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Effect of Different Concentration of Cellulose Nanocrystals Comprising Hydroxyethyl Cellulose / Poly(Vinyl Alcohol) as a Bone Tissue Engineering Scaffold

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Abstract. In this study, biodegradable scaffolds based on hydroxyethyl cellulose (HEC) (5 wt%) and poly (vinyl alcohol) (PVA) (15 wt%) with different percentages of celullose nanocrystal (CNC) (1 and 7 wt%) were fabricated by lyophilization method to get highly porous scaffolds. These scaffolds were made water insoluble by cross-linking *via* heat treatment. The morphology and thermal properties of HEC/PVA/CNCs scaffolds were characterized by using Scanning Electron Microscope (SEM) and Thermogravimetric Analysis (TGA). The morphological study showed that both prepared scaffold have highly porous structures with good pore interconnected structure. It was observed that thermal properties of scaffolds increased significantly as the concentration of CNCs increased. Cytotoxicity studies on scaffolds were carried out by utilizing human fetal osteoblast (hFOB) cells using DAPI nuclear stain and then confirmed using SEM. hFOB cells were able to attach and spread on all scaffolds. Incorporated CNCs as reinforcing nanofiller on scaffolds promising a superior functionality in bone tissue engineering.

Introduction

Tissue engineering scaffolds provide a better choice in order to repair and regenerate damaged tissues by mimicking the structural and functional profile of the natural extracellular matrix (ECM). ECM are composed into two phases; organic and inorganic, where the inorganic phase consisting primarily of nano-hydroxyapatite(n-HA) crystals, and the organic phase consisting mainly of type I collagen and small amount of ground substance including glycosaminoglycans (GAGs), proteoglycans and glycoproteins [1]. An ideal scaffold should have the appropriate surface chemistry, porosity, and biocompatibility in which optimizing to reduce inflammation and immune response, and mechanical properties. These characteristics are important to integrate them with the native host tissue inside the body. Nowadays, porous scaffolds are commonly used to heal damaged bone tissue over traditional scaffolds; autografting or allografting [2]. The fabrication of scaffolds has been extensively explored for decades. Preparation of porous scaffolds involved some methods such as gas-forming foam, three-dimensional printing, solvent casting/particulate leaching, freeze-drying and electrospinning [3]. Lyophilization or freeze-drying is a dehydration process used to overcome the instability of nanoparticles suspension, increasing its shelf-life and simultaneously facilitating its handling and storage [4].

There are numerous scaffolds have been widely produced from a variety of biomaterials and manufactured using many different kind of fabrication techniques. Biomaterials was known as material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body. Typically, biomaterials consist of three individual groups which are ceramics, synthetic polymers and natural polymers. Each of these individual biomaterial groups has its own specific advantages and disadvantages, due to that there has been an extremely rapid growth of biomaterials especially in tissue engineering application [5]. Many types of scaffolds are made as hydrogel, a 3D flexible network of natural or synthetic polymer that is

insoluble in water [6]. Natural polymers such as polysaccharides (starch, alginate, chitosan) or proteins (soy, collagen, fibrin gels) were commonly used to promote cell adhesion and function but may be immunogenicity and hard to control the mechanical and biodegradability properties. Meanwhile, synthetic polymers such as polycaprolactone (PCL) [7] and poly(lactic-co-glycolic acid) (PLGA) possess relatively good mechanical, biocompatible and biodegradable features [8]. Cellulose is a crystalline, renewable and abundant natural polysaccharide, which is widely used for the reinforcement of the polymeric matrices [9]. CNCs has drawn an increasing attention from researchers due to its abundant sources and multiform applications especially in tissue engineering. They are typically rigid rod-like crystalline nano-particles, and exhibit many appealing properties such as high crystallinity, large aspect ratio, biodegradability, light weight, large surface area, and nanoscale dimension [10].

The aim of this work was to study the effect of different concentration of CNCs as nanofillers in HEC/PVA scaffolds produced by freeze-drying technique that offer the best performance as bone tissue engineered scaffolds. A novel biomimetic HEC/PVA/CNC scaffolds have gained less attention as no reliable evidence about the development, which gave us the motivation to carry out this study. To this aim, the morphology and thermal properties of scaffolds were characterized, and the cytotoxicity of scaffolds *in vitro* was assayed to investigate the potential of the produced scaffolds as substrates for bone tissue engineering applications.

Materials and Experimental

Materials. HEC and glutaraldehyde (GA) solution (25%) was purchased from Merck-Schuchardt, Germany. PVA (Mw 95,000) was purchased from ACROS, New Jersey, USA. Human fetal osteoblast (hFOB), SV4D large T antigen transfected was supplied from American Type Culture Collection (ATCC), USA. All chemicals were characterized by analytical purity and applied without further treatment. All the solutions were prepared using Millipore water.

Solution preparation. HEC (5 wt%) was prepared by dissolving 5 g of HEC powder in 100 ml of Millipore water and stirred until the solution became clear and thickened. Meanwhile, PVA (15 wt%) was prepared by dissolving 15 g of PVA granules in 100 ml of Millipore water and stirred at 80 °C for 2 h until completely dissolved. Then, HEC and PVA solution were mixed together with ratios 50:50 and then stirred overnight to get a homogeneous mixture. Finally, different concentration of cellulose suspension were added into the HEC/PVA solution and stirred until completely blended. Compositions of HEC/PVA modified with CNCs are presented in Table 1.

Table 1. Compositions of HEC/PVA modified with CNCs

Composition of CNCs in HEC/PVA				
HEC [ml]	PVA [ml]	HEC/PVA [%]	CNC [wt %]	CNC [ml]
75	75	50:50	1	1.5
			7	10.5

Freeze-drying. The aqueous polymeric solutions were transferred into falcon tube and allowed to stand in a deep freezer at -80 °C for 24 h. Then, the frozen samples were lyophilized in a freeze-dryer (FreeZone 6 Liter Benchtop Freeze Dry System, Labconco), at -50 °C for 72 h. After that, the produced scaffolds were cross-linked *via* heat treatment at 80 °C in 30 minutes and kept in dry cabinet for further use.

Morphology study. SEM (ZEISS EVO 50) was used to study the surface of scaffolds. The scaffolds were fractured in the liquid nitrogen and sputter coated with a thin layer of platinum. The average pore size of the scaffolds was observed from the SEM micrographs using ImageJ software.

Thermal study. Thermal stability of scaffolds was evaluated using TGA, Toledo STAR-1 (Mettler, Switzerland). About 5 mg samples each was analysed at heating rate of 10 °C/min under a nitrogen flow as purge gas. The test temperatures were ranged from 30 to 750 °C.

Cell Culture Studies

hFOB cell culture. hFOB cells lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 5% penicillin/ streptomycin in cell culture flask. The osteoblast culture was maintained in a humidified atmosphere of 5% carbon dioxide (CO_2) and 95% air at 37 ºC. After reaching 80-90% confluency, the cultured cells were trypsinized by TrypLE solution, and used for the in vitro experiment.

hFOB cell seeding. All prepared scaffolds were punched into small disks with 10 mm diameter, soaked in 100 % ethanol for 24 h, and then sterilized under UV light for 3 h and subsequently immersed in DMEM solution. All changes on scaffolds were observed including the pH of media before and after five days kept in the incubator. Finally, the scaffolds were washed thrice with sterile PBS. hFOB cells were seeded in 24-well (10×10^3 cells/well) plates coated with prepared scaffolds together with DMEM solution in each well and incubated at 37 ºC with 5% CO_2**.**

Cell proliferation study. DAPI (4,6-diamidino-2-phenylindole) staining were carried out to detect nucleus cells. The cell-scaffolds were washed three times in PBS solution. DAPI stain solution was added to cover the cells and incubated for 5 minutes, protected from light. The stain solutions were removed and the cell-scaffolds were washed again in PBS solution. The images of the cell were observed under fluorescent microscope.

Cell morphology study. The morphology of hFOB cells grown on scaffolds were observed under SEM at an accelerating voltage of 10 kV. Briefly, hFOB cell in scaffolds were fixed with 3% GA for 30 min. Thereafter, the scaffolds were dehydrated in a graded series of ethanol and air dried by keeping the samples in a fume hood.

Results and Discussion

Morphology of scaffolds. SEM images presented in Fig. 1 shows surface morphology of HEC/PVA/CNC (1, 7 wt%) porous scaffolds. In tissue engineering, the scaffolds should have high porosity and sufficient distribution of pore size to support cell adhesion and proliferation. As shown in Fig.1, it can be observed that as the concentration of CNC increased, the structure of scaffolds become rougher in which more complex density. That would be beneficial as a rough surface may provide abundant points for cell attachment and three-dimensional porous structure of scaffold will be favorable for nutrients and metabolic waste exchange [11]. Modification of the scaffold with a variation amount of CNC clearly affects the surface morphology of the scaffolds in which resulted in a significant reduction in the average pore size but still favorable for cell attachment.

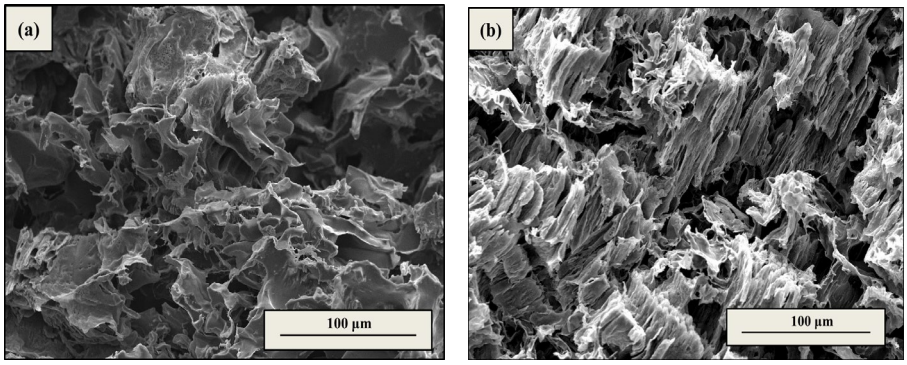

Fig. 1. SEM micrographs of interconnected porous scaffolds for HEC/PVA incorporated with (a) 1 wt% and (b) 7 wt% of CNC

TGA measurements. The porous scaffolds of HEC/PVA/CNC were analyzed using TGA to compare the degradation characteristics among them. The derivative thermogravimetric analysis (DTA) illustrates the detection of maximum weight loss rate for the components. The thermograms and DTA curves of scaffolds were represented in Fig. 2. The details of the decomposition step and percentage mass loss were summarized in Table 2. It can be seen that the thermal stability increased

and the total weight loss of scaffolds decreased when concentration of CNC increased. The initial decomposition in the range of 25-100 °C could be assigned to water evaporation. The weight loss of the organic component in the HEC/PVA/CNC scaffolds occurred mostly in the range of 150–400 °C. After all, adding CNC into blended polymer of HEC/PVA will enhance the degradation of HEC and PVA polymer and hence give effects to the thermostability of the scaffolds.

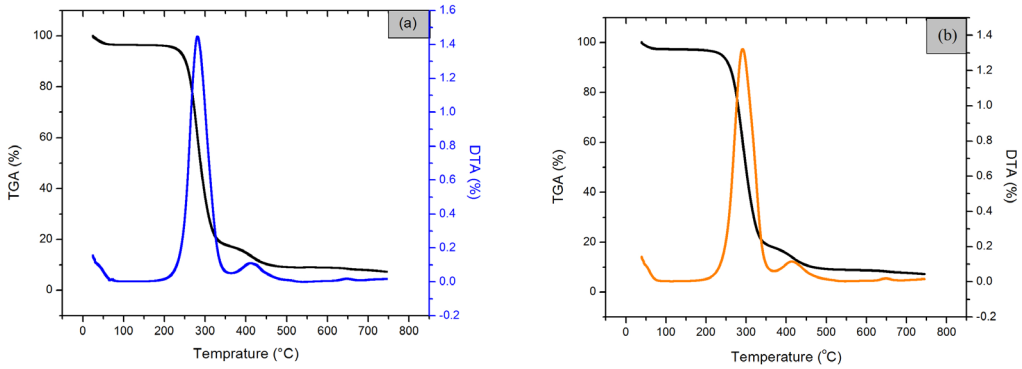

Fig. 2. TGA and DTA curves for scaffolds for HEC/PVA incorporated with (a) 1 wt% and (b) 7 wt% of CNC

Table 2. TGA and DTA for HEC/PVA/CNC (1, 7 wt%) scaffolds

Sample	Region of decomposition	Temperature (°C)			Weight loss (%)	
		T_{start}	T_{end}	T_{peak}	Partial	Total
HEC/PVA/CNC (1 wt%)	1st	25	190	140	5.438	97.548
	2nd	190	387	290	71.26	
	3rd	387	511	422	20.85	
HEC/PVA/CNC (7 wt%)	1st	39	192	124	3.119	92.829
	2nd	124	359	291	79.09	
	3rd	359	509	418	10.62	

Cell Scaffold Interaction Study

Cell proliferation on scaffolds. Cell adhesion and proliferation are important criterion for evaluating the biocompatibility of biomaterials. The quantitative analysis of DAPI staining of hFOB cells cultured on scaffolds under fluorescent microscope were observed. After 7 days of incubation, it can be seen clearly that a slightly higher amount of blue dots are presented in HEC/PVA/CNC (7 wt%) scaffolds which represent more cell were presented.

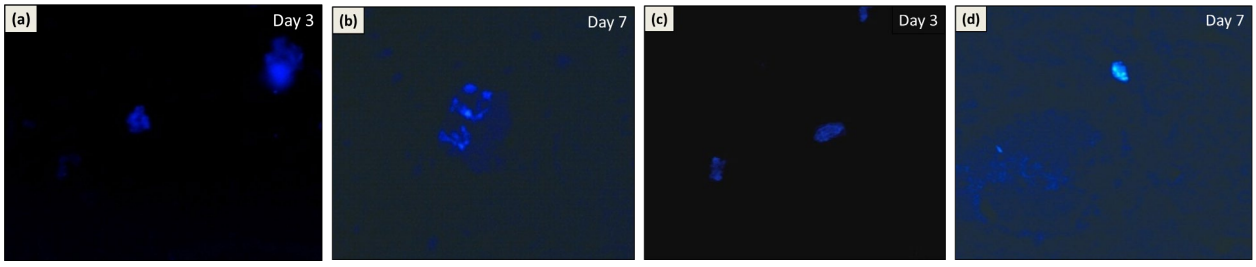

Fig. 3. DAPI stained osteoblast cells grown in scaffolds for HEC/PVA incorporated with (a,b) 1 wt% and (c,d) 7 wt% of CNC at day 3 and 7

Cell morphology study of hFOB cell. SEM micrographs of the cell-scaffolds at different culture times are illustrated in Fig.4. After 3 days of cell culture, it was observed that round shape morphology of osteoblast cells get adhered on the surface of all scaffolds. After that, the cells appeared to be more elongated in which covered almost the entire porous scaffold's surface at 7 days of cell culture. These results revealed that both scaffolds were suitable for attachment and growth of hFOB cell.

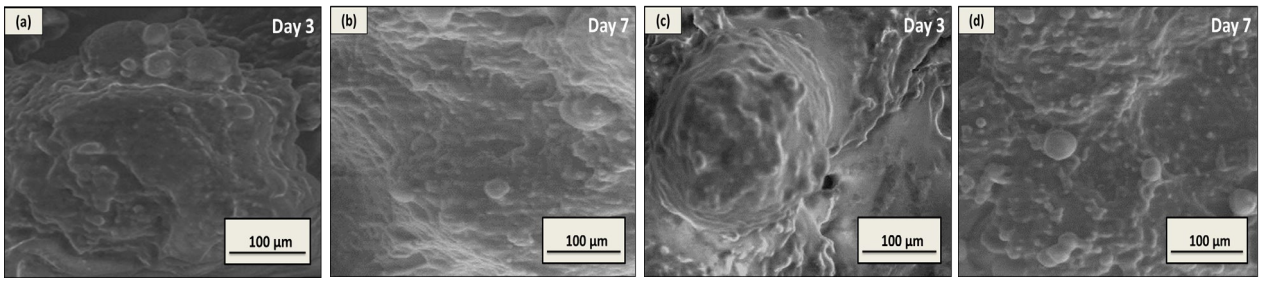

Fig. 4. hFOB cells-scaffold images for HEC/PVA incorporated with (a,b) 1 wt% and (c,d) 7 wt% of CNC at day 3 and 7

Conclusion

A novel three-dimensional biomimetic HEC/PVA/CNC porous scaffold were prepared *via* freeze-drying technique, which was confirmed to be with high porosity and wide range of pore size distribution to mimic a favorable environment for hFOB cells attachment and proliferation. As the concentration of CNC increased, the surface of scaffolds tended to be rougher and the thermal properties increased significantly. DAPI assay results showed that HEC/PVA/CNC (7 wt%) become the best viability hFOB cells cultured *in vitro*, most excellent morphology of hFOB cells seeded and a significantly increasing in cell proliferation. In our opinion, CNC could act as reinforcing nanofillers and HEC/PVA/CNC scaffolds will have a great potential as an excellent scaffold for the bone tissue engineering.

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