

Novel Biopolymers/Functionalized Multi-Walled Carbon Nanotube Composite Scaffolds for Cardiac Tissue Engineering

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ABSTRACT: This work introduces the novel gelatin/chitosan blend scaffolds containing different amounts of functionalized multi-walled carbon nanotubes (f-MWCNTs) up to 0.1wt%, which were prepared by freeze drying (freezing and lyophilization). The composite scaffolds were characterized by Fourier transformed infrared spectroscopy (FTIR) to distinguish the functional groups and different bonds in the structure of composite, and field-emission scanning electron microscope (FE-SEM) to evaluate the morphology of scaffolds. The scaffolds with the porosity of 89–93% and pore size of 40–200 μ m could be obtained by freezing at -20 °C and subsequent lyophilization. The porosity and swelling ratio of scaffolds were decreased, but the pore diameter was increased with an addition of f-MWCNTs. The electrical conductivity of incorporated scaffolds showed a significant increase with f-MWCNTs at an amount of 0.05wt%, and could achieve to those of the heart muscle. Compressive mechanical properties of the scaffolds revealed that the incorporation of f-MWCNTs led to significantly stiff the biopolymeric scaffold. The findings indicate that these novel fabricated composite scaffolds have microstructurally and electrically the potential to use in cardiac tissue engineering applications.

KEYWORDS: *Cardiac tissue engineering; Chitosan; Electrical properties; Functionalized multi-walled carbon nanotube; Gelatin*

INTRODUCTION

Carbon nanotubes (CNTs) have been considered as one of the novel nanomaterials for biomedical research and development because of their remarkable physical and electrical properties as well as their high biocompatibility when used in low contents. These features led CNTs to be used for biosensing [1], molecular delivery [1-3], electrochemical detection of biological species [1,4], and tissue scaffolding [5-8]. Two forms of CNTs, i.e. single-walled carbon nanotubes (SWCNTs), and multi-walled carbon nanotubes (MWCNTs) have been used for aforementioned applications. There are also more subdivisions according to the shape features and functional groups on their surfaces. Among them, MWCNTs functionalized with carboxyl groups are the most important type for tissue regeneration [9]. Tissue engineering scaffolds have been designed to act as a temporary, artificial extracellular matrix which supports cell attachment and proliferation, as well as three dimensional tissue formation [5-11]. An ideal scaffold should mimic the natural extracellular environment of the tissue to be regenerated. The inherent nature of the extracellular matrix is in the scale of nanometer. Although CNTs behave like an inert matrix to support cell proliferation, their combination with other natural or synthetic materials as bio composites

have attracted many interests to use in tissue engineering applications [5,13].

Natural polymers such as collagen, gelatin and chitosan have a low immunogenic potential, with a bioactive cue to interact with the host tissue. Gelatin and chitosan are suitable biopolymers for tissue engineering because of their high biocompatibility and antibacterial characteristics [12]. They have been used to prepare composite scaffolds with using different reinforcements [14-15]. Gelatin contains Arg-Gly-Asp (RGD)-like sequence which can improve cell adhesion and migration, as well as forms a polyelectrolyte complex [11].

Chitosan is a linear hydrophilic polysaccharide and generally obtained by deacetylation of chitin. It has been used in many industrial and medical applications, and has received much attention in biological fields [16-17]. Chitosan is an attractive biocompatible, biodegradable, and non-toxic natural biopolymer. In addition, Chitosan can be used as a modifier because it has abundant $-NH_2$ and $-OH$ functional groups which can react with bioactive molecules [16,18,19]. Among the biopolymers, Gelatin has a potential to be a modifier for improving the mechanical or biological properties of chitosan with different ranges of blending. Incorporation of CNTs into the above unique biopolymers may produce novel composite scaffolds that would have suitable characteristics for tissue engineering applications [16-17]. The hydrophobicity of the CNTs limits their application in biology.

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In order to use CNTs for biomedical applications, they should be surface-functionalized. There have been remarkable progresses in functionalization of CNTs by the chemical treatments or using various synthetic or natural polymers to endow them a hydrophilic surface. Acid treatment of CNTs, and non-covalent or covalent bonding between CNTs and polymers are some examples of this process [18-21].

Chitosan and gelatin have desirable chemical effects onto the surface of CNTs to improve their hydrophilicity, resulting high dispersion and long-term stability of CNTs in the solution mixture. These biopolymers can obtain a hydrophilic surface on the CNT surface for covalent adsorption, or ionic bindings with bioactive molecules. The combination of CNTs with biopolymers is one of the most successful methods to improve hydrophilicity of CNTs.

In cardiac tissue engineering, as a new field of tissue regeneration, two forms of products as a patch or an injectable material have been suggested to repair heart defects [22].

In this regard, the native extracellular matrix and biopolymers have been considered as the appropriate templates. The chitosan and its polyelectrolyte with gelatin have been investigated as the cardiac patches [23]. The most important feature in this field is to maintain the transfer of an electrical signal to cardiomyocytes (heart cells). As the biopolymers are the insulator, the use of biocompatible materials providing electrical conduction would be necessary. The CNTs incorporated in relatively high concentration (up to 5 %wt) or in a very low concentrate (e.g. 100 ppm) in chitosan have been used to prepare conductive materials [24,25]. The SWCNTs was used in chitosan and chitosan/gelatin (1:1) scaffolds for cardiac applications [24]. In this study, we use the functionalized multi-walled carbon nanotubes (f-MWCNTs) at low concentrations (0.025, 0.05 and 0.1wt%) incorporated in a gelatin/chitosan scaffold with a weight ratio of 2:1 to develop composite scaffolds for cardiac tissue engineering applications. The biopolymer used in this work is a gelatin-based biopolymer with the chitosan as the additive to form a polyelectrolyte.

MATERIALS AND METHODS

Materials

Multi-walled carbon nanotubes functionalized with carboxyl groups (the outer diameter of 8–15 nm, the inner-diameter of 3–5 nm, the length of ~30-50 μm , and the purity >95 wt. %) were purchased from Neutrino Co. Medium molecular weight chitosan was supplied by Sigma Aldrich. Gelatin and acetic acid were purchased from Merck Co (Germany).

Preparation of composite scaffolds

Gelatin was dissolved in deionized water at 40 °C by a magnetic stirrer. Chitosan powder (2% w/v) was dissolved in a 2% (v/v) acetic acid solution. The gelatin solution was

added to the chitosan one as the final solution had a gelatin to chitosan weight ratio of 2:1. The obtained solution was homogenized by stirring for 2h. To prepare homogenous solutions with different fractions of f-MWCNTs (0, 0.025, 0.05 and 0.1 wt%), f-MWCNTs powder was ultrasonically dispersed in deionized water for 3h. Subsequently, the homogenous gelatin/chitosan solution was added drop by drop to the f-MWCNTs solution, while the mixture was being ultrasonicated (in an ultrasonic bath). The gelatin/chitosan/f-MWCNTs solution was vigorously mixed using a magnetic stirrer for 12 h to obtain a homogeneous mixture. The obtained solution was transferred to a freezer at -20 °C for 12 h. Then, it was lyophilized by a freeze dryer (Operon- FDB-5503-model) for 48 h. In order to stabilizing the products and to neutralize the acetate, the scaffolds were immersed in a 10% NaOH solution for 24h and then lyophilized again.

Characterization

The prepared composites were characterized by Fourier-transformed infrared spectroscopy (PerkinElmer, Frontier model) in the wavenumbers of 4000–400 cm^{-1} . The microstructure of composite scaffolds was evaluated by a field-emission scanning electron microscope (FE-SEM, Hitachi-S4160, Japan).

The porosity of scaffolds was measured by liquid-displacement method using ethanol as the immersion solution, according to the ref [26]. In order to measure the swelling ratio, dry scaffolds were weighed (W_d) and then immersed in distilled water for one and two weeks. After removing the scaffolds, they weighed (W_w). The swelling ratio was determined by the equation $(W_w - W_d)/W_d$.

The electrical conductivity of dry scaffold was measured by using a picoammeter source at room temperature. It was determined from volume resistivity which is dependent on the applied voltage, the thickness of scaffold, and the area of electrodes.

RESULTS AND DISCUSSION

The procedure suggested in this work could tend to produce solutions with well-dispersed f-MWCNTs into the gelatin/chitosan solution, as illustrated in Figure1. Zhang et al. [18] investigated the dispersion of carbon nanotubes with using derivatives of chitosan. The results showed that the homogeneous solutions of CNTs were very stable and did not show any sedimentation in 3 months, which indicates strong interaction between CNTs and chitosan. It was proved that the chitosan and gelatin can yielded a homogenous distribution of carbon nanotubes throughout the composite scaffolds [25].

In this work, the scaffolds were produced by freezing and then lyophilization process. Initial experiments were performed by freezing blend solutions at -20°C to minimize separation of three components. After freeze drying, the formed scaffolds showed uniform distribution of the components. Figure 1 also shows the prepared scaffolds

after freeze drying and neutralization. The scaffolds with higher concentrations of MWCNTs appear darker.

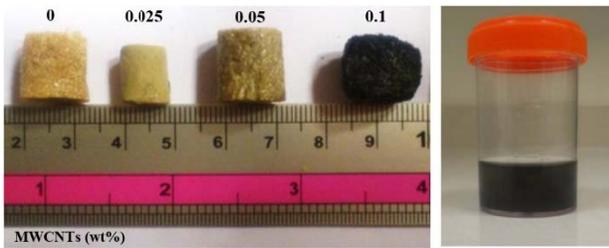


Fig. 1. Homogeneous dispersion of f-MWCNTs in the gelatin/chitosan solution, and the prepared scaffolds after freeze drying and neutralization

FE-SEM micrograph of the specimens is observed in Figure 2. It can be seen a porous microstructure with interconnected pores. There was no significant difference in the morphology of porous structure.

Figure 2 shows that the scaffolds had macro- and micropores in the range of 40–200 μm . Table 1 indicates that the pore size of scaffolds was increased with incorporation of f-MWCNTs.

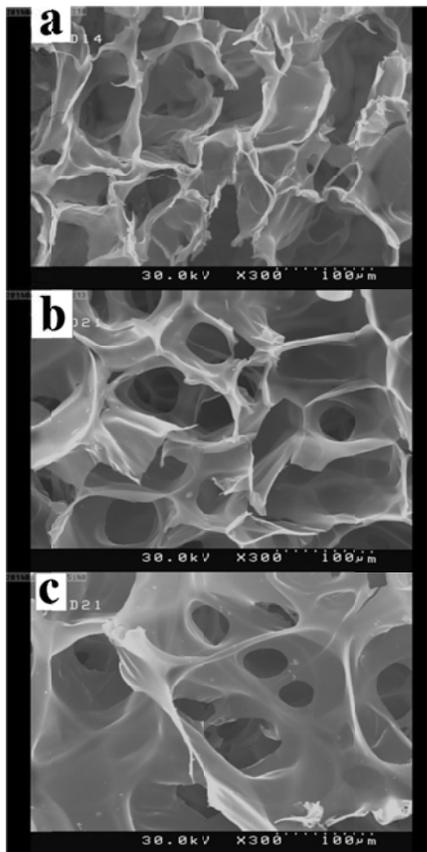


Fig. 2. FE-SEM micrographs of gelatin/chitosan/f-MWCNT scaffolds. All scaffolds were prepared by freezing solutions at -20°C and subsequent lyophilization. (A) 0.025, (B) 0.05, and (C) 0.1 wt. % of f-MWCNT

Table 1

Pore diameter (μm) of gelatin/chitosan scaffolds with different amounts of f-MWCNTs.

f-MWCNTs (wt%)	pore diameter (μm)
0.025	40-100
0.05	60-120
0.1	100-200

Venkatesan et al [27] characterized the carbon nanotube-grafted chitosan-natural hydroxyapatite (HA) composites for bone tissue engineering and showed that uniform dispersion of HA and f-MWCNT was obtained in chitosan matrix with interconnected porosity of 70–200 μm for chitosan/HA and 46–200 μm for f-MWCNT-grafted chitosan/HA. In general, pore size and porosity are the two most important morphological parameters of scaffolds. Macropores ($>50 \mu\text{m}$) and micropores ($< 50 \mu\text{m}$) influence tissue and cell functions, respectively [28]. The scaffold pores should be interconnected to support cell migration and proliferation. As can be seen in Figure 2, the incorporation of f-MWCNTs resulted to form scaffolds with a more regular structure and a thicker pore wall. The circular, interconnected pores with a diameter in the range of 20-60 μm were also observed in the scaffolds with a higher concentration of f-MWCNTs (i.e. figure 2b and c). It is believe that the circular pores can provide an appropriate pathway of celltransport due to their larger area than cells [24]. When the CNT concentration is increased, the viscosity of the polyelectrolyte is also increased. This causes the formation of ice crystal with a slower rate which led to exert a compressive force on neighbouring crystals. This compaction gives rise to form a more circular arrangement of the pores.

The composite scaffolds have been developed for regeneration of various types of tissues using synthetic and/or natural polymers. The scaffolds with typical pore size of $>100\mu\text{m}$ were investigated for soft tissue regeneration such as liver, muscle, blood vessel and cartilage tissues. In cardiac tissue applications, the myocardial cells are in the range of 10-100 μm [29]. Therefore, the produced scaffolds can morphologically conduct the cardiac cells. It should be note that the pore size of biopolymeric scaffolds is determined with the conditions of phase separation process [30]. The phase separation of polymeric solutions is occurred by different methods such as lyophilization (freeze drying) or freeze gelation [8]. In the latter method, instead of freeze drying, the phase separated biopolymer solution exposed to gelation [8]. The conditions of freeze drying such as freezing temperature, cooling rate or cooling directionality are influenced the morphology of scaffolds (e.g. pore diameter, pore shape, interconnectivity, anisotropic structure). For instance, the faster cooling rate of chitosan solutions is resulted scaffolds with smaller pores [30]. It is also shown that the lower freezing temperature of chitosan

solutions led to form scaffolds with smaller pores. Lau and Cooney showed that the scaffolds prepared from chitosan solution and MWCNT (2.5 wt%) using freeze drying method had a pore size of 16 μm [25]. In our work, the freezing temperature was the same as one used in aforementioned study, but the mean pore size was larger than 40 μm .

This may be attributed to the using slower cooling rate in our work which led to form large pores.

In another study, Pok et al prepared freeze-dried scaffolds in the system chitosan/gelatin/ SWCNTs at the same cooling rate of this work but a lower freezing temperature (i.e. -55°C compared to this work of -20°C) [24].

This may led to form scaffolds with smaller pores than those produced in our work. On the other hand, it was showed that in the scaffolds of chitosan/gelatin system prepared at the conditions comparative to our work, the pore size was increase with the gelatin to chitosan ratio [31].

In the Pok et al work [24], the polyelectrolyte with a weight ratio of chitosan to gelatin was 1:1 but in this work this ratio was selected as 2:1.

Therefore, it is expected that the pores of scaffolds prepared in this work have a larger mean diameter compare to the results of Pok et al.

Figure 3 shows that the porosity of scaffolds were in the range of 89-95%. The highly porous scaffolds with interconnectivity would be suitable from the point of view of cell penetration, blood supply and transport of wastes. A slightly decrease in porosity was resulted with an addition of f-MWCNTs.

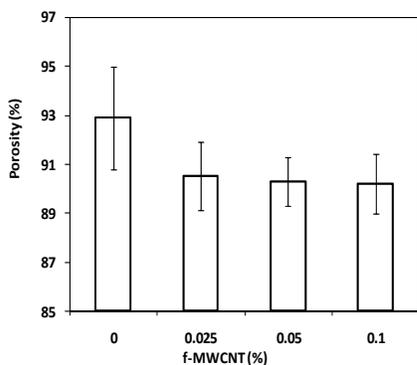


Fig. 3. Porosity of the gelatin/chitosan scaffolds incorporated with different amounts of f-MWCNT

The homogenous dispersion of f-MWCNTs in the porous structure of blend scaffolds can be seen in Figure 4. The diameter of f-MWCNTs was approximately estimated around 23 nm.

It is believed that the increased thickness may be attributed to the presence of gelatin and/or chitosan [25] which cover the nanotubes.

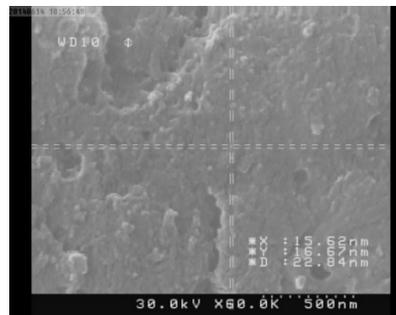


Fig. 4. FE-SEM micrograph of the f-MWCNTs dispersed in the gelatin/chitosan scaffold

In order to illustrate intermolecular interactions between components and to define the changes of functional groups and different bonds in the structure of composites with different contents of f-MWCNTs, FTIR spectrum was taken in the range of wavenumber $4000\text{--}400\text{ cm}^{-1}$.

Figure 5A shows FTIR spectrum of the f-MWCNT powder.

As can be seen, the bands of 1705 cm^{-1} and 1212 cm^{-1} indicate C–O and C=O stretching of carboxylic acid [32]. This proves the functionalization of the MWCNT powder with –COOH groups. In Figure 5B, FTIR spectrum of the gelatin/chitosan scaffold without f-MWCNT addition shows a broad peak at 3332 cm^{-1} due to the stretching of –NH and it is broad due to hydrogen bonds of polysaccharides.

Two bands at 1241 and 1086 cm^{-1} were due to C–N and C–O vibrations of gelatin, respectively [33].

The peak at 1336 cm^{-1} was attributed to N–H and C–N vibrations of amide III in chitosan and/or C–H vibrations of gelatin [33].

Two absorptions bands at 1549 and 1650 cm^{-1} were assigned to N–H bending vibrations of amide II in chitosan and C–O stretching for gelatin, respectively [34].

Figure 5C shows the FTIR spectrum of the gelatin/chitosan /f-MWCNTs scaffold. It depicts a new strong absorbance at 1655 cm^{-1} which might be indicating that the carboxylic group (–COOH) of f-MWCNTs has reacted with the amine (–NH₂) group of chitosan and converts it to amide (–NHCO–) group. This strong absorbance can prove the presence of f-MWCNTs in the structure of fabricated scaffold.

The swelling ratio of the scaffolds after one and two weeks immersion in water is represented in Figure 6. An addition of f-MWCNT to the chitosan led to decrease the swelling ratio.

This behavior may be helpful because the biopolymers in the body encounter swelling which causes deterioration of mechanical properties.

The results of electrical conductivity of scaffolds show that the incorporation of f-MWCNT was caused to a significant increase in conductivity, in particular the addition of higher amounts of f-MWCNT (figure 7).

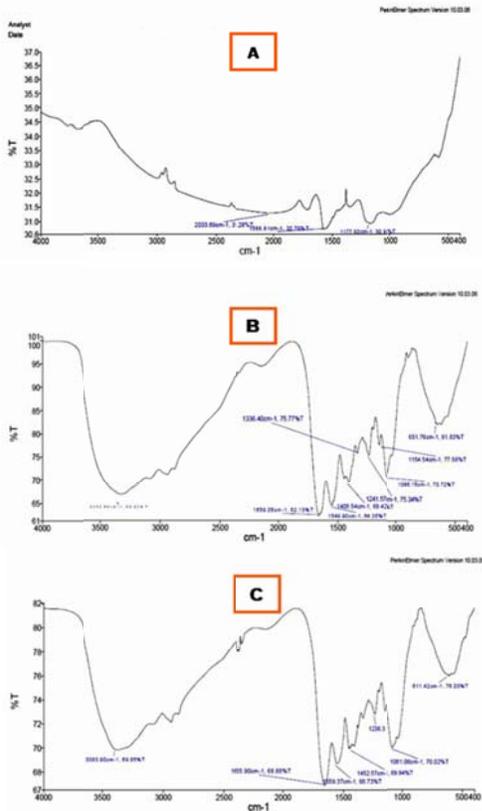


Fig. 5. FTIR spectrum of (A) f-MWCNTs powder, (B) gelatin/chitosan (2:1) scaffold, and (C) gelatin/chitosan/ f-MWCNTs (0.05wt%) composite scaffold

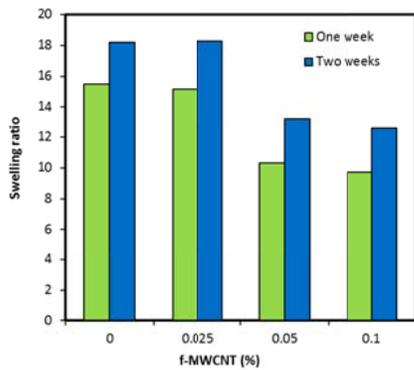


Fig. 6. Swelling ratio of the scaffolds after one and two weeks immersion

This indicates that the carbon nanotubes could bring the electrical conductivity to a value to mimic the native of heart muscle conductivity of 0.1 S/m [35-36]. Lau et al. [25] have used high concentrations of MWCNTs (up to 5wt%) in chitosan scaffolds. They found a percolation threshold of 2.5 wt%. It should be noted that there is a critical percolation threshold density of CNTs which establishes an electrical contact between particles [25]. It is suggested that in biopolymeric scaffolds, the electrical conductivity is provide by direct contact between CNTs

[25]. From the point of biocompatibility view, and due to the toxicity of CNTs, to find the minimum concentration of CNTs that surpass a percolation threshold is also important. In this work, we use much lower concentrations of MWCNTs compare to Lau et al work [25], but in a gelatin/chitosan scaffold instead of chitosan one. The results indicate that there is a threshold of 0.05wt% f-MWCNTs in a gelatin/chitosan matrix with a ratio of 2 to 1. This difference may be attributed to the presence of gelatin. It was proved that the incorporation of MWCNTs into the gelatin- based hydrogels provides an enhanced electrical conduction for the system [37-38].

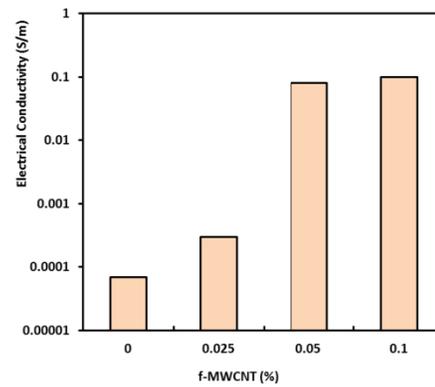


Fig. 7. Electrical conductivity of gelatin/chitosan scaffolds with different amounts of f-MWCNT

Figure 8 shows the stress-strain curve of compression tests for the scaffolds.

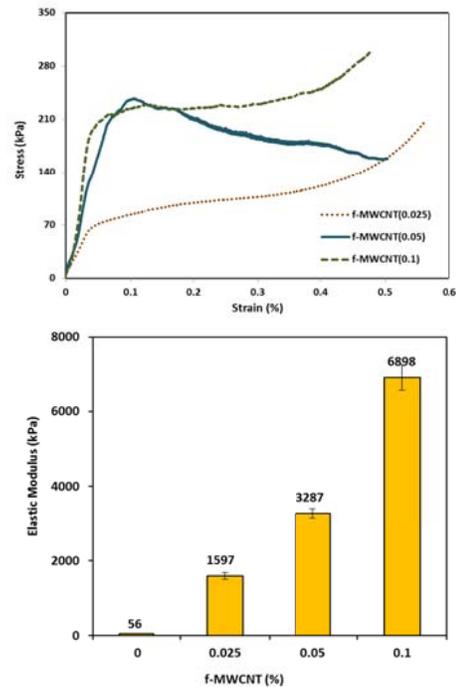


Fig. 8. Compressive mechanical properties of dry gelatin/chitosan/f-MWCNT scaffolds

The common three stages in the curve of compression test can be observed [39]. The elastic modulus of scaffold was determined from the first stage (elastic region) of curve.

The addition of f-MWCNT to biopolymeric scaffolds led to significantly enhance the stiffness of scaffolds such that an addition of 0.1wt% f-MWCNT caused to 120-fold increase in modulus.

It should be noted that the stiffness of muscle tissue has been usually reported in hydrated condition, and then its modulus magnitude was in the range of 8-15kPa [40]. The specimens in this work were mechanically evaluated in compression and in dry condition.

Therefore, it is expected that the measured modulus has much more magnitude than those of hydrated one. The results indicate that the incorporation of f-MWCNT led to stiff the biopolymers.

Recently, the dynamic mechanical properties of hydrated chitosan/carbon nanofibers scaffolds were measured. A storage modulus as the stiffness of scaffold around 28 kPa was reported [40].

In summary, this initial work shows that the f-MWCNT addition in an amount of 0.05wt% to gelatin/chitosan scaffolds prepared by freeze drying could produce suitable highly porous structures to conduct the cells such as myocardial cells, with an appropriate electrically conductivity for cardiac tissue engineering applications.

CONCLUSIONS

Functionalized multi-walled carbon nanotubes, f-MWCNTs, up to 0.1 wt% (0.025, 0.05 and 0.1wt%), were incorporated to the natural, polymeric matrix to obtain a suitable composite scaffold for cardiac tissue engineering applications. The composite scaffolds of gelatin/chitosan with homogeneously dispersed f-MWCNTs obtained by lyophilization showed a highly porous microstructure with the pores in the range of 40–200 μm and circular, interconnected pores of 20-60 μm inward the scaffolds containing f-MWCNTs higher than 0.025wt% that are favourable for attachment and growth of myocardial cells. The pore diameter was increased with addition of f-MWCNTs. However, the porosity of scaffolds was slightly decreased with addition of f-MWCNTs. The swelling ratio of scaffolds decreased with an addition of f-MWCNTs. The electrical conductivity of biopolymeric scaffolds was significantly increased with f-MWCNTs at a threshold of 0.05wt% which is suitable for establishing heart muscle conductivity. The introduction of MWCNTs into the gelatin/chitosan (2:1) system led to stiff the scaffold. The use of f-MWCNTs in tissue engineering represents a challenging but potentially rewarding opportunity to develop the next generation of engineered biomaterials.

REFERENCES

- [1] Harrison BS, Atala A. Carbon nanotube applications for tissue engineering. *Biomaterials*. 2007 Jan 31;28(2):344-53.
- [2] Spizzirri UG, Hampel S, Cirillo G, Nicoletta FP, Hassan A, Vittorio O, Picci N, Iemma F. Spherical gelatin/CNTs hybrid microgels as electro-responsive drug delivery systems. *International journal of pharmaceuticals*. 2013 May 1;448(1):115-22.
- [3] Cirillo G, Vittorio O, Hampel S, Spizzirri UG, Picci N, Iemma F. Incorporation of carbon nanotubes into a gelatin–catechin conjugate: innovative approach for the preparation of anticancer materials. *International journal of pharmaceuticals*. 2013 Mar 25;446(1):176-82.
- [4] Liu Z, Tabakman S, Welsher K, Dai H. Carbon nanotubes in biology and medicine: in vitro and in vivo detection, imaging and drug delivery. *Nano research*. 2009 Feb 1;2(2):85-120.
- [5] Sahithi K, Swetha M, Ramasamy K, Srinivasan N, Selvamurugan N. Polymeric composites containing carbon nanotubes for bone tissue engineering. *International journal of biological macromolecules*. 2010 Apr 1;46(3):281-3.
- [6] Carson L, Kelly-Brown C, Stewart M, Oki A, Regisford G, Luo Z, Bakhmutov VI. Synthesis and characterization of chitosan–carbon nanotube composites. *Materials letters*. 2009 Mar 15;63(6):617-20.
- [7] Abarrategi A, Gutiérrez MC, Moreno-Vicente C, Hortigüela MJ, Ramos V, López-Lacomba JL, Ferrer ML, del Monte F. Multiwall carbon nanotube scaffolds for tissue engineering purposes. *Biomaterials*. 2008 Jan 31;29(1):94-102.
- [8] Wang SF, Shen L, Zhang WD, Tong YJ. Preparation and mechanical properties of chitosan/carbon nanotubes composites. *Biomacromolecules*. 2005 Nov 14;6(6):3067-72.
- [9] Newman P, Minett A, Ellis-Behnke R, Zreiqat H. Carbon nanotubes: their potential and pitfalls for bone tissue regeneration and engineering. *Nanomedicine: Nanotechnology, Biology and Medicine*. 2013 Nov 30;9(8):1139-58.
- [10] Liu X, Smith LA, Hu J, Ma PX. Biomimetic nanofibrous gelatin/apatite composite scaffolds for bone tissue engineering. *Biomaterials*. 2009 Apr 30;30(12):2252-8.
- [11] Huang Y, Onyeri S, Siewe M, Moshfeghian A, Madhally SV. In vitro characterization of chitosan–gelatin scaffolds for tissue engineering. *Biomaterials*. 2005 Dec 31;26(36):7616-27.
- [12] Li H, Wang DQ, Liu BL, Gao LZ. Synthesis of a novel gelatin–carbon nanotubes hybrid hydrogel. *Colloids and Surfaces B: Biointerfaces*. 2004 Jan 15;33(2):85-8.

- [13] Battigelli A, Ménard-Moyon C, Da Ros T, Prato M, Bianco A. Endowing carbon nanotubes with biological and biomedical properties by chemical modifications. *Advanced drug delivery reviews*. 2013 Dec 31;65(15):1899-920.
- [14] Nouri-Felekori M, Mesgar AS, Mohammadi Z. Development of composite scaffolds in the system of gelatin– calcium phosphate whiskers/fibrous spherulites for bone tissue engineering. *Ceramics International*. 2015 May 31;41(4):6013-9.
- [15] Mohammadi Z, Mesgar AS, Rasouli-Disfani F. Reinforcement of freeze-dried chitosan scaffolds with multiphasic calcium phosphate short fibers. *Journal of the mechanical behavior of biomedical materials*. 2016 Aug 31;61:590-9.
- [16] Liu Y, Lim J, Teoh SH. Review: Development of clinically relevant scaffolds for vascularised bone tissue engineering. *Biotechnology advances*. 2013 Oct 31;31(5):688-705.
- [17] Shieh YT, Yang YF. Significant improvements in mechanical property and water stability of chitosan by carbon nanotubes. *European polymer journal*. 2006 Dec 31;42(12):3162-70.
- [18] Zhang J, Wang Q, Wang L, Wang A. Manipulated dispersion of carbon nanotubes with derivatives of chitosan. *Carbon*. 2007 Aug 31;45(9):1917-20.
- [19] Wu Z, Feng W, Feng Y, Liu Q, Xu X, Sekino T, Fujii A, Ozaki M. Preparation and characterization of chitosan-grafted multiwalled carbon nanotubes and their electrochemical properties. *Carbon*. 2007 May 31;45(6):1212-8.
- [20] Bandyopadhyaya R, Nativ-Roth E, Regev O, Yerushalmi-Rozen R. Stabilization of individual carbon nanotubes in aqueous solutions. *Nano letters*. 2002 Jan 9;2(1):25-8.
- [21] Spitalsky Z, Tasis D, Papagelis K, Galiotis C. Carbon nanotube–polymer composites: chemistry, processing, mechanical and electrical properties. *Progress in polymer science*. 2010 Mar 31;35(3):357-401.
- [22] Venkatesan J, Ryu B, Sudha PN, Kim SK. Preparation and characterization of chitosan–carbon nanotube scaffolds for bone tissue engineering. *International journal of biological macromolecules*. 2012 Mar 1;50(2):393-402.
- [23] Venkatesan J, Qian ZJ, Ryu B, Kumar NA, Kim SK. Preparation and characterization of carbon nanotube-grafted-chitosan–natural hydroxyapatite composite for bone tissue engineering. *Carbohydrate Polymers*. 2011 Jan 10;83(2):569-77.
- [24] Clemens van Blitterswijk, *Tissue Engineering*, Academic Press, 2008, p 405–408.
- [25] Davis ME, Hsieh PC, Grodzinsky AJ, Lee RT. Custom design of the cardiac microenvironment with biomaterials. *Circulation research*. 2005 Jul 8;97(1):8-15.
- [26] Yan J, Wu T, Ding Z, Li X. Preparation and characterization of carbon nanotubes/chitosan composite foam with enhanced elastic property. *Carbohydrate polymers*. 2016 Jan 20;136:1288-96.
- [27] Kakkar P, Verma S, Manjubala I, Madhan B. Development of keratin–chitosan–gelatin composite scaffold for soft tissue engineering. *Materials Science and Engineering: C*. 2014 Dec 1;45:343-7.
- [28] Salem KS, Lubna MM, Rahman AM, NurNabi M, Islam R, Khan MA. The effect of multiwall carbon nanotube additions on the thermo-mechanical, electrical, and morphological properties of gelatin–polyvinyl alcohol blend nanocomposite. *Journal of Composite Materials*. 2015 May ;49(11):1379-91.
- [29] You JO, Rafat M, Ye GJ, Auguste DT. Nanoengineering the heart: conductive scaffolds enhance connexin 43 expression. *Nano letters*. 2011 Aug 3;11(9):3643-8.
- [30] Liao B., Zhang D, Bursac N. Functional cardiac tissue engineering. 2012 Mar;7(2): 187-206.
- [31] Martins AM, Eng G, Caridade SG, Mano JF, Reis RL, Vunjak-Novakovic G. Electrically conductive chitosan/carbon scaffolds for cardiac tissue engineering. *Biomacromolecules*. 2014 Jan 28;15(2):635-43.