MWCNTs and SWCNTs based Nanocomposites for Cartilage and Bone Tissue Regeneration

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ABSTRACT

Cartilage and bone defects, which are caused by a variety of reasons such as traumatic injuries, osteoarthritis, osteoporosis or bone cancers, represent common and severe clinical problems. Developments in nanotechnology and tissue engineering have provided promising ways to repair and replace damaged cartilage and bone [1-3]. Since human cartilage and bone extracellular matrices are nanostructured, novel biomimetic nanomaterials are desirable for cartilage and bone regenerations [4, 5]. Thus, the objective of this research is to create novel biologically inspired tissue engineered cartilage and bone scaffolds via two types of carbon nanotubes and nanocrystalline hydroxyapatite (nHA) for improving bone and cartilage regeneration. Our results showed that nanocomposites containing magnetically synthesized B-SWCNTs had superior cytocompatibility properties when compared to non-magnetically synthesized SWCNTs and blank controls. B-SWCNTs have much smaller diameters and are twice as long as their non-magnetically prepared counterparts, indicating that the dimensions of carbon nanotubes can have a substantial effect on osteoblast functions. For the cartilage regeneration, through electrospinning we designed a series of novel 3D biomimetic nanostructured scaffolds based on H_2 treated MWCNTs, poly L-lysine and biocompatible poly(L-lactic acid) (PLLA) polymers. Our *in vitro* human bone marrow mesenchymal stem cell (MSC) differentiation results demonstrated that incorporation of the biomimetic MWCNTs and poly L-lysine coating can induce more chondrogenic differentiations of MSCs than controls.

Keywords: Carbon Nanotubes, Cartilage, Bone, Tissue Engineering, Biomimetic

1 INTRODUCTION

Although orthopedic implants, allografts, and autografts have been used to treat various orthopedic defects caused by trauma or disease, these traditional methods of treatment are complicated by the possibility of infection, improper healing from invasive surgeries, insufficient bone donations to seal gaps completely, and donor site morbidity .[1, 6, 7] Developments in nanotechnology and tissue engineering have provided promising ways to repair and replace damaged bone [3, 8]. Human bone tissue is a nanocomposite with both organic and inorganic components. Therefore, it is desirable to design a biomimetic three-dimensional nanostructured tissue-engineered scaffold that can mimic the natural extracellular bone matrix and provide an environment for new bone regeneration which is favorable to the cell. In this study, biologically inspired nHA was synthesized via a hydrothermal treatment method. This treatment method can produce small grain sizes with high crystallinity of geometrically shaped biomimetic nHA rods at relatively low temperatures but under higher pressures. Another important nanomaterials that was studied here are SWCNTs and MWCNTs. Considering their excellent mechanical properties, cytocompatibility, and electrical properties, they have received a lot of attention for bone tissue engineering [9-15]. Their nanotubular geometry also simulates the extracellular matrix in bone and cartialge. Carbon nanotubes formed using arc discharge have extremely high mechanical strength and flexibility, with elastic modulus values of 1 TPa [10, 13]. Therefore, in this study, an arc discharge method was adopted to synthesize SWCNTs. More importantly, we applied a magnetic field during arc discharge synthesis in order to produce more biomimetic dimensions and fewer defects in the SWCNTs. SWCNTs were then incorporated as a third phase in our chitosan/nHA hydrogels to promote osteogenesis further.

Modifications to the porosity of tissue engineered scaffolds has been another important way for improved cartilage regeneration. In native cartilage tissue, the extracellular matrix forms naturally porous, nano structured environments that promote cell adhesion, proliferation and differentiation [16, 17]. Therefore, it is important to construct a scaffold that mimics the scale and structure of the cartialge ECM [18]. In this study, we will also engineer a series of novel biomimetic cartilage constructs with nano to micro structure via wet electrospinning and nanomaterials (i.e., MWCNTs). We will investigate if the mechanical and cytocompatibility properties of electrospun polymer scaffolds for cartilage repair could be enhanced, with the addition of nanomaterials. It was also a goal to evaluate if the nano scaffolds modified with a cell-favorable molecule (i.e., Poly-L-lysine) can effectively control chondrogenic differentiation of human bone marrow mesenchymal stem cells (MSCs) *in vitro*.

2 MATERIALS AND METHODS

2.1 Nanomaterial Synthesis and Scaffold Fabrication for Bone Scaffolds

The N-SWCNT (non magnetically treated) and B-SWCNT samples were both fabricated using an arc discharge method. NHA was prepared by hydrothermally treating amorphous hydroxyapatite in the Parr system at 200°C for 20 hours. The final particles were rinsed with distilled and deionized water three times, dried overnight at 80°C, and ground with a mortar and pestle to obtain fine particles.Unmodified chitosan scaffolds (controls) were prepared by adding chitosan (Sigma-Aldrich) to 2% acetic acid and stirring for one hour to dissolve the chitosan fully. The solution was homogenized by sonicating for 10 minutes, then distributed in a cylindrical mold and frozen at -80°C for several hours. The samples were lyophilized in a Labconco freeze-dry system overnight to create interconnected porous structures. Scaffolds with nHA concentrations of 5, 10, and 20 wt% were prepared as well. Scaffold, SWCNT and nHA morphologies were evaluated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

2.2 Nanomaterial Synthesis and Scaffold Fabrication for Cartilage Scaffolds

As-synthesized MWCNTs were obtained from Shanghai Xinxing Chenrong Technology Development CO., LTD. The MWCNTs were synthesized by the floating-catalyst technique in chemical vapor deposition process. Dimethylbenzene (C_8H_{10}) and Thiophene (C_4H_4S) served as carbon sources and the Iron atom from Ferrocene ($Fe(C_5H_5)_2$) was used as catalyst for the growth of MWCNT. The synthesis processes were carried out in a cylindrical chamber with temperature of 1100 ^oC in hydrogen environment. The as-synthesized MWCNTs were further treated by H_2 heating. The processes for H_2 heating treatment is to put the as-synthesized MWCNT in a mixture of hydrogen and nitrogen environment in the temperature of 800 ^oC for two hours and then turn off hydrogen supply and cool down samples naturally. The H_2 treatment removes amorphous carbon and nanohorns encapsulating metal catalyst nanoparticles and MWCNT, making tubes more uniform. The morphologies of these tubes, both treated and untreated, were evaluated using SEM.

For all experiments, biocompatible poly L-lactic acid (PLLA), purchased from Sigma Aldrich was used as the base polymer to be electrospun. Fibers were fabricated using an in house setup, consisting of a syringe pump, Harvard Apparatus variable voltage supply and an aluminum collector plate. The proliferation and differentiation study used PLLA dissolved in pure DCM at 18% weight by volume, which was then wet-electrospun into a coagulation bath of methanol. The optimized working distance (i.e., 18cm) were used here. More importantly, PLLA scaffold were electrospun with solutions containing 0.5% w/v untreated MWCNTs, 0.5% w/v H_2 treated MWCNTs and 1% w/v H_2 treated MWCNTs. All samples were mixed using ultrasonication for 75 minutes, with the nanotubes being sonicated in the solvent first for 45 minutes in order to assure uniform distribution.

3 RESULTS

3.1 Nanomaterials and Scaffolds Characterization

Figure 1 shows the SEM and TEM images of syntheiszed nanomaterials and bone/cartialge nano scaffolds used in this study.

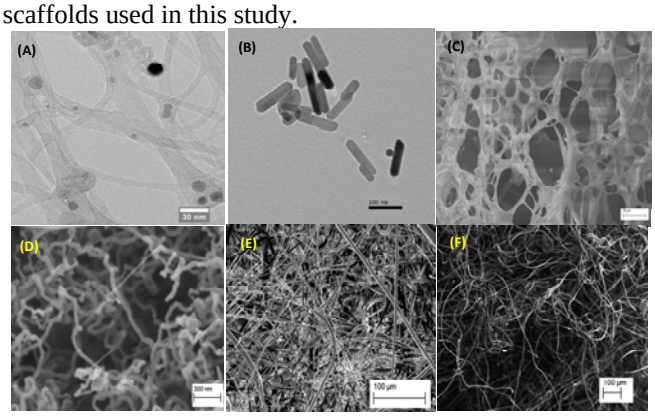

Figure 1. TEM images of (A) single-walled carbon nanotubes with magnetic field of 0.06 Tesla (B-SWCNT); (B) hydrothermally treated nHA. SEM images of (C) 3D porous chitosan with nHA and magnetically synthesized B-SWCNTs; (D) H_2 treated MWCNTs; (E) 0.5% H_2 treated MWCNTs in PLLA scaffold; and (F) 1% H_2 treated MWCNTs in PLLA scaffold.

3.2 Bone Scaffold Results

In this study, we not only fabricated a nanostructured chitosan scaffold by incorporating hydrothermally treated biomimetic nHA, but also designed a novel scaffold with both nHA and magnetically/nonmagnetically synthesized SWCNT. The result of the present study demonstrate for the first time that when combining biologically inspired nHA chitosan hydrogels with both types of SWCNT, osteoblast adhesion can be greatly augmented (data not shown). Moreover, Figure 2 shows greatly enhanced bone cell proliferation in the 20% nHA in chitosan and 20% nHA+B-SWCNTs in chitosan scaffolds when compared to chitosan controls after 1, 3, and 7 days of culture. In particular, osteoblast density in 20% nHA + B-SWCNT in chitosan was highest when compared with the other experimental groups. These results suggest that the addition of SWCNT to a nHA chitosan scaffold may have a synergistic effect of improving their cytocompatibility properties, thereby making them promising for bone

regeneration. Essentially, all of the scaffolds incorporating SWCNT or nHA showed enhanced osteoblast adhesion compared with chitosan controls without nHA and SWCNT. These results can be explained by considering how the surface properties and interior structure of chitosan scaffolds were changed by embedding them with different nanomaterials. The nanostructured nHA and SWCNT contribute to changes in the chitosan surface to amplify nanoroughness and improve the surface area of the whole scaffold. It is well known that

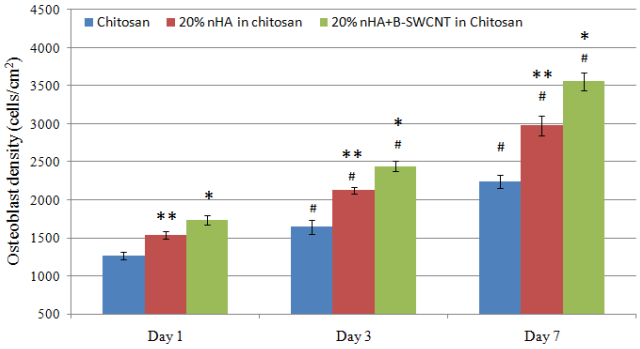

Figure 2. Enhanced osteoblast proliferation on nHA and B-SWCNT chitosan nanocomposites. Data are mean ±SEM; n = 9. *p<0.05 when compared to all other scaffolds at respective days; **p<0.05 when compared to chitosan controls at respective days; and #p<0.05 when compared to respective scaffolds at 1 day.

electrical stimulation can promote osteogenesis at defective bone sites so B-SWCNT, with their improved electrical conductivity and excellent cytocompatibility properties, hold great potential for application in bone tissue engineering.

3.3 Cartilage Scaffold Results

After the addition of MWCNTs of both species, the PLLA scaffolds' Young's modulus increased dramatically when compared to a pure PLLA control (Data not shown). And all MWCNT reinforced scaffolds were within the range of native articulate cartilage (~0.75 to 2 MPa depending on locations) [19-22]. This shows that the incorporation of just a small amount of MWCNTs can greatly increase the mechanical properties of a tissue engineering scaffold to within biomimetic regimes.

Moreover, the differentiation study showed increased chondrogenic differentiation activity of MSCs in these nanostructured scaffolds *in vitro*. There was a dramatic increase in GAG content at one and two weeks on the scaffolds containing poly-L-lysine coated MWCNTs PLLA scaffolds, and among those samples the H_2 treated tubes performed the best (Figure 3).

The positively charged poly-L-lysine can create an electrostatic interaction with negative charged GAG for improved GAG nucleation in the scaffold. This implies that

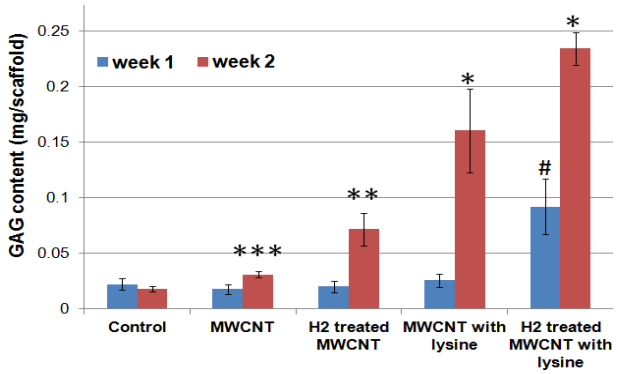

Figure 3. Significantly improved glycosaminoglycan (GAG) synthesis of hMSCs in all MWCNT (0.5%) embedded in PLLA scaffolds after two weeks. Data are means ± SEM, n=9. #p<0.05 all other samples at week 1; *p<0.05 compared to all other samples at week 2; **p<0.05 compared to control and MWCNTs without H2 treatment; and ***p<0.05 compared to controls.

the surface coating of the nanotube scaffold (to decrease hydrophobicity) had the greatest impact on GAG synthesis *in vitro*, greater than the nano surface topography contribution of MWCNTs.

4 DISSCUSSION AND COLUSIONS

The advances in tissue engineering require more sophisticated materials both to characterize and grow tissues. For this purpose, carbon nanotubes/fibers are emerging candidates. Although the use of carbon nanotubes in tissue engineering is at its infancy, they have been considered exciting alternatives as templates for tissue growth, drug delivery agents and in bio-sensory applications. Carbon nanotubes mimic the dimensions of the constituent components of tissues, where cells are accustomed to interacting with nano fibrous proteins. This property makes them excellent candidates for invoking positive cellular responses when employed as implants [15]. In addition, the superior mechanical properties of carbon nanotubes are efficient for their usage as a secondary phase for high load bearing applications. Their electrical properties make them a potential choice in neural applications where signal transfer between growing axons necessitates electrical conductance. The unique chemical properties they possess permit them to be functionalized with different chemical groups, which further promote cell growth.

In our study, we implemented a small concentration of homogenously distributed CNTs which have been H_2 purified and poly-L-lysine coated for the chondrogenic differentiation of MSCs for the first time. Our results show the significant beneficial effects of these nanostructured scaffolds in directing stem cell differentiation *in vitro*. We believe that our scaffolds are advantage for cellular growth because the nanotubes are modified to have cell-favorable hydrophilic poly-L-lysine coated surfaces, are

homogenously dispersed and imbedded in solid microfibrous structures, which creates a stable and advantageous environment for cellular activity. Moreover, this study demonstrated that our synthesized nHA and B-SWCNTs with nanoscale-biomimetic features created a cell-favorable environment to improve osteoblast functions, thus, making them intriguing materials for further study in orthopedic applications.

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