

Fabrication of Tissue Engineering Scaffolds Using Marine Bioactive Materials for Diverse Applications

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ABSTRACT

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Scaffolds are polymeric matrices employed in delivering cells and drugs into human body. The scaffold matrices can be fabricated using natural or chemical polymers. In the present study, natural polymers and biomineralized materials extracted from marine sources have been used in the fabrication of the scaffolds. Marine biomaterials are highly biocompatible and they have been used for medicinal purposes. Hence the study focuses on fabricating a novel molluscan nacre incorporated scaffold, using biomaterials like κ -carrageenan from *Kappaphycus alvarezii*, collagen from *Sepia lycidas* and chitosan from shrimp shell by freeze-drying method. Due to the osteogenic and dermal regeneration properties of nacre, this novel scaffold can be used for tissue engineering applications. The extracted natural polymers used in fabricating the scaffolds were analyzed by Fourier Transform Infrared Spectroscopy (FTIR). The FTIR analysis of the sulfated polysaccharide κ -carrageenan showed strong absorbance peaks along 926 cm^{-1} and 1250 cm^{-1} which correspond to 3,6 anhydro-d-galactose and sulphur bonds respectively whereas the deacetylated polysaccharide chitosan showed peaks along the 3428 cm^{-1} and 1635 cm^{-1} corresponding to OH groups and secondary amine groups. FTIR analysis of collagen exhibited characteristic peaks along 3432 cm^{-1} and 1641 cm^{-1} indicating the presence of the amine groups and hydrogen bonds. The structural morphology of the fabricated scaffold has been analyzed by Scanning Electron Microscopy (SEM). These scaffolds have wide scope in diverse areas and will be a useful model of 3D cell culture for engineering edible meat, tissues and organs.

ADDITIONAL INDEX WORDS: 3D cell culture, edible meat, marine biomaterials, nacre, scaffold, tissue engineering.

INTRODUCTION

Tissue engineering is a field of science that combines cells, scaffolds and bioactive materials to create functional three-dimensional tissues (Garg *et al.*, 2012). This interdisciplinary field involves thrust areas of cell biology, material science, chemistry, molecular biology, engineering and medicine. The scaffold plays a major role in tissue engineering. Hence fabricating a biocompatible scaffold is a key factor for the success of engineering a tissue. Scaffolds are polymeric matrices where the cells adhere, grow and remain viable. The main aim of using a scaffold in cell culture is to provide *in vivo* microenvironment to the cells in laboratory conditions. These porous structures comprise the biochemical, biophysical and biomechanical cues that help in the cell proliferation, maintenance, differentiation and functioning (Sala *et al.*, 2013). Scaffolds are fabricated from a variety of natural and synthetic biomaterials. The biocompatible nature of the scaffold depends upon the biomaterial used. Natural biomaterials extracted from plants, animals and microbes may be either protein based or polysaccharide-based (Ha *et al.*, 2013).

Commonly used natural biomaterials are collagen, alginate, chitosan and hyaluronic acid. The marine environment houses exotic sources of marine biomaterials. The major sources of marine biomaterials like collagen, chitosan and carrageenan are from fish, molluscs, crustaceans and algae. Most of the marine biomaterials are highly biocompatible and have found application in the field of tissue engineering (Venkatesan and Kim, 2011).

Collagen is one among the most abundant proteins present in both vertebrates and invertebrates. Collagens are constituted with the repeated triplets Gly-X-Y of glycine and two other amino acids, where proline and hydroxyproline (Hyp) are the most common. They act as a ligand for the cell receptors found on the surface of the cells. Scaffolds fabricated using collagen facilitates the adherence, proliferation and growth of the cells *in vitro* and has the ability to allow the adherence and proliferation of cells within the scaffold (Silva *et al.*, 2014). Chitosan is a cationic polymer obtained from chitin comprising copolymers of β (1 \rightarrow 4)-glucosamine and N-acetyl-D-glucosamine. The amino and hydroxyl groups of the chitosan can be modified chemically which provides high stability to the molecules and makes it metabolizable by the human system. Chitosan can mimic the structural characteristics of the glycosaminoglycans (Sharma and Chandy, 1990). Carrageenan is a sulphated polysaccharide of the galactans group with alternating 1,3 and 1,4 linked

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galactose residues. Carrageenan possesses sulphate groups which mimic the chondroitin sulphate component of the extracellular matrix (ECM). This component of the ECM helps in cell differentiation (Li *et al.*, 2014). Nacre is a biomineralized material secreted by the mantle cells of the molluscs. They are calcified structures that form the inner lustrous layer. It is made up of the aragonite tile surrounded by organic matrix. The organic matrix of the nacre is composed of the polysaccharides and proteins. Water-Soluble Matrix (WSM) of nacre is also known to help in osteogenic regeneration and can be employed in treatment of burn injuries (Moutahir *et al.*, 2001).

In the present study, the biomaterials used in the fabrication of the scaffold are carrageenan from *Kappaphycus alvarezii*, chitosan from crustacean shells, collagen from skin of *Sepia lycidas* and WSM of nacre from shells of *Pinctada fucata*. They were employed in the fabrication of the scaffold with a view to represent various components of the ECM.

METHODS

Extraction and Characterization of the Biomaterials

Chitosan was extracted from the shrimp exoskeleton by a modified procedure of Toan (2009). Fresh shrimp shells of *Penaeus sp.* were collected from the local market of Tuticorin, Tamil Nadu. Exoskeleton of the shrimp was separated and washed with fresh water. The collected shell was then dried in shade for 24 hours. Dried shells were taken and then ground well using a blender, followed by demineralization using 3% hydrochloric acid, deproteination using 4% sodium hydroxide and deacetylation using 60% sodium hydroxide. The resultant flakes were dried and characterized.

Collagen was obtained from the outer skin of *Sepia lycidas* (Nagai *et al.*, 2001). The cuttle fish was collected from the Tharuvaikulam Landing Centre of Tuticorin district of Tamil Nadu. The skin was separated and treated with 0.1M sodium hydroxide for three days followed by treatment with acetic acid. The acid solubilized collagen was separated from the acid by salting out technique and the precipitate was dialyzed and lyophilized. The lyophilized protein sample was then characterized.

Carrageenan was isolated from the macro algae *Kappaphycus alvarezii* by the method of Istini, Ohno, and Kusunose (1994). The algae were collected from the Mullakadu coast of Tuticorin district and dried. The dried samples were treated with 6% potassium hydroxide and boiled to obtain a gelatinous liquid which was then filtered. The filtered liquid was treated with absolute alcohol and the resultant precipitate was dried and characterized.

The extracted biomaterials were characterized using Fourier Transform Infrared Spectroscopy (FT-IR). Infrared spectra were taken for the synthesized biomaterials using FTIR to determine the functional groups present in the biomaterials. The FTIR Spectra was measured using Perkin Elmer Spectrum GX2000. The FTIR spectra were recorded in the transmittance mode over the wave range of 4000 cm^{-1} - 400 cm^{-1} . The FTIR Spectra were interpreted as per Coates (2006).

The biomineralized material, nacre, was extracted from the shells of the pearl oyster, *Pinctada fucata* and powdered. Two grams of shell powder was dissolved in 200 ml PBS and mixed overnight. The mixture was centrifuged and the supernatant was

lyophilized. The lyophilized Water Soluble Matrix (WSM) was used in the fabrication of the scaffold.

Preparation of the Collagen Chitosan Carrageenan WSM (CCCW) Scaffold

Collagen (0.5%) and chitosan (0.5%) were dissolved in 1% acetic acid, mixed in equal volumes (1:1) under aseptic conditions and stored. Carrageenan (1%) was prepared by dissolving in 0.15M potassium chloride solution. The prepared carrageenan was mixed with the collagen chitosan mixture in the ratio of 7.5:2.5 and stirred continuously for 12 hours under constant speed. After 12 hours, the mixture was added with 1% WSM. The mixture was again mixed for several hours. After mixing, the polymeric composite was poured into a glass beaker and frozen at -30°C overnight. The frozen polymeric mixture was then lyophilized for 8 hours. Following this, the scaffold was further treated with ethanol for crosslinking and again lyophilized. The final scaffold was used for analysis.

Physiochemical Properties

The chemical characteristics of the CCCW scaffold was determined by FTIR spectroscopy and the thermal properties were determined by means of the Thermogravimetric analysis. The experiment was done at a heating rate of $10^{\circ}\text{C}/\text{min}$ within a temperature range of 30°C - 350°C . The microstructure and morphology of scaffolds were analysed using a Scanning Electron Microscope (TESCAN Vega3 SEM). Dried scaffolds were sputter-coated with gold at 40mA (JEOL-JFC1200E) prior to observing under SEM.

Swelling Ratio

Six scaffolds of same weight were taken (W_0) and immersed in 2 ml of PBS and incubated at 27°C . The swelling ratio was observed at a time interval of 24 hours up to the next 144 hours. The scaffolds were taken out from the buffer and weighed. The excess buffer on the surface of the scaffold was removed by wiping it with a sterile tissue paper and the final weight of the scaffold was noted (W_F). The swelling ratio of the scaffold was calculated using the following equation;

$$\text{Swelling Ratio} = (W_F - W_0)/W_0 \quad (1)$$

Degradation Rate

Five scaffolds of same weight were taken (W_0) and immersed in 2 ml of MEM medium and incubated at 27°C for 120 hours at a time interval of 24 hours. The scaffolds were taken out from the medium. The resultant scaffold was dried and the final weight of the scaffold was noted (W_F). The degradation rate of the scaffold was calculated using the following equation;

$$\text{Degradation Rate} = (W_0 - W_F)/W_F \quad (2)$$

Protein Absorption Capacity

The protein absorption capacity of the scaffold was determined by soaking the scaffold in standard protein solution of known concentration. Bovine Serum Albumin (BSA) was used as the standard protein and the protein absorption capacity of the scaffold over time was estimated. The scaffold was soaked for a total of 120 hours and it was taken out at a time

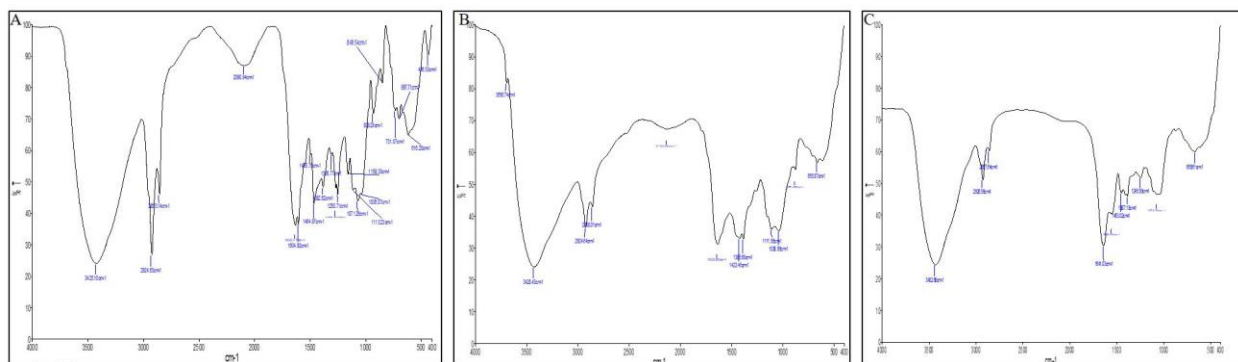


Figure 1. FTIR spectra of extracted biomaterials A) carrageenan, B) chitosan, C) collagen.

interval of 24-hour intervals. The proteins adsorbed onto the surface of the scaffold were washed with PBS twice followed by soaking the scaffold in 1% SDS for two hours to elute the absorbed protein. The solution was then taken and read at 280 nm to determine the total protein absorbed.

Isolation and Maintenance of Fish Muscle Cells

The white muscle cells of the fish were isolated from *Trachinotus blochii* by a modified procedure of Dodson *et al.* (2008). The fish was anesthetized and sacrificed. The surface of the fish was sterilized using 70% ethanol, followed by PBS containing antibiotics. The white muscle from fish was excised, rinsed well in PBS containing antibiotics and again washed in sterile PBS with antibiotics by centrifuging at 10000 rpm for 10 minutes. The procedure was repeated twice. The washed tissue was then minced well, treated with collagenase enzyme at 27°C for 2 hours, centrifuged and the pellet was taken and washed twice with PBS and once with PBS containing antibiotics. The supernatant from the above step was taken and centrifuged. The resulting pellet was suspended in medium (MEM) containing 15% FCS and incubated at 27°C with 5% CO₂. The cells were then passaged continuously and maintained.

Biocompatibility of Scaffold with Muscle cells of *Trachinotus blochii*

A twelve well plate with MEM medium was taken and the scaffolds were placed in each well of the plate, after being heated at 60°C for 30 minutes and exposed to UV radiation. A volume of 0.5ml (1x10⁵ cells ml⁻¹) of muscle cells was added into the wells with the scaffolds. The viability of the cells was monitored routinely by Trypan Blue Dye Exclusion Assay (Tolnai, 1975) and the compatibility of the cells with the scaffolds was determined.

RESULTS

Isolation and Characterization of the Marine Bioactive Compounds

The marine bioactive compounds, carrageenan, chitosan, collagen and WSM of the nacre were extracted and characterized by FTIR spectroscopy. The yield of carrageenan extracted from *K. alvarezii* was about 36.5% (w/w) of the total weight. The FTIR spectrum of the extracted carrageenan showed peaks corresponding to different functional groups (Figure 1A).

The peak at 3425.10 cm⁻¹ indicated the presence of O-H groups. The peaks along 2924.63 cm⁻¹ and 2855.14 cm⁻¹ showed the presence of C-H groups. The peak at 2090.94 cm⁻¹ showed the presence of thiocyanite ions. The peak at 1632.47 cm⁻¹ showed the presence of N-H groups. The peak at 1604.92 cm⁻¹ indicated the presence of C-O groups. The peaks along 1495.75 cm⁻¹ and 1464.97 cm⁻¹ indicated the presence of NH³⁺ and C=S groups respectively. The peak along 1382.62 cm⁻¹ showed the presence of C-H groups. The peaks at 1268.47 cm⁻¹ and 1250.71 cm⁻¹ confirmed the presence of C-O groups. The peak at 1158.33 cm⁻¹ showed the presence of C-H groups. The peak along 1071.26 cm⁻¹ and 1036.07 cm⁻¹ showed the P-O-C groups and S=O groups, respectively. The characteristic peak along 849.54 cm⁻¹ indicating the bending of secondary sulphate groups on C4 of the galactose sugar and 929.29 cm⁻¹ showing the stretching of C-O group due to the presence of the 3,6 anhydro-galactose in the compound (Pereira *et al.*, 2009) confirms that the extracted compound is κ -carrageenan (Chopin and Whalen, 1993).

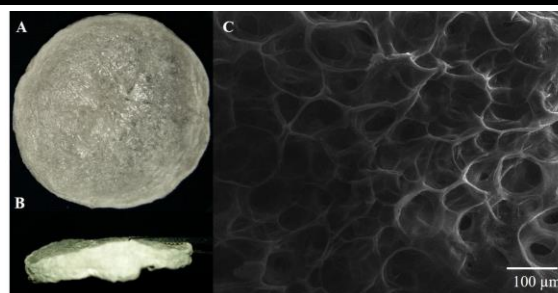


Figure 2. A) Photographic image of the fabricated CCCW scaffold, B) Side view of scaffold showing thickness of the scaffold, C) Microstructure of CCCW scaffold observed under Scanning Electron Microscope.

The shrimp shells were processed for the production of chitosan and the yield was about 20% (w/w) of the total weight. The FTIR spectrum of the extracted chitosan was analysed using FTIR spectroscopy (Figure 1B). The spectrum showed peaks along 3428.43 cm⁻¹ which was due to the presence of O-H and N-H groups in the chitosan. The peaks along 2924.64 cm⁻¹ and 2858.01 cm⁻¹ showed the presence of C-H groups. The weak peak at 2134.45 cm⁻¹ showed the C=C groups. The peak along

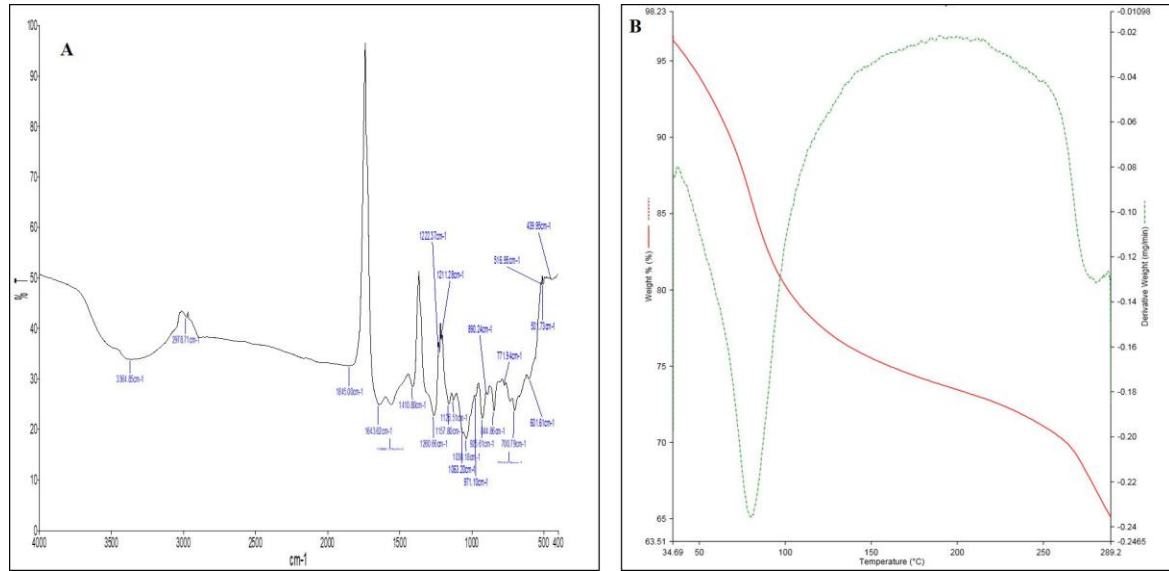


Figure 3. A) FTIR spectrum of the CCCW scaffold, B) Thermogram of CCCW scaffold.

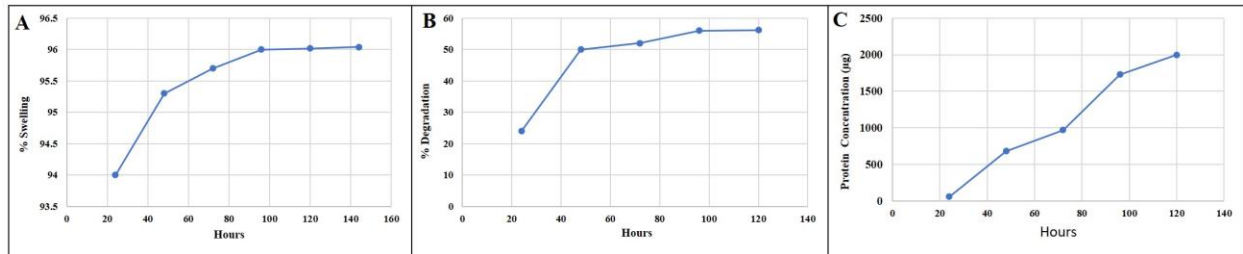


Figure 4. A) Graph showing the swelling ratio of CCCW scaffold in PBS (pH 7.4), B) Graph showing the degradation of the scaffold in the medium, and C) Graph showing the protein absorption rate of the scaffold.

1635.95 cm^{-1} showed the presence of C=C groups. The peaks along 1423.45 cm^{-1} and 1385.68 cm^{-1} showed the presence of methyl groups. The characteristic peaks along 1635.95 cm^{-1} indicating the presence of Carbonyl group (Teli and Sheik, 2012) and 3428.43 cm^{-1} indicating the presence of amide group (Varun *et al.*, 2017) were observed.

Further the presence of peaks along 877.27 cm^{-1} in the obtained spectrum indicates the Pyranose sugar stretching showing that the polysaccharide was Chitosan. The yield of collagen extracted from the skin of *S. lycidas* was about 2.5% (w/w). The FTIR spectrum of collagen (Figure 4C) showed peaks along 2926.56 cm^{-1} which was due to the CH_2 group in the extracted compound.

The peaks along 2857.64 cm^{-1} and 1641.03 cm^{-1} indicated the presence of CH_2 group and C=O groups, respectively. The peak along 1553.69 cm^{-1} and 1453.02 cm^{-1} indicated the presence of NH bend and CH_2 bend, respectively. The peaks along 1387.13 cm^{-1} , 1245.93 cm^{-1} and 1074.76 cm^{-1} showed the presence of wagging of CH_2 group, NH group and C-O group, respectively. These peaks were closely in correspondence to peaks observed

in other collagens. Hence, it is inferred that the extracted protein is collagen.

Fabrication of the Scaffold

The polymeric mixtures used in the fabrication of the scaffolds are usually oppositely charged. However, carrageenan, chitosan and collagen when mixed form strong insoluble precipitates. This was prevented by adding potassium chloride to the mixture. The fabricated scaffold was white in colour and inelastic in nature. The wet scaffold was softer and showed elasticity. The thickness of the scaffold was found to be 1.5 mm.

Physicochemical Properties

The chemical composition of the scaffold analyzed using the FTIR analysis showed the changes in absorption peaks of different functional groups. The shifts in the peaks were mainly due to the interaction of the various functional groups. Similarly, the Thermogram of the scaffold also showed its thermal stability under various temperatures. The microstructure of the scaffolds observed under Scanning Electron Microscope revealed the

porous nature of the scaffold. The scaffold had pore size ranging from 19-44 μm in diameter.

Swelling Ratio

Swelling ratio of the scaffold was estimated by incubating the scaffold at 27°C in PBS. Figure 4A shows the swelling profile of the scaffold. The swelling ratio of the scaffold increased rapidly to 94% after 24 hours of incubation. A gradual increase in the swelling ratio was observed after 48 hours. The swelling ratio reached its saturation after 96 hours.

Degradation Rate

The degradation of the scaffold was analyzed by incubating the scaffold in MEM medium. The degradation of the scaffold was observed for 5 days. The degradation rate of the scaffold is shown in Figure. 4B. About 24% of the scaffold was found to be degraded after 24 hours and 56% of the scaffold after 96 hours. The rest of the scaffold was intact even after two weeks.

Protein Absorption Capacity

The protein absorption into the scaffold increased gradually over time (Figure 4C). A maximum of 2000 μg of protein was found to be absorbed on to the scaffold at the 120th hour. The porosity of the scaffold also plays a major role in its protein absorption capacity and the protein absorption also reaches a saturation point. However, the scaffold protein absorption rate was appreciable and seemed to meet the requirement necessary for a tissue engineered scaffold.

Biocompatibility of Scaffold with Muscle cells of *Trachinotus blochii*

The isolated fish cells were inoculated along with the fabricated scaffold and the compatibility was assessed. The viability of the cells was checked continuously and was found to be greater than 90% for 120 hours. The cells were seen to surround the scaffold (Figure 5) and did not move away thereafter. The scaffold promoted the growth of fish cells, and formation of the muscle fibers was seen on the fifth day (Figure 5F) indicating its biocompatibility with the fish cells.

DISCUSSION

The CCCW scaffold fabricated using the extracted marine biomaterials were found to be biocompatible, with enhanced swelling and protein absorption properties. The polysaccharide present in the scaffold influences the properties of the scaffolds to a greater extent. Li *et al.* (2014) reported that carrageenan can improve the swelling ratio and mechanical strength of the scaffold. The larger the water holding capacity of the scaffold the greater will be the efficiency of the scaffold *in vitro*. In this study, swelling ratio increased up to 96 hours. The higher absorption of water by the scaffold was mainly due to the water absorption capacity of the carrageenan, which is present in larger amounts in the scaffold.

Protein adsorption capacity of the scaffold is a parameter for the tissue engineering application, as the scaffold must have the capacity to absorb proteins from the medium thus facilitating the cells to utilize the protein and grow within the scaffold. The proteins may also include the growth factors and other proteins which are supplemented through the medium. Chen *et al.* (2016)

reported that incorporation of nacre in the scaffold enhances the protein absorption capacity of the scaffolds. Liu, Huang, and Feng (2013) incorporated pearl powder into the scaffolds. After the addition of the pearl powder a change in the pore size of the scaffold was observed. They reported that the pearl powder in the scaffold improved the mechanical properties of the scaffolds. Hence WSM of nacre was used in this study to infer its impact on the scaffold.

Other polysaccharides such as alginate when incorporated into the scaffold improve the degradability of the scaffold (Chen *et al.*, 2016). The carrageenan component of the scaffold plays a major role in decelerating the degradation rate of the scaffold. Controlled degradability of the scaffold is also necessary for an efficient scaffold.

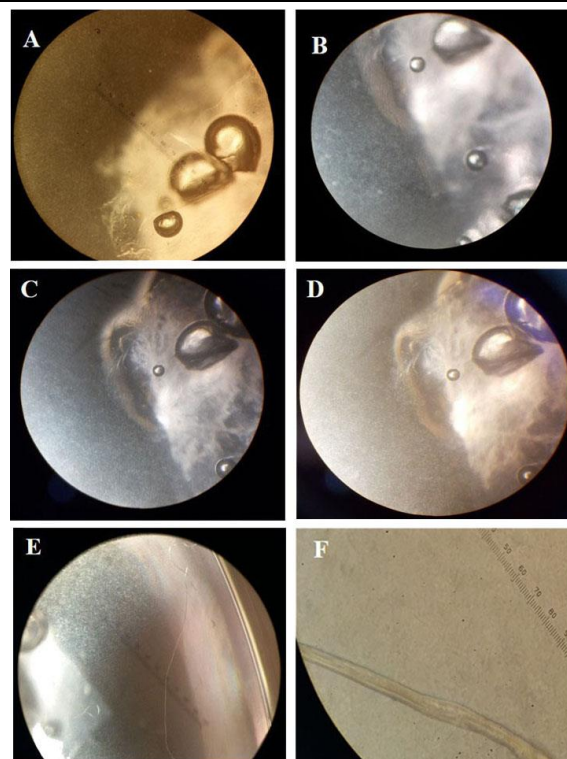


Figure 5. Observation of scaffold with cells under phase contrast microscope (4X magnification) at different time intervals A) 24 hours B) 48 hours C) 72 hours D) 96 hours E) 120 hours F) Appearance of muscle fibers after 120 hours of incubation (40X).

The scaffolds composed of collagen and chitosan are known to be biocompatible and are used for diverse applications. Ma *et al.* (2003) proposed a freeze-dried collagen chitosan porous scaffold for skin tissue engineering. Ahn *et al.* (2010) reported a collagen scaffold with precisely controlled pore structure for skin tissue engineering in which the keratinocytes and fibroblast cells were dispersed within the scaffold. Li *et al.* (2014) showed that the carrageenan chitosan gelatin scaffold was suitable for the attachment and proliferation of the adipose-derived mesenchymal cells. Apart from the biomedical application, the scaffolds can also be used for various other applications. Since

the scaffold is made up of biocompatible and edible biomaterials, the application may extend to the food industry.

In vitro meat production is a recent concept in food biotechnology, and is also referred to as cellular agriculture, where the agricultural products are produced from plant or animal cells rather than the whole organism. They can involve either growing the edible part by means of an explant culture or by three-dimensional culture of tissue (Rubio *et al.*, 2018). The process of production of the meat *in vitro* involves the culturing of the muscle cells in a three-dimensional network. Edible biomaterial-based scaffolds can be used in the *in vitro* meat production process for the three-dimensional cultivation of the tissue. In the current study, the materials used in the fabrication of the scaffolds are food related biomaterials. Carrageenan, chitosan and collagen are used in the food industry, and hence a scaffold fabricated using these materials were evaluated for compatibility with fish muscles as an initial step towards edible application. The biomaterials used in the fabrication of the scaffold were all derived from the wastes such as crustacean shell and skin of fish. The shells of crustaceans are considered as waste material by the food processing companies and approximately 60,000-80,000 tons of shell fish wastes are disposed by these industries (Tarafdar and Biswas, 2013). The skin of cuttle fish is treated as waste at homes, fish shops and fish processing factories (Krishnamoorthy *et al.*, 2017). Hence, they also prove to be economically viable.

In the present cell-based fish approach, the muscle cells of *Trachinotus blochii* were isolated and cultured along with the fabricated scaffolds. Earlier reports suggest that cells of marine molluscs and crustaceans can also be maintained *in vitro*. George and Dhar (2010) reported the maintenance of fibroblast cells and ovary-derived epithelial cells of crustaceans. Similarly, the cells of abalone were successfully maintained in M199 medium (Suja and Dharmaraj, 2005). Torres *et al.* (2016) cultured the muscles of fish to study the myostatin function in fish. The cells of other marine organisms can also be co-cultured with the scaffold for meat production.

Enrione *et al.* (2017) reported the growth of the *Mus musculus* muscle cells (C2C12) in the scaffold for meat production. A cost effective medium for the culturing of cells and identification of a plasticizer suitable for the type of cell used are necessary for the successful production of *in vitro* meat. The present study is a stepping stone for the production of marine food by means of the cellular agriculture.

CONCLUSION

The CCCW scaffold possesses all the characteristics required for an ideal scaffold. The scaffold production cost also proves to be cost effective as the materials used for the extraction of these marine bioactive compounds are industrial waste products. The scaffold supports the growth of fish muscle cells and can be used in the *in vitro* meat production. Further research and modifications are required. Modifications in the scaffold can also be made for better pore size and adherence of the cells to the scaffold. The successful growth of the cells in the scaffold can be beneficial in production of sea food without intense aquaculture systems. The current study is an initial study that shows the biocompatibility of the scaffold with the fish muscle cells.

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