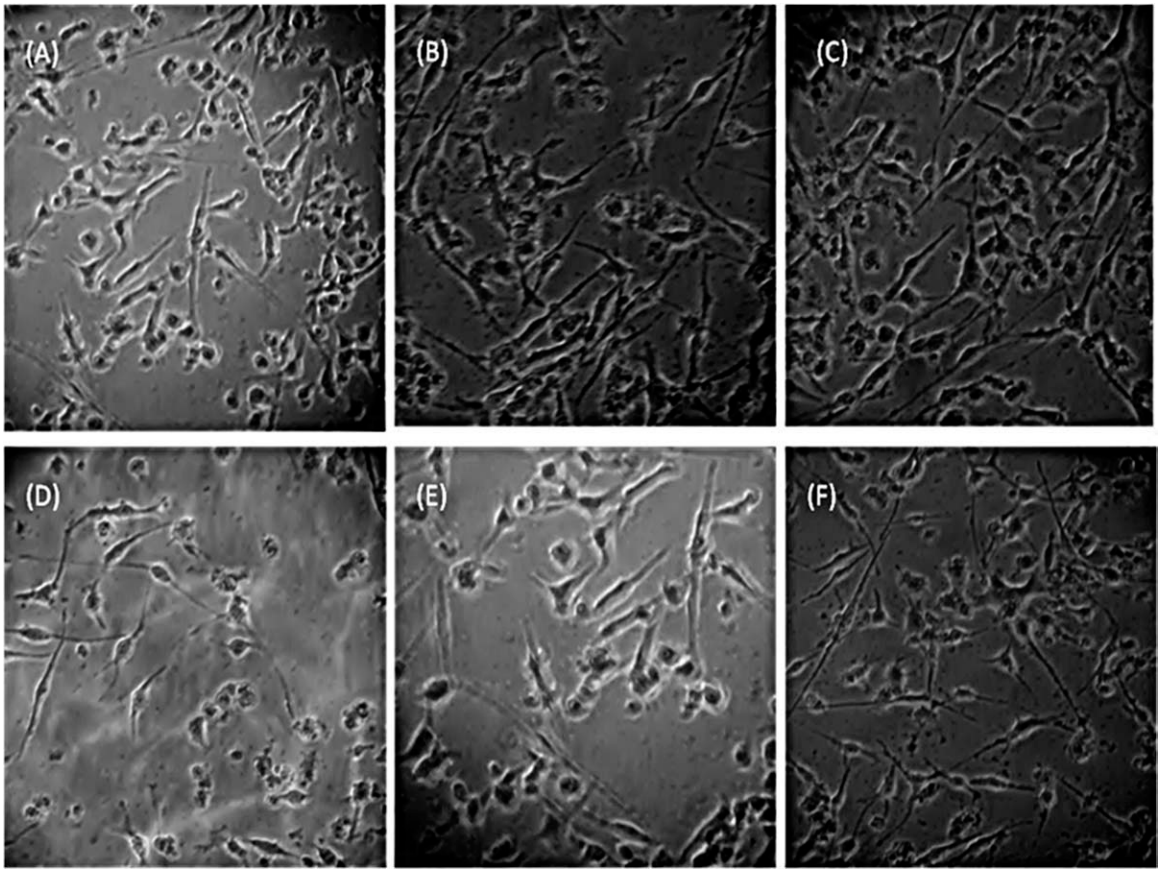

Figure 12. Phase contrast microscopy showing L929 mouse fibroblast cells growing over scaffold in tissue culture plate; A-C cell: scaffold construct at 24 h, 48 h, and 72 h, respectively; D-F: Control at 24 h, 48 h, and 72 h, respectively (Magnification: 20× for all images).

microscopy of Calcein AM stained beads showing viable green colored cells almost evenly distributed on surface as well as within the beads as shown in Figure 13.

Assessment of Cell Proliferation by DNA Quantification. Analysis of DNA content in cell-scaffold construct showed a significant enhancement comparative to initial time points (Figure 14). The DNA content was increased as a function of time, indicating a high cellular proliferation has occurred on the composite scaffold. The DNA biochemical analysis was corroborated by MTT assay (Figure 10) showing that

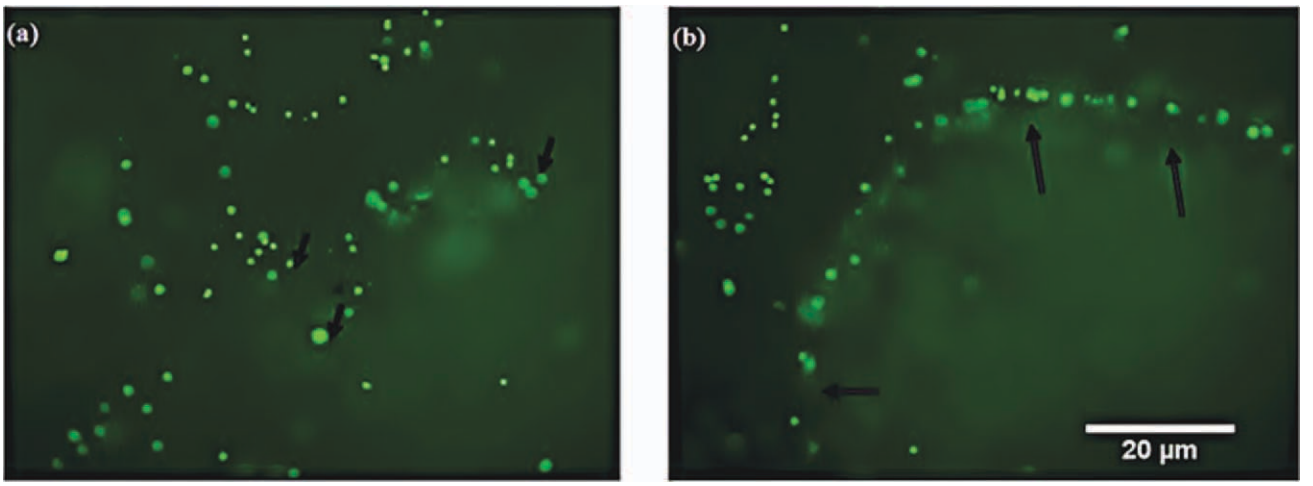

Figure 13. Fluorescent microscopy of calcein AM stained scaffold showing cells (a) inside the scaffold and (b) on the surface (100×). The arrow shows the viable cells that has acquired the characteristic green fluorescence due to staining. (Scale bar = 20 μm for both figures). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

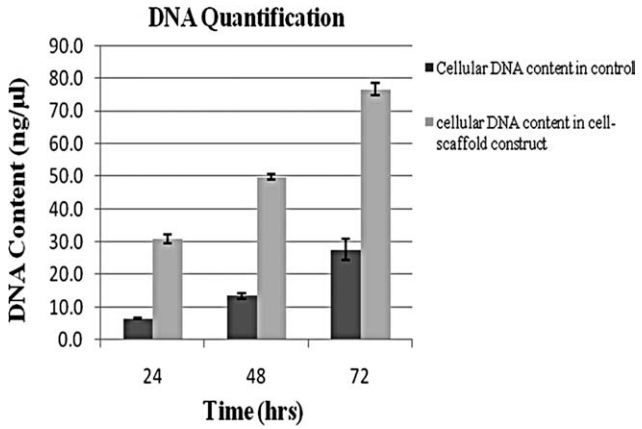

Figure 14. Changes in DNA content of L929 mouse fibroblasts cultured on the composite scaffold, at different time intervals.

cells are not only viable but also showed significant proliferation in composite scaffold. These results illustrate that the porous composite scaffold fabricated here by using natural polymer combination of chitosan/gelatin and alginate is a suitable 3D support for cellular growth and proliferation.

CONCLUSIONS

A natural origin tripolymer scaffold from chitosan, gelatin and alginate was fabricated by applying foaming method without adding any foam stabilizing surfactant. Previously, in foaming method of scaffold fabrication, toxic surfactants were used to stabilize the foam, but in this work, it had been possible to restrict the use of surfactants in foaming method, which can create better environment for cellular response and viability. The natural tri-polymer used here, have structural similarities with natural extracellular matrix of the tissues and have excellent cell adhesion properties, and because of these properties, the tri-polymer composite scaffold would probably be an ideal and promising candidate for tissue engineering applications. The thriving exploration of the natural tri-polymer combination might eliminate the need of synthetic polymers for scaffold fabrication that often are less biocompatible and biodegradable as compared to natural polymers.

ACKNOWLEDGMENTS

The authors are grateful to the Council of Scientific and Educational Research (Grant No. 27(0222)/1 O/EM R-II dated May 31, 2010), India, for funding this research work. They are thankful to Mr. C. K. Prashant and Mr. S. K. Srivastava, Research Scholars, Dept. of Pathology, AIIMS for their cooperation in carrying out the biocompatibility experiments.

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