

Evaluation of Extracted Chitosan from *Portunus Pelagicus* for the Preparation of Chitosan Alginate Blend Scaffolds

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Abstract In recent years there is a growing need in generating a biocompatible and cost effective porous scaffold for tissue engineering purposes. Therefore, this study focused on conversion of the shell waste of locally available crab variety *P. pelagicus* (Blue swimming crab) into the chitosan scaffold. As the poor mechanical strength of chitosan limits its usage in tissue engineering, it was blended with alginate. The scaffolds were prepared by the freeze gelation method which requires less time and minimum energy, with fewer residual solvent and easier to scale up. To the best of our knowledge there are no reports on scaffold preparation from the extracted chitosan, blended with alginate by freeze gelation method. The biological properties of chitosan-alginate scaffolds (Cts–Alg) were evaluated and compared with those of chitosan scaffolds. The prepared scaffolds were characterized by SEM, swelling property, in vitro enzymatic degradation, and hemo, biocompatibility properties. Chitosan-alginate scaffolds had an average pore size of 40 μm and tensile strength of 0.564 ± 0.0018 % MPa. Its swelling ratio was 27.5 ± 0.28 %, with mass loss percentage of 10 ± 0.33 % after 4 weeks of degradation. It has exhibited good hemocompatible properties too. Mouse fibroblast 3T3 cells were able to adhere and proliferate well in the blended scaffold. All these results indicated that chitosan-alginate scaffold is a suitable alternative substitute for tissue engineering.

Keywords Tissue engineering · Scaffold · Chitosan · Alginate · Biocompatibility

Introduction

With growing shortage of organ donors and increasing need for transplantation, tissue engineering gives hope to patients who desperately require tissue/organ substitutes [1]. It seeks to develop biological substitutes that replace and restore various tissues. Scaffolds are 3D porous matrices that are utilized in tissue engineering as frameworks to seed and grow the cells into tissues. The scaffold used for tissue engineering should possess characteristics like biocompatibility, biodegradability at the ideal rate corresponding to the rate of new tissue formation with optimal mechanical property, adequate porosity and morphology for the transport of cells, gases, metabolites nutrients and signal molecules both within the scaffold as well as between the scaffolds [2]. The choice of the biomaterial used for scaffold preparation determines the success of its application. The biomaterial used for scaffold preparation must have the ability to promote cellular interactions and tissue development besides providing the required mechanical and physical properties [3–5].

Materials for scaffold production include metals, ceramics, polymers (natural and synthetic) and their combinations. Metals and ceramics possess disadvantages for tissue engineering applications as they lack degradability in a biological environment, and their very limited processability [6]. On the other hand, synthetic polymers like PLA, PCL etc. are artificial macromolecular substances, originating from non renewable petroleum resource [7]. In this context, considerable attention has been given to chitosan because of its low cost, accessibility and availability,

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antimicrobial activity, low toxicity and biocompatibility [8–13].

Export of processed and frozen crab products is the backbone of seafood export in India. The blue swimmer crab *Portunus pelagicus* (L.) represents a valuable component of crustacean fishery and contributes up to 90 % of the crab landings in India. Among the maritime states, Tamilnadu ranks first in crab landings [14]. It is estimated that the industrial processing of these crab varieties generates 1354 tonnes of wastes every year in India [15] and 70 % of crabs are discarded as waste during marine food product processing [16]. Moreover, the waste generated from the worldwide production and processing of shellfish poses a serious problem and threat to the environment. Therefore, much of the research is focussed on utilisation of this waste and converting them into useful products such as chitin. Chitosan is obtained by the deacetylation of chitin, which is predominantly found in the exoskeleton of crustaceans. It is mainly composed of units of glucosamine linked to N-acetyl glucosamine through β -glycosidic bonds [17]. Although chitosan, an undisputed biomaterial has many distinctive properties like antimicrobial activity, non toxicity, biocompatibility, biodegradability, remarkable affinity to proteins and cell adhesion [18], but still, it lacks mechanical stability, to overcome this it was blended with alginate, another biodegradable substance extracted from seaweed *Sargassum* sp. Due to its negative charge, it is able to chemically bond with positively charged chitosan to form a superior quality scaffold material with enhanced stability [19]. Though several reports are available for the preparation of chitosan blend scaffolds using commercial chitosan, the present study is focussed on the extraction of chitosan from *P.pelagicus* and evaluation of prepared chitosan (Cts) and chitosan-Alginate (Cts–Alg) scaffolds for tissue engineering purpose.

Materials and Methods

Preparation of Chitosan

Shells of *Portunus pelagicus* were collected from south-east coast of Rameshwaram seashore. The collected shells were packed in plastic bags and stored in $-20\text{ }^{\circ}\text{C}$ until it is used for extraction. Chitin and chitosan were prepared from crab shell waste following the method of Takiguchi [20]. The exoskeleton of crab was washed with tap water followed by demineralization by adding 300 ml of 2N hydrochloric acid. Excess acid was drained off and the sample was washed thoroughly with distilled water. It was then dried in hot air oven at $60\text{ }^{\circ}\text{C}$. Deproteinisation was carried out by adding 300 ml of 1N sodium hydroxide to

the filtrate sample at $80\text{ }^{\circ}\text{C}$ for 24 h with constant stirring. After 24 h, excess NaOH was removed. The sample was washed with water and filtered till the wash liquid showed neutral pH. The filtrate was dried at $40\text{ }^{\circ}\text{C}$, to obtain chitin it was then deacetylated by adding 250 ml of 40 % NaOH, heated under reflux for 6 h at $40\text{ }^{\circ}\text{C}$ with constant stirring to which 200 ml of 10 % acetic acid was added and was kept for 12 h at room temperature with constant stirring. Dissolved sample was re-precipitated by adding 40 % NaOH and the pH was adjusted to 7. The sample was then centrifuged at 10,000 rpm to obtain chitosan.

Preparation of Chitosan- Alginate Blend Scaffold by Freeze Gelation

Chitosan extracted from crab shell waste was blended with alginate. Scaffolds were prepared by freeze gelation method [21]. The materials were dissolved in 0.1 M acetic acid. The obtained polymer solution was placed in a fourteen cm petridish and frozen at $-20\text{ }^{\circ}\text{C}$ for 24 h. Both the mixture was blended until homogenous. The frozen chitosan-alginate solution was immersed in a NaOH/ethanol aqueous solution to adjust its pH to allow for the gelation of polymer and later allowed to evaporate in a the vacuum dessicator for overnight.

Characterization of Scaffold

SEM Analysis

The scaffolds were prepared for the SEM by sputter coating them with gold. It was observed under Scanning electron microscopy (SEM), specifically the JEOL 7000JSM 6390 Japan, with a 15 kV applied voltage to analyze the structure of the prepared scaffolds [22].

In vitro Enzymatic Degradation

The absolute dry weights (W_0) of the chitosan scaffolds were measured after which the samples were placed in PBS buffer solution at a $37\text{ }^{\circ}\text{C}$. To this $5\text{ }\mu\text{g/ml}$ of lysozyme was added. Then the samples were placed in shaker with temperature set at constant $37\text{ }^{\circ}\text{C}$ for 4 weeks for the measurement of enzymatic degradation. At the end of period, the scaffolds were freeze-dried for 24 h and the weight loss ratio was calculated using the following equation [22]:

$$\text{Weight loss(\%)} : \frac{W_0 - W_t}{W_0} \times 100 \quad (1)$$

Three specimens were tested for each sample and their average values were used for data analysis.

Swelling Ratio

The swelling ratio of the scaffolds was calculated by measuring the weight of the scaffold before and after immersion in 0.05 M buffer PBS pH 7.4 at a temperature of 37 °C. The swelling ratio of the scaffold was defined as the ratio of weight increase (W_s) with respect to the initial weight (W_d) of dry sample [23]

$$E_s = \frac{W_s - W_d}{W_d} \quad (2)$$

Mechanical Properties of Scaffolds

Mechanical properties, viz., ultimate tensile strength of the dried scaffolds were measured using Universal Testing Machine (Tinius Oisen H10 ks) at a crosshead speed of 50 mm min⁻¹ at 25 °C and 10 N load cell. All the mechanical tests were performed with dried samples and were examined in triplicates [24].

Blood Compatibility Studies

All the procedures were carried out after ethical approval from institutional ethics subcommittee.

Hemolysis Assay

The hemolysis test was performed as recommended by ISO10993-4 [25]. Blood was collected in heparin-coated siliconized vials from healthy volunteers. The scaffolds were equilibrated in normal saline for 30 min at 37 °C before testing. These scaffolds were incubated in a siliconized tube containing 10 ml of heparinized blood. 9 ml of heparinized blood diluted with 1 ml PBS was taken as a negative control. Heparinized blood diluted with 9 ml distilled water without the scaffold was taken as a positive control. The content were gently mixed and incubated at 37 °C for 1 h. The samples were centrifuged and the absorbance of the supernatant measured at 545 nm using UV–visible spectrophotometer

$$\text{Hemolysis (\%)} = \frac{[\text{Abs}(\text{sample}) - \text{Abs}(-\text{ve control})]}{[\text{Abs}(+\text{ve control}) - \text{Abs}(-\text{ve control})]} \times 100 \quad (3)$$

Platelet Adhesion Test

The platelet adhesion study was performed according to International standard 10993-4 [26]. Whole blood was taken from healthy volunteer in sterile plastic tubes containing 3.8 % sodium citrate in PBS to prevent coagulation. Samples were centrifuged at 1300 rpm for 10 min at 4 °C

to collect the platelet rich plasma (PRP). The films were punched into circular shape and placed in 24-well polystyrene plates sterilized with 75 % ethanol and rinsed thrice with PBS and equilibrated in PBS for 1 h. PRP was warmed to 37 °C for 30 min or 120 min and films were rinsed three times with PBS to remove the weakly-absorbed platelets. The platelets on the scaffolds were observed under the microscope after incubation.

Protein Adsorption Study

For protein adsorption studies, sterile scaffolds were incubated in 10 % serum (in PBS) for 120 min at 37 °C and washed gently with PBS followed by distilled water to remove weakly adhered proteins. These scaffolds placed in 1 % SDS were shaken vigorously to dislodge the adsorbed protein and the solution was centrifuged to remove particulate or insoluble matter, if any. Finally the amount of protein was assayed by the Lowry method [27].

Biocompatibility Study

Mouse 3T3 Fibroblast Culture

Experiments were performed with NIH/3T3 fibroblasts, obtained from Aravind Medical Research Foundation (AMRF) Madurai, Tamilnadu. The cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) (Invitrogen-GIBCO BRL, Grand Island, NY) containing 10 % fetal bovine serum (US origin from HyClone, Logan, UT) and 100 U/ml penicillin and 100 pg/ml streptomycin (Invitrogen-GIBCO BRL, Grand Island, NY) at 37 °C in a humidified atmosphere of 5 % CO₂.

Seeding of 3T3 Cells on Scaffolds

To evaluate the biocompatibility of the scaffolds on 3T3 cells, the uniformly cut (1 × 1 mm) and weighed chitosan scaffolds were placed in a 96 well plate in triplicates. 50 μl of DMEM was added and the matrices were kept for sterility check overnight at 37 °C. Before seeding the cells, the scaffolds were washed twice with phosphate-buffered saline (PBS), once with DMEM and were placed in 96-well plates. The scaffolds were seeded with 5 × 10³ 3T3 fibroblast cells. DMEM culture media was added and the cells were cultured at 37 °C in a humidified 5 % CO₂ incubator for 24 h after which the viability of cells was assessed using trypan blue assay [28].

Viability by Trypan Blue Exclusion Test

Trypan blue solution was prepared by dissolving trypan blue powder (Sigma Aldrich, St. Louis, MO, USA) in

DPBS to make a 0.4 % solution and the dye solution was then filtered to remove any undissolved particles. 10 µl of the single cell suspension was taken in a 50 µl micro centrifuge tube and was mixed with 10 µl of 0.4 % trypan blue dye solution. After keeping for 3 min at RT, 10ul of the suspension was loaded onto haemocytometer chamber. The viable (unstained) and non-viable (blue stained) cells were counted separately. The number of cells per ml and percentage of viable cells were given by the formula:

$$\text{Viable cell (\%)} = \frac{(\text{No. of viable cells per ml})}{\text{Total number of cells per ml}} \times 100$$

Result and Discussion

Morphology of the Scaffolds

Being a cationic polymer, chitosan is an appealing choice of biomaterial for scaffold production. It is recognised as a versatile biomaterial because of its non-toxicity, biocompatibility and biodegradability [29]. They possess numerous interesting physicochemical and biological properties, ideal for scaffold preparation. In addition to this, as a marine product, chitosan has many advantages compared with other biomaterials prepared from bovine or porcine source which have the risk of transmission of prions and other pathogens to humans. In contrast, marine-derived products are safe for human use due to the species barrier [30]. Poor mechanical strength and stability of chitosan limits its application in tissue engineering. In order to circumvent this problem, it is blended with alginate, a natural anionic polymer. Freeze gelation is one of the attractive options for fabrication of porous scaffolds as it helps to create a scaffold that emulates the properties of the natural tissue. Porous 3D scaffolds of Cts and Cts–Alg blends were prepared using chitosan obtained from the shell waste of crab by freeze gelation technique. All the scaffolds prepared were flexible and smooth. Cts scaffold had little deformation compared to Cts–Alg blend in both wet and dry conditions (Fig. 1). SEM images revealed the

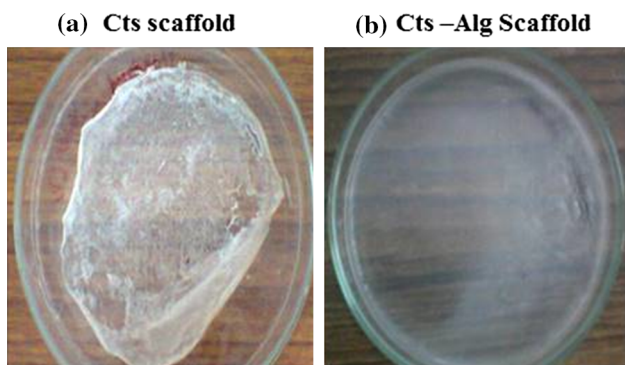


Fig. 1 Scaffold prepared by freeze-gelation method

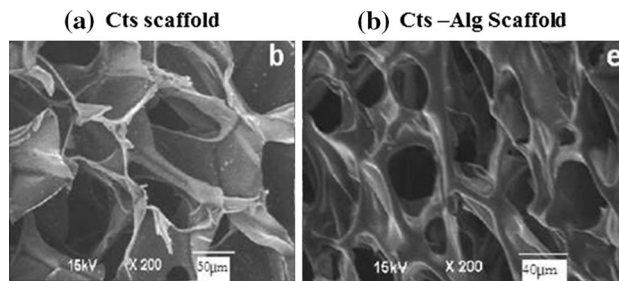


Fig. 2 SEM micrograph of prepared scaffolds

3D pore microstructures and were heterogeneous with well interconnected pores in both scaffolds. The mean diameter of pores on both the scaffold formulation was found in the range of 40–50 µm (Fig. 2), which is more suitable for cellular infiltration and interaction since the size of fibroblast cells is about 10–30 µm. Therefore it is assumed that pore size of the scaffolds were sufficient for nutrients to enter into the cell, to allow cells to migrate, to release metabolic products produced from cells and to diffuse oxygen [31].

In vitro Enzymatic Degradation Study

Degradation of the scaffold is one of the key considerations in its design and fabrication. Ideally, the degradation rate of scaffolds should match the rate of new tissue formation which allows a smooth transition of load transfer from scaffolds to tissue [32]. Chitosan polymer exhibits degradation in vivo by several proteases such as lysozyme, papain, pepsin etc. Their biodegradation by product is non-toxic oligosaccharides of variable length (glycosaminoglycans and glycoproteins). It is subsequently incorporated to metabolic pathways and excreted [33]. The mass changes of both Cts and Cts–Alg scaffolds were observed during the degradation process. The mass loss of Cts scaffold was 36 ± 0.57 % after 4 weeks which was higher than

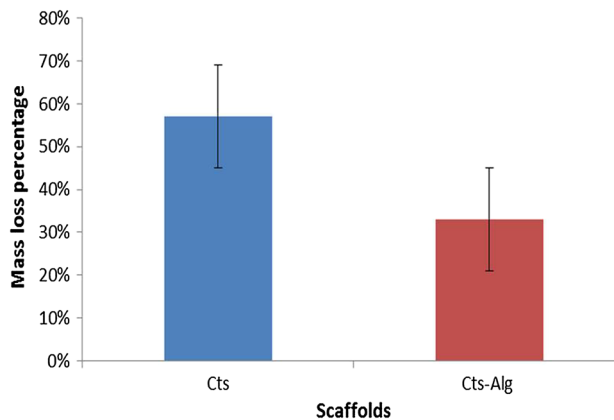


Fig. 3 In vitro enzymatic degradation study of scaffolds

Cts–Alg scaffolds ($10 \pm 0.33 \%$). The weight loss curves indicate that Cts scaffolds degrade at a faster rate compared to Cts–Alg scaffolds throughout the degradation process (Fig. 3). In this study, mass loss percentage in Cts–Alg blend scaffolds was lower compared to Cts. This is due to the presence of high number of interconnecting pore structure on the scaffold. Moreover the presence of alginate made Cts–Alg scaffold more susceptible to enzymatic degradation due to better accessibility of cleavage sites by the enzymes. Furthermore the hydrophilic nature of alginate also contributed to a higher degradation of Cts–Alg scaffolds, as it enhances the interaction of the biomaterial with the enzymatic solution [34].

Swelling Property of Scaffolds

Diffusion and exchange of nutrients (e.g. oxygen) and waste throughout the entire scaffold are related to the swelling properties of the scaffolds [29]. The absorption ability of the scaffolds was determined with swelling ratio. The swelling ratio of Cts and Cts–Alg scaffold were $60 \pm 1.15 \%$ and $27.5 \pm 0.28 \%$ respectively (Fig. 4). The swelling ratio of Cts scaffold was greater than Cts–Alg blend scaffold. The water absorption property of a scaffold is influenced by the nature of the polymer. Chitosan polymer absorb large amount of water due to the presence of abundant number of hydrophilic groups and swell considerably. This is conquered by addition of a biopolymer alginate which enhances the inter pore connectivity resulting in enhanced rigidity and strength to the scaffolds, allowing it to absorb solution without swelling [35].

Mechanical Property of Scaffolds

Mechanical property of the scaffold materials is one of the fundamental properties for any scaffold material in the biomedical application point of view. Thus, confirming the

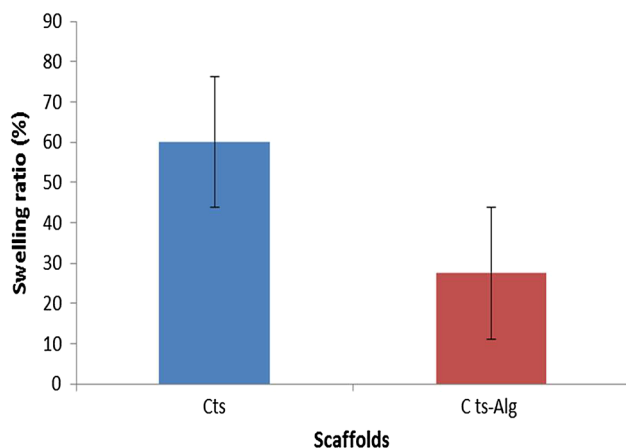


Fig. 4 Swelling properties of scaffolds

mechanical strength of the scaffolds is essential especially when it comes to scaling up the scaffolds for translational purposes [36]. From the results, it was observed that the mechanical strength of the Cts–Alg scaffold ($0.564 \pm 0.018 \text{ MPa}$) was higher than Cts scaffold ($0.408 \pm 0.052 \text{ MPa}$). High tensile strength (MPa) values is attributed enhanced cross-linking than that of the native polymer chitosan (Fig. 5). The mechanical stability of the scaffolds for tissue engineering is necessary to maintain the cell differentiation and proliferation by withstanding various stresses incurred during implantation in vivo and culture in vitro [37]. It is well known that the tensile strengths of porous structures have been reported to be in the range of 0.03–0.06 MPa [38, 39]. The Cts–Alg scaffolds exhibited enhanced tensile strength than that of Cts scaffolds and also found to be suitable for tissue engineering applications.

Blood Compatibility Studies

Hemolysis Test

The biocompatibility, especially blood compatibility, is the most important property with regard to biomedical materials. When the polymeric scaffold comes in contact with blood it must not induce thrombosis, thromboembolisms, antigenic responses, destruction of blood constituents, plasma proteins, and so forth [40]. Hemolysis test was done to determine the extent of exosomatic hemocytolysis of the biomaterial. The hemolysis levels of the scaffold samples are summarized in Table 1. The OD values of the positive and negative controls were 1.66 and 0.02. These values are regarded as 0 and 100 %, respectively, when calculating the relative red cell toxicity. The hemolysis rates of Cts and Cts–Alg scaffold samples were 1.4 and 0 %, respectively.

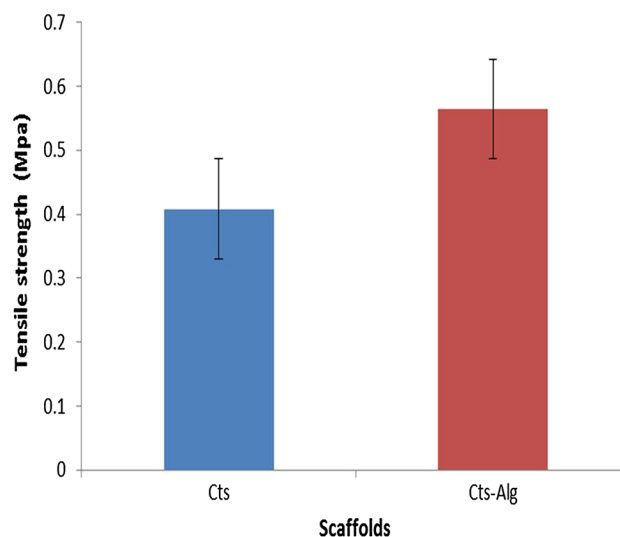


Fig. 5 Mechanical properties of scaffolds

Table 1 Results of hemolysis test

Sample	Optical density at 545 nm	Haemorrhage (%)	Hemolysis (%)
Positive control (Distilled water)	1.66	100	0–2
Negative control (Normal saline)	0.02	0	
Chitosan scaffold	0.02	1.4	
Chitosan-alginate blend scaffold	0.03	0	

In our investigation the values obtained for hemolysis test are lower than the criterion set by ISO 10993-4 (reference <5 %), which strongly suggests that these scaffold materials have no potential to induce hemolysis.

Platelet Adhesion Test

Platelet adhesion on the surface of the biomaterial is the most essential character in evaluating the hemocompatibility of the scaffolds. It is an important test to evaluate the compatibility of blood with natural membranes. When blood contacts a foreign material, plasma protein are adsorbed onto the material surface and provoke adhesion of platelet. Chitosan, the only pseudonatural polycationic substance when it forms electrostatic complexes with natural polymers such as alginates they are used as antithrombogenic material [41]. The number of adhered platelets was lower in Cts–Alg scaffolds compared to Cts scaffolds, indicating decreased platelet adhesion and increased hemocompatibility [42]. (Figure 6a, b). Platelet spreading and aggregation are markers of platelet activation and thought to be a major mechanism by which biomaterial thrombogenicity is transduced [43]. In the present investigation numerous platelets were observed on Cts compared to Cts–Alg blend films after a 120-min exposure to platelet-rich plasma (PRP).

Protein Adsorption Studies

The initial adsorption of protein onto a biomaterial surface plays a key role in determining how the body responds to an implanted biomaterial. Cts–Alg blend scaffold adsorbed higher amount of protein ($35 \pm 1.45 \mu\text{g/ml}$) compared to Cts scaffold ($16 \pm 1.15 \mu\text{g/ml}$) (Fig. 7). In our study, we observed that protein adsorption was greater in Cts–Alg scaffolds compared to Cts scaffold. The porous architecture of the scaffold acts as a selective substrate, enhancing cell–ECM interactions, protein specificity and adsorption of the scaffold [44]. Furthermore the structural changes that occurred during freeze gelation process during the preparation of scaffold contributed to significantly higher surface area in Cts–Alg scaffold thereby enhancing protein adhesion.

Biocompatibility Testing Using 3T3 Fibroblast Cells

Cell Attachment on Scaffolds

Cell viability is an important parameter in tissue engineering and culture studies to evaluate the effect of environmental conditions on cell behavior [45]. The trypan blue exclusion test is a simple, rapid and inexpensive method to assess cell viability in response to environmental insult.

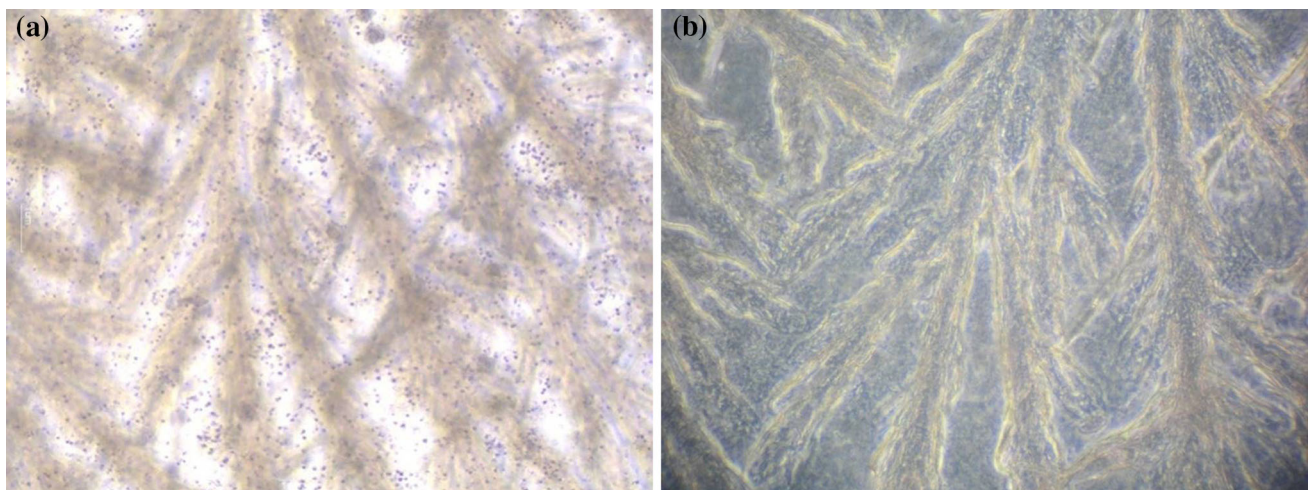


Fig. 6 **a** Confocal microscopic image of platelet adhesion on Cts scaffold. **b** Confocal microscopic image of platelet adhesion on Cts–Alg scaffold

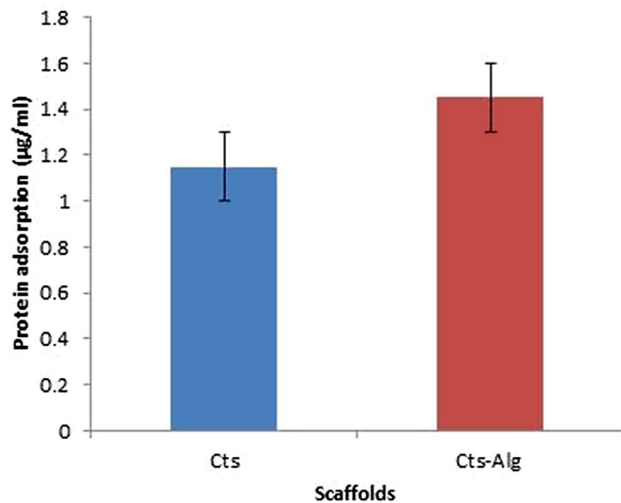


Fig. 7 Protein adsorption study of scaffolds

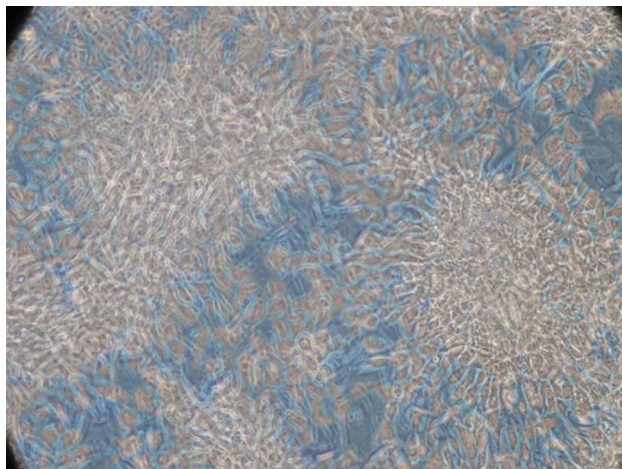


Fig. 8 Microscopic image of trypan blue assay for mouse fibroblast 3T3 cells on Cts scaffold

Cell viability and biocompatibility assessments were carried out using the trypan blue assay. The attachment of the fibroblast 3T3 cells onto the prepared scaffold took 4 h after seeding of cell. The cells were cultured on the both Cts and Cts–Alg films for 3 days, and the cell morphology, adhesion, viability, proliferation were determined. Trypan blue dye exclusion analysis of the cultured 3T3 cells revealed better proliferation and viability ($94.6 \pm 2.5\%$) when grown in Cts–Alg blend scaffolds compared to Cts scaffolds alone ($83.2 \pm 3.5\%$) (Figs. 8, 9). The dye exclusion test is based on the ability of viable cells to be impermeable to trypan blue dye. When membrane integrity of the cells is compromised, there is uptake of the dye into the cells so that viable cells, which are unstained, appear clear with a refractile ring around them and non viable cells appear blue colored with no ring [46]. In trypan blue assay Cts–Alg scaffold materials exhibited very good

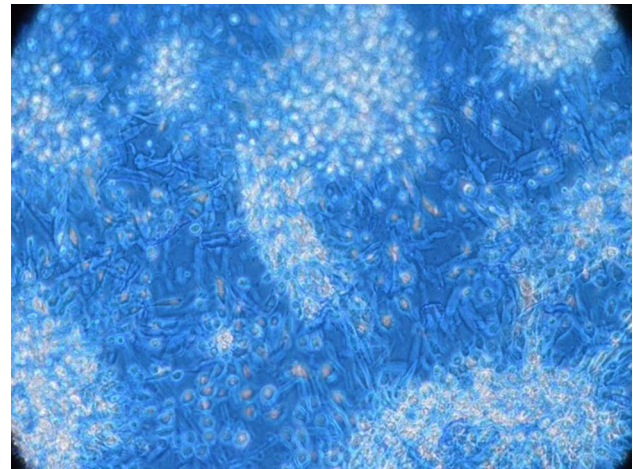


Fig. 9 Microscopic image of trypan blue assay for mouse fibroblast 3T3 cells on Cts–Alg scaffold

biocompatibility with decreased cell death. Results of trypan blue assay with mouse fibroblast 3T3 cells showed that freeze gelled Cts–Alg scaffold supported the adhesion and proliferation of more number of cells compared to Cts scaffold. The results are in agreement with previous reports suggesting that alginate-based chitosan hybrid biomaterials provides excellent supports for fibroblast adhesion and viability [47].

Conclusion

The present study described the preparation and fabrication of biomatrices by blending chitosan with alginate in order to enhance both physical and biological properties of scaffolds. The prepared Cts–Alg scaffolds were porous and exhibited suitable tissue engineering properties compared to that of Cts scaffold. This study also proved that Cts–Alg scaffolds enhanced fibroblast proliferation compared to those of chitosan scaffolds. The obtained data so far present an acceptable perspective for the use of chitosan–alginate blend in tissue engineering. This study would be further extended to determine the behaviour of the cells in the scaffold.

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