

## **Polyelectrolyte Complex of Chitosan and $\kappa$ -Carrageenan as Potential Scaffold for Tissue Engineering**

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### **ABSTRACT**

Chitosan,  $\kappa$ -carrageenan scaffolds were fabricated by forming their polyelectrolyte, followed by crosslinking  $\kappa$ -carrageenan with calcium chloride. The scaffolds were reinforced with hydroxyapatite of nano and micron sizes. Most stable scaffolds were formed when chitosan and  $\kappa$ -carrageenan were in 1:1 molar ratio, and when 1 wt% nano-hydroxyapatite was used as the reinforcing agent. The scaffolds in dry form were sponge-like, flexible, and strong enough to be handled in dry conditions without undergoing any deformation. Scanning electron microscopy (SEM) revealed that the scaffolds were porous at 79-95% porosity, depending on the type of hydroxyapatite used. The scaffolds swelled moderately and showed slow rate of degradation in the presence of lysozyme under human physiological condition. The extent of swelling and degradation was influenced by the type of hydroxyapatite incorporated. The scaffolds also supported the growth of BT-20 cells, proving that they are not cytotoxic.

*Keywords:* Chitosan,  $\kappa$ -carrageenan, hydroxyapatite, scaffold

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## INTRODUCTION

Fabrication of scaffolds from engineered biomaterials has been gaining much attention in the field of biotechnology as it offers a physiologically and biochemically suitable method of enhancing and restoring natural tissue. With the increasing number of calamities involving bone fracture and skin injury, numerous investments and research are being conducted on the engineering of biodegradable scaffolds for various applications, specifically those that are designed for tissue engineering or regenerative medicine.

A scaffold is a three-dimensional framework that primarily functions as a support system for cells to attach, proliferate, and grow, thereby allowing them to develop and perform their differentiated functions. Once the cells begin to undergo proliferation, the scaffold naturally degrades and its components are absorbed by the body, eventually providing space for the cells to further regenerate and for the new tissue cells to organize and take form. In order to facilitate tissue regeneration, certain biological and structural requirements must be taken into consideration. An ideal scaffold should be biocompatible with interconnected pore network to allow the migration of cells, oxygen, and nutrients. It should facilitate cell adhesion, proliferation, differentiation, and biodegradability at a controlled rate and should have sufficient mechanical strength to support the cells.

Although synthetic polymers, such as polylactic acid, polyglycolic acid, and polyprolactone (Wayne et al. 2005; Yeong et al. 2010; Giuliani et al. 2014; Santoro et al. 2016), are used in tissue engineering, there has been a growing interest in the use of natural polymers over synthetic polymers as material for scaffolds. Their relatively low cost and wide availability, as well as their biological and chemical similarities to bodily tissues which reduce the risk of rejection by the body, give them an advantage over synthetic polymers. Some of the natural polymers, such as pullulan, dextran, hyaluronic acid, and collagen, have been studied as scaffolding material (Chen et al. 2012; Sun and Mao 2012; Cutiongco et al. 2014; Amrita et al. 2015).

Apart from the abovementioned natural polymers, chitosan (CS) has been widely explored as a scaffolding material component (Nettles et al. 2004; Rodríguez-Vázquez 2015). It has been established that this polymer aids in osteochondral tissue regeneration (Lahiji et al. 2000; Abarrategi et al. 2010), and has considerable antibacterial activity against a broad range of bacteria (Benhabiles et al. 2012).

So far, most of the CS scaffold have been fabricated by using either genipin (Zhang et al. 2013) or glutaraldehyde (Monteiro and Airoidi 1999) as the crosslinking components in the scaffold formation. However, the disadvantage of using genipin is that the crosslinking reaction is pH-sensitive, whereas glutaraldehyde is considered as cytotoxic. This project explored a novel route to synthesize CS scaffolds without the use of genipin and glutaraldehyde.

Carrageenan is a biopolymer extracted from marine red seaweeds. It is a linear heteropolysaccharide consisting of alternating 1,4- $\alpha$  and 1,3- $\beta$ -D-galactopyranose, and 3,6-anhydro-D-galactopyranose units. Among several types of carrageenans which differ based on the number and position of ester sulfate groups,  $\kappa$ -carrageenan (CRG) is known to produce strong and rigid gels in the presence of sodium, potassium, and calcium ions. Carrageenan alone or in combination with other polymers has been widely studied for various applications. Polyelectrolyte complexes of CS and carrageenan as vehicles for controlled release applications (Piyakulawat et al. 2007; Briones and Sato 2010; Pinheiro et al. 2012) are well-documented. Carrageenan has also been explored as a component of biocomposite scaffold for bone tissue engineering (Luís et al. 2007; Lim et al. 2010; Araujo et al. 2014).

Hydroxyapatite (HA) is one of the most stable forms of calcium phosphate and is a major component (60-65%) of natural bones and hard tissues found in the body. In tissue engineering, HA-based scaffolds exhibit excellent mechanical strength and osteoconductive properties. Thus, many studies extensively use HA as a reinforcing agent in biodegradable polymeric scaffolds (Oliveira et al. 2006; Han et al. 2010; Peter et al. 2010; Pathi et al. 2011; Mondal et al. 2016; Lei et al. 2017).

The group has made an innovative approach of fabricating scaffold from semi-interpenetrating network (semi-IPN) polyelectrolyte complex (PEC) of CS and CRG. Since CS is a polycation and CRG is a polyanion, they form PEC when in contact with each other. CS and carrageenan chains were allowed to penetrate each other, followed by crosslinking the carrageenan with calcium ions ( $\text{Ca}^{2+}$ ). This approach was adopted to avoid the use of harmful crosslinkers, such as glutaraldehyde. CS is expected to contribute towards the strength of the scaffold, whereas carrageenan is expected to provide the crosslinkable sites. Further reinforcement to the scaffolds was provided through the incorporation of HA.

## MATERIALS AND METHODS

### Materials

CS, sodium azide (99%), HA were purchased from Sigma-Aldrich. CRG was purchased from Rico. Calcium chloride was obtained from J.T. Baker. All the chemicals and reagents were used without further purification. For the cell culture experiment, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. Minimum Essential Medium (MEM) Eagle, Roswell Park Memorial Institute (RPMI) medium, fetal bovine serum (FBS), sodium bicarbonate (7.5 wt%), L-glutamine (200 mM in saline), and Pen-Strep Sol. (penicillin 10000 units/mL and streptomycin 10 mg/mL) were provided by Biological Industries.

### METHODS

#### PEC with Various Ratios of CS and CRG

To synthesize a PEC of CS-CRG scaffold with CS:CRG in 1:1 molar ratio, 0.2 g of CS (mass of each repeat unit: 160.15 g/mol) was dissolved in 50 mL 1% acetic acid, and 0.5 g of CRG (mass of each repeat unit: 440.2 g/mol) was dissolved in 50 mL deionized water. Both the solutions were mixed and stirred together in a beaker at room temperature using a magnetic stirrer for one hour to obtain a homogenous solution. This solution was transferred to a vial and was placed in a refrigerator to freeze for 24 hours at  $-15^{\circ}\text{C}$ , and then freeze-dried for 48 hours at  $-40^{\circ}\text{C}$  and  $200 \times 10^{-3}$  mBar using the Freezone 4.5 Labconco instrument. This procedure was repeated to obtain a set of freeze-dried samples prepared with CS:CRG in molar ratios of 1:1, 2:1, 5:1, 10:1, and 1:2.

#### Synthesis of Scaffolds in the Presence of HA

In order to fabricate HA-reinforced scaffold with CS and CRG in the stoichiometric ratio of 1:1, 0.2 g of chitosan was dissolved in 50 mL of 1% acetic acid, and mixed with 0.005 g (1 wt% w.r.t. the scaffold) of micro-HA ( $\mu\text{HA}$ ). The dispersion was stirred for 6 hours, followed by slow addition of CRG solution (0.5 g of CRG in five mL of water) with continuous stirring. The mixture was stirred for another 30 minutes before the addition of 0.025 g of  $\text{CaCl}_2$ . The mixture was stirred for another 30 minutes and transferred to a vial. The vial was placed in a refrigerator

at -15°C for 24 hours and lyophilized for 48 hours using Freeze Dry System/Freezone 5.4. Labconco instrument (-40°C,  $200 \times 10^{-3}$  mBar).

The same procedure was followed to prepare the scaffolds with 0.005 g of nano-HA (nHA), except that the CS-nHA mixture was stirred for one hour instead of six hours. Scaffolds without any HA were also fabricated as control.

### Stability Test for the PECs in Aqueous System

Stability of the semi-IPN with different CS to CRG ratios was evaluated in aqueous medium. A total of 0.05 g of each type of semi-IPN was immersed in 15 mL deionized water for a week at room temperature. The morphological changes over the period of one week were monitored.

### Swelling Behavior

Each type of dry scaffold (0.10 g) was completely submerged in a closed vial containing 15 mL of deionized water and placed on a shaker. After a certain period of time, water was decanted out of the vial, and the scaffold was pat-dried to remove excess water from its surface before the actual weight determination. The percent swelling of the systems were computed using equation (1).

$$\% \text{ Swelling} = \frac{\text{Weight of Swelled Hydrogel} - \text{Weight of Dry Hydrogel}}{\text{Weight of Dry Hydrogel}} \times 100 \quad (1)$$

### Porosity Measurement

Porosity of scaffolds was measured through the liquid displacement method. Absolute ethanol with density ( $\rho$ ) of 0.785 g/mL was used as a displacement liquid because it can easily penetrate the scaffolds without inducing shrinking or swelling as it serves as a non-solvent for the scaffolds.

The experiment was performed as per the procedure reported by Xu et al. (2012). All the samples were cut into cylindrical shape using a thin blade cutter, and their corresponding diameter and thickness were measured using a ruler. The thickness of the samples ranged from 1-1.5 cm, while the diameter ranged from 0.9-1.3 cm. Volume of the scaffolds ( $V_{\text{scaffold}}$ ) was calculated using these values. Weight of each

sample ( $W$ ) was recorded before placing them in vials containing ethanol. The vials were sonicated for 30 minutes to allow ethanol to fully impregnate the scaffolds. The wet weights of the scaffolds ( $W_1$ ) were measured after taking them out from the containers. Equation (2) was used to calculate the percent porosity (%P) of the scaffolds.

$$\%P = \frac{W_1 - W}{\rho \cdot V_{scaffold}} \times 100 \quad (2)$$

### Degradation Study in the Presence of Lysozyme

Scaffolds were weighed ( $W_i$ ), sterilized in PBS buffer for 15 minutes at 100°C, and placed inside the pomade jars. After sterilization, the PBS buffer was replaced by 15 mL of freshly prepared PBS solution with 10 µg/mL of lysozyme. This PBS with lysozyme solution was refreshed weekly. The samples were collected after 14 and 28 days. The collected samples were washed with distilled water before subjecting them to freeze drying. The freeze-dried scaffolds were weighed ( $W_f$ ), and equation (3) was used to calculate the percent weight loss (%W) of the samples.

$$\%W = \left[ \frac{W_i - W_f}{W_i} \times 100 \right] \quad (3)$$

### Cell Culture Study

BT-20 human breast-cancer cells (ATCC® Number: HTB-19TM) were purchased from the National Health Research Institutes (NHRI), Taiwan. BT-20 cells were incubated in flasks with MEM medium at 37°C, 5% CO<sub>2</sub>, and 95% humidified atmosphere, and were sub-cultured every three days. Each 100 mL of MEM was supplemented with 10 mL FBS, 2 mL NAHCO<sub>3</sub>, 1 mL L-glutamine, and 1 mL Pen-Strep Sol. (penicillin 10000 units/mL and streptomycin 10 mg/mL).

BT-20 cells were seeded onto 24-well Lab-Tek slides at a density of 5 × 10<sup>4</sup> cells per well with the presence of the cell matrix. After incubation at 37°C with 5% CO<sub>2</sub> for 24 hours, the medium was removed. After rinsing with PBS, the cultured cells were fixed with 3.7% paraformaldehyde in PBS for 10 minutes, followed by rinsing with PBS three times. The fixed cells were then permeated with 0.2% Triton X-100 in PBS for 10 minutes. The samples were then rinsed with PBS three times and incubated with 500 nM phalloidin-TRITC for F-actin staining and 100 nM DAPI for nuclei staining. Fluorescent images of cells were captured with a Nikon TE2000-U camera. The excitation/emission wavelengths were 405/461 nm for DAPI and 561/572 nm for phalloidin-TRITC.

### **Fourier Transform Infrared (FTIR) Spectroscopy**

The different components of the scaffold were characterized using Shimadzu IR Affinity-1 FTIR Spectrophotometer. A dried sample was ground along with potassium bromide (KBr) powder and pressed mechanically to a thin disc. For each sample spectrum, a 40-scan interferogram was collected in terms of percent transmittance (%T) with a  $2\text{ cm}^{-1}$  resolution from the  $4000\text{-}400\text{ cm}^{-1}$  region in the IR Solution software.

### **Scanning Electron Microscopy**

The microstructures of the scaffolds were viewed using a scanning electron microscope (SEM). A small portion of the bulk scaffold was carefully removed using tweezers without compressing the material. The sample was then gently placed onto a carbon film and sputtered with gold using a JEOL JFC 1200 gold fine coater before analyzing with JSM-5310 scanning microscope (SEM) at 10 kV under different magnifications.

## **RESULTS AND DISCUSSION**

### **Stability Test for the CS-CRG PECs in Aqueous System**

It is critical that CS and CRG form a stable PEC through the electrostatic interaction of cationic CS and anionic CRG to render strength to the semi-IPN scaffold. A scaffold is expected to have enough strength to maintain its structural integrity to support cell growth. Hence the CS:CRG molar ratio at which the PEC retains its structural integrity in aqueous medium for a prolonged period was determined.

As shown in Figure 1, after two weeks of immersing of the PECs in deionized water, the PEC with 1:1 molar ratio of CS to CRG maintained its structural integrity, whereas the other combinations fell apart. Stability of 1:1 molar ratio formulation can be attributed to the complete neutralization of polycationic CS and polyanionic CRG. Other formulations, wherein either CS or CRG was in excess, attracted extra amount of water and disintegrated.

As seen in Figure 1, the scaffold with CS and CRG in the stoichiometric ratio of 1:1 was able to maintain its structural integrity for two weeks, and hence, this ratio was maintained for the synthesis of all the scaffolds.

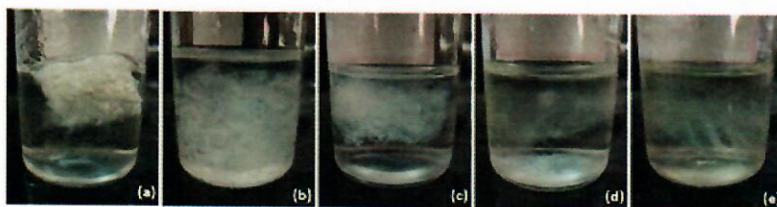


Figure 1. CS-CRG PEC with CS:CRG in molar ratio of (a) 1:1, (b) 1:2, (c) 2:1, (d) 5:1, and (e) 10:1 in deionized water after two weeks.

### Scaffold Synthesis

Once stable PEC of CS and CRG was formed in the aqueous medium, CRG was subjected to crosslinking by using  $\text{CaCl}_2$  to form the scaffold. The reaction is schematically represented in Figure 2.

As shown in Figure 3, the scaffold formulations were white in the dry form, with sponge-like texture, strong enough to be handled in dry conditions without deformation, and can be compressed.

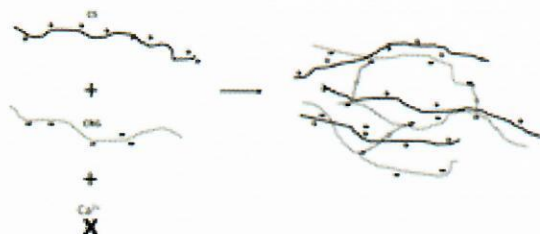


Figure 2. Schematic representation of scaffold formation.

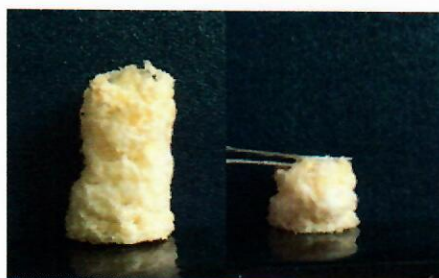


Figure 3.  $\text{Ca}^{2+}$ -crosslinked CS-CRG scaffold after freeze drying (a) without any compression and (b) under compression.



Figure 4 compares the FTIR spectrum of the CS-CRG scaffold with CS. The characteristic peaks of the amide I and amide II was identified at  $1640\text{ cm}^{-1}$  and  $1562\text{ cm}^{-1}$ , respectively for CS. In addition to these, a broad band at  $\sim 3400\text{ cm}^{-1}$  was also identified due to the overlapping stretching vibrations from the  $-\text{NH}_2$  and  $-\text{OH}$  groups of CS. CRG exhibited a broad peak at  $1252\text{ cm}^{-1}$ , corresponding to the sulfate groups. Peaks at  $923\text{ cm}^{-1}$  and  $848\text{ cm}^{-1}$  corresponded to the 3,6-anhydrogalactose and galactose-4-sulfate of CRG. The FTIR spectrum of the complex proved the presence of both CS and CRG peaks. Shift in the position of some of the peaks were also observed: for instance, the amide peaks of CS shifted from  $1640\text{ cm}^{-1}$  and  $1562\text{ cm}^{-1}$  to  $1630\text{ cm}^{-1}$  and  $1550\text{ cm}^{-1}$ . The sulfate peak of CRG shifted from  $1252\text{ cm}^{-1}$  to  $1249\text{ cm}^{-1}$  and the intensity of the peak was reduced as well. These observations are similar to the observations reported by Araujo et al. (2014) for the FTIR spectra.

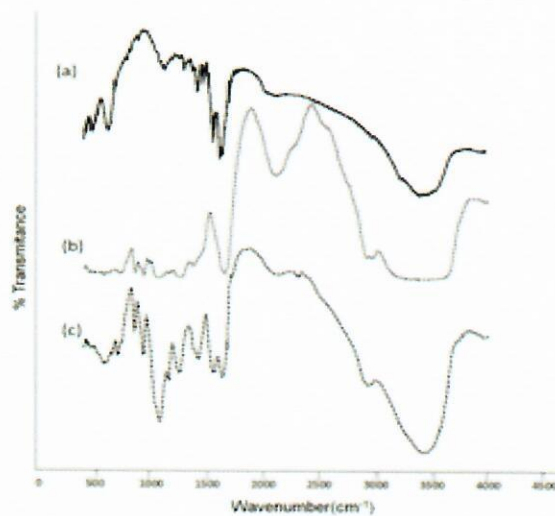


Figure 4. FTIR spectrum of (a) CS, (b) CRG, and (c) CS-CRG scaffold.

### Morphology

An interconnected porous morphology and homogeneous microstructure is desirable for a scaffold as it facilitates unobstructed flow of cells and vital nutrients, thus promoting cell growth and proliferation. SEM image of the freeze-dried CS-CRG scaffold showed a porous and interconnected network (Figure 5).

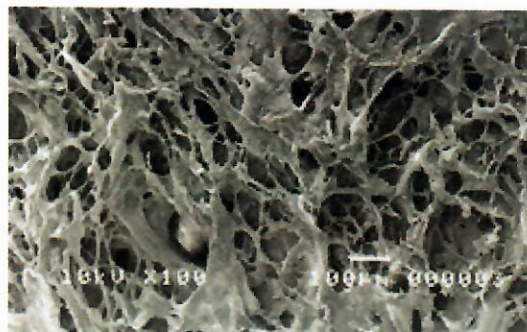


Figure 5. SEM micrograph of CS-CRG reinforced with 1 wt% nHA.

However, the scaffold that was fabricated by mixing 1 wt%  $\mu$ HA with CS solution for one hour showed the presence of  $\mu$ HA (Figure 6a) on the surface of the network, indicating inefficient incorporation of  $\mu$ HA in the scaffold network. Extending the stirring time to six hours significantly improved the incorporation of the  $\mu$ HA in the scaffold (Figure 6b), suggesting that the stirring time has a profound influence on scaffold synthesis. On the other hand, as seen in Figure 6c, a stirring time of one hour for the 1 wt% nHA-reinforced scaffold was found to be sufficient for the incorporation of nHA in scaffold network.

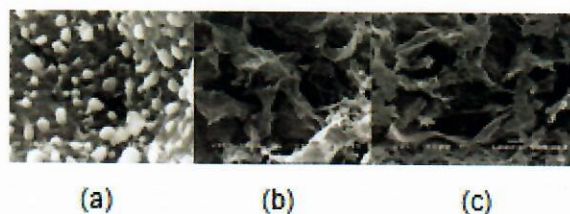


Figure 6. SEM micrographs of scaffolds reinforced with (a) 1 wt%  $\mu$ HA after one hour of mixing, (b) 1 wt%  $\mu$ HA after six hours mixing, and (c) 1 wt% nHA after one hour of mixing.

### Porosity

Porosity is an important aspect of an ideal scaffold as a porous structure with intercommunicating pores facilitates efficient regrowth of the bone and the migration of nutrients to support the regrowth. However, too much porosity might compromise the mechanical strength of the scaffold. In order to assess the porosity of the CS/CRG/HA scaffolds, liquid displacement test was performed. The results are summarized in Table 1.

The scaffolds without HA exhibited a porosity of 85%. Addition of 1 wt% nHA reduced the porosity of the scaffolds, confirming its firmer structure that could improve the mechanical strength while promoting cell growth. In contrast to 1 wt% nHA, 1 wt%  $\mu$ HA significantly increased the porosity of the scaffolds, thus it might not be able to provide adequate reinforcement to the scaffold.

**Table 1. Porosity for different scaffold formulations**

Sample	Average $V_{\text{scaffold}}$ (cm <sup>3</sup> )	Average W (gm)	Average $W_1$ (gm)	%P*
CS/CRG/no HA	1.6	0.11	1.18	85
CS/CRG/1 wt% $\mu$ HA	0.98	0.06	0.80	95
CS/CRG/1 wt% nHA	1.8	0.09	0.09	79

\*Each point is the mean of two readings,  $\rho_{\text{EtOH}}$ : 0.785 g/mL.

### Swelling Behavior

Swelling behavior of the scaffolds with and without HA was studied. As seen in Figure 7, the scaffolds with nHA swelled less than the CS-CRG scaffold without HA, implying that nHA fortifies the scaffold structure. However, the scaffolds with  $\mu$ HA swelled more than its CS-CRG counterpart. The poor dispersion of  $\mu$ HA in CS-CRG PEC generated additional voids between fillers, allowing excess water to penetrate inside the scaffold and contributing towards maximum swelling.

Porosity and swelling behavior trends indicate that the size of reinforcing agent has an impact on the properties of the scaffolds.

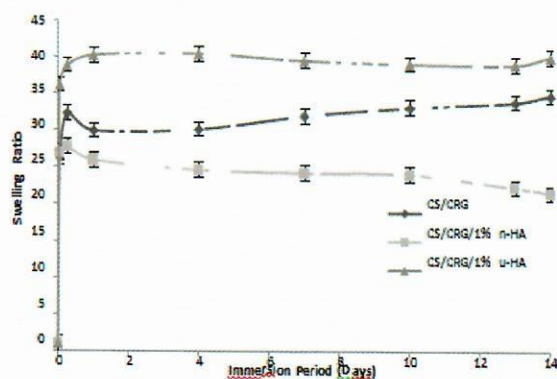


Figure 7. Swelling behavior of CS-CRG scaffold with and without HA. Each data point represents the average of three samples.

### Degradation Behavior in the Presence of Lysozyme

Scaffolds are expected to promote cell growth followed by degradation at a controlled rate to create space for the growing cells; hence, it is critical to assess the degradation profile of the scaffold. Scaffolds were subjected to degradation in the presence of lysozyme, and their weight loss as function of time was studied. Lysozyme is the enzyme responsible for degradation of CS inside human body (Tomihata and Ikada 1997; Verheul et al. 2009).

No significant deformation of the scaffolds was observed for almost 10 days. After 14 days, scaffolds with 1 wt%  $\mu$ HA underwent maximum weight loss, whereas the scaffolds with 1 wt% nHA showed minimal weight loss. This observation is similar to the swelling behavior trend. The results also reveal that scaffolds degraded partially after 28 days. Since these scaffolds undergo slow degradation, they should be able to facilitate the cell growth at a controlled rate for a prolonged period. Table 2 summarizes the weight loss of the scaffolds as a function of time.

**Table 2. Weight losses (degradation) of scaffold in PBS buffer in the presence of lysozyme**

Sample	Day	% Weight lost*
CS/CRG/no HA	14	34
	28	62
CS/CRG/1 wt% $\mu$ HA	14	53
	28	59
CS/CRG/1 wt% nHA	14	26
	28	39

\*Each point is a mean of two readings

### Cytotoxicity

Cell culture studies are important for determining the cytotoxicity of the scaffold and assessing its performance for tissue engineering applications. Through fluorescence image analysis, the presence of BT-20 cells marked by the blue dye was detected for 1 wt % nHA scaffold (Figure 8). *In vitro* cell adhesion and proliferation on the nanocomposites occur without changes in the viability and cytoskeleton formation, proving that the fabricated scaffold is not cytotoxic. This observation also confirms that the fabricated scaffolds exhibited sufficient surface area and an interconnected porous structure that promotes cell adhesion, proliferation, and the flow of nutrients.

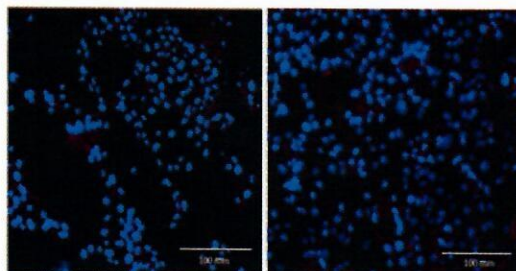


Figure 8. SEM micrographs of (a) non-reinforced and (b) 1 wt% nHA-reinforced scaffolds seeded and cultured with BT-20 cells for three days.

## CONCLUSION

This study established a novel method of fabrication of CS scaffold, wherein PEC of CS and CRG is formed, followed by crosslinking CRG with  $\text{CaCl}_2$ . The process thus avoids the use of glutaraldehyde as the crosslinker. HA in the form of particles in nano and micron sizes were incorporated in the scaffold by dispersing them in CS-CRG solution prior to crosslinking. It was easier to disperse nHA than  $\mu\text{HA}$ . The scaffolds were porous and swelled in pH 7 buffer. Percent porosity and swelling were minimal for the scaffolds with nHA compared to scaffolds without HA or with 1 wt%  $\mu\text{HA}$ . nHA scaffolds were also most resistant towards degradation in the presence of lysozyme, and formed a stable porous scaffold which was further fortified by HA. Reduction in the size of HA from micron to nano size led to the formation of a homogenous blend with CS-CRG in a shorter time span. The size reduction of HA also improved. The said scaffolds can also support adhesion and proliferation of BT-20 cells and are noncytotoxic. Overall, the results suggest that these scaffolds have optimal properties and the potential of promoting cell growth. Hence, they can be further explored as benign and viable scaffolds for tissue engineering.

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