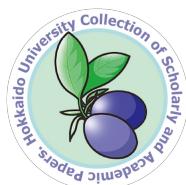




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Carbon nanomaterials for bone tissue regeneration

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ABSTRACT : Carbon nanomaterials (CNMs), including carbon nanotubes carbon nanohorns and graphene, have gained great attention in the scientific community due to their unique physico-chemical properties. These could also be promising in many biomedical-related fields. In particular, CNMs have been studied for bone tissue engineering, because of their low toxicity. In this review, the advantages of using carbon-based materials in bone tissue engineering are presented. In this study osteoblast cell culture scaffold was achieved using carbon nanotubes and functionalized carbon nanotubes for bone regeneration and is described. The biocompatibility of the CNMs for bone and mechanism of the bone formation by CNMs will also be discussed.

Key Words : carbon nanotube, carbon nanohorn, graphene, osteoblast, bone

Introduction

Bone bioengineering is rapidly progressing to accelerate bone cell growth and proliferation and expand or replace bone tissue. This approach is important to achieve stable implantation of dental materials for patients with using regeneration medical techniques. Carbon nanomaterials (CNMs), such as carbon nanotubes (CNTs), graphene and carbon nanohorns (CNHs), have been studied for biomedical applications due to unique physical property, chemical stability, and biocompatibility.^{1, 2)} These properties can offer possibility for novel scaffolds and appropriate stimuli to induce growth factors for bone regeneration. Herein, current trends and advanced researches of CNMs are overlooked with demonstrating their promising characteristics for the novel biomaterials for bone bioengineering.

1. CNTs for osteoblast cell culture scaffold

CNTs are cylindrical carbon material possessing nanometer diameters. The chemical stability and mechanical strength of CNTs have fascinated researchers

for their unique components of scaffolds on tissue engineering.³⁾ Aoki et al reported that osteoblast like cells adhered strongly on the CNTs sheet.⁴⁾ Warowicka *et al* reported that MWCNT alignment increased expression levels of integrin, talin and fibronectin which are involved in cell attachment.⁵⁾ Das *et al* reported that COOH-MWCNT and COOH-SWCNT acted as better cues for osteogenic differentiation.⁶⁾ Siqueira *et al* reported that Poly (D, L-lactide acid, PLLA) superhydrophilic vertically aligned carbon nanotubes:nano-hydroxyapatite

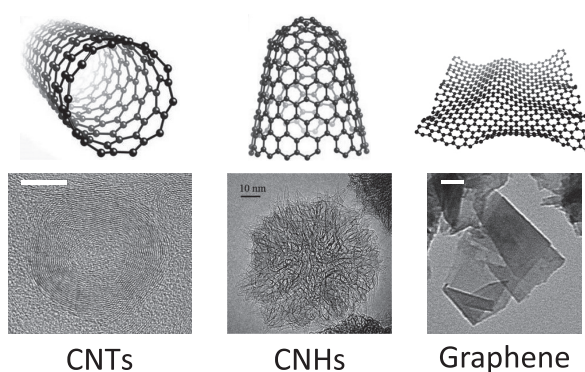


Fig. 1 Schematic models and TEM observation of carbon nanomaterials.

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(PDLLA/VACNT-O : nHAp) scaffolds showed higher calcium precipitation compared to the PDLLA control.⁷⁾ MWCNT-coating of polylactic acid (PLLA) achieved the enhancement of surface wettability and promoted the attachment of human osteosarcoma cell by its improved wettability at an early stage⁸⁾.

Based on the previous studies on the conventional two-dimensional (2D) cell culture,⁹⁾ importance of 3D cell culture has attracted great attention because it can mimic the real situation for cell adhesion, growth and differentiation *in vivo*. Adipose-derived human MSCs showed good cell viability, attachment, proliferation, and infiltration in MWCNT and SWCNT 3D- macroporous scaffolds fabricated using a novel radical initiated thermal cross-linking method that covalently cross-links CNTs.¹⁰⁾ 3D porous CNT scaffold showed significantly higher cell proliferation, better osteoconductivity, and more bone generation with rhBMP-2.¹¹⁾ On the 3D-scaffolds of collagen sponge in which the surface is homogeneously coated by CNTs, cell adhesion and differentiation of osteoblasts were promoted by CNTs (Fig 2).¹²⁻¹⁴⁾ It showed sharp contrast with the surface of non-treated collagen

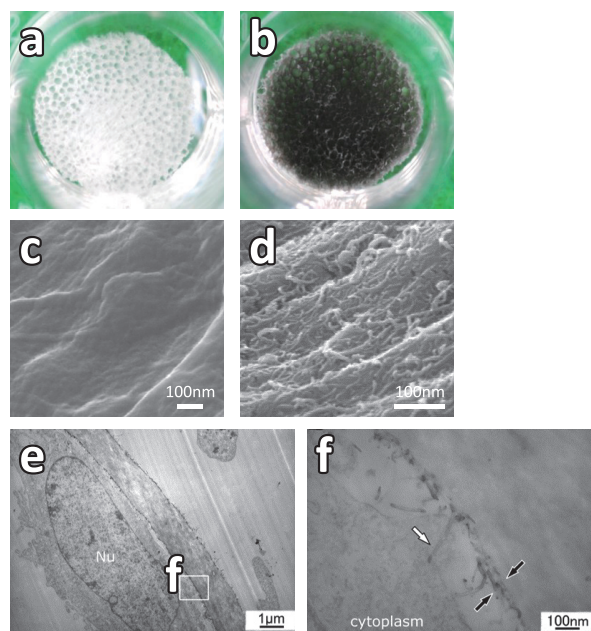


Fig. 2 The whole shape of collagen sponge (a) and the CNT-coated sponge (b), SEM images of the surface of collagen sponge (c) and the CNT-sponge (d). modified from Carbon 2011, 49, 3285. TEM observation of a cell adhering to the MWCNT-coated sponge after 3 days of incubation. Low magnification (e). High magnification at the surface of the MWCNT-coated sponge (f). Nu, nucleus. MWCNT-coated sponge surface, black arrow ; MWCNTs penetrating plasma membrane, white arrow. modified from J. Electron Microscop. 2010, 59, 448

sponge, which is not readily attached by cells because of incompatibility of the surface environment for cell adhesion. CNT-coating method is so convenient that does not require any special apparatus and unconventional agents. Uniquely, the CNTs-coated 3D-scaffolds are capable to enhance bone regeneration (Fig. 3).¹⁵⁾ The rapid vertical 3D-adhesion on the CNTs-coated scaffold can promote the differentiation of rat primary osteoblasts, resulting in remarkable bone formation in the scaffolds. The bone morphology in the scaffolds suggests that the tight osteoblast adhesion and early differentiation on the CNTs maintain the shape of 3D-scaffold. CNTs take a role to strengthen the mechanical stability of the scaffold to keep the pore shape in the scaffold favorable for bone formation.

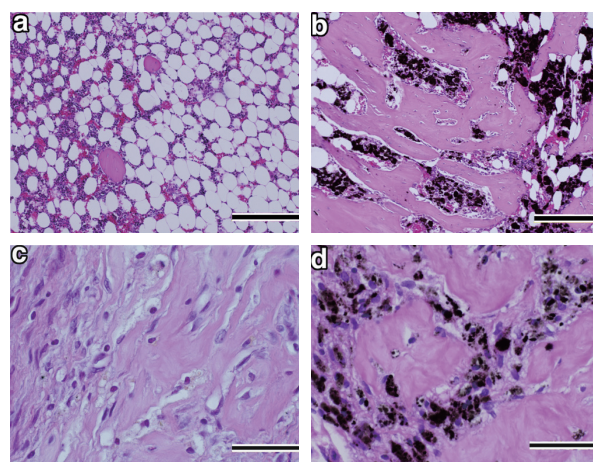


Fig. 3 Histology at 56 days after implantation of the collagen sponge (a) and CNT-coated sponge (b) in the rat bone marrow. HE stain. Small parts of bone remained in with the collagen sponge while the newly formed bone around the MWCNT-coated sponge has not absorbed. Histology at 28 days after implantation of each sponge with osteoblasts cultured for 1 day in the subcutaneous tissue. After implantation of the uncoated sponge, flattened bone-like tissue is observed (c) while the bone formed in the transplanted sponge coated with MWCNTs maintains the original shape of the sponge even after the sponge walls have been absorbed (d). modified from Carbon 2011, 49, 3288-9

2. Biocompatibility of the CNMs in bone tissue applications

For bone tissue applications, CNMs are only thought to pose health risks when migrating from the desired site of implantation. Such behavior is problematic since CNTs in dispersion/detached from a substrate show a different toxicological profile to those in composites or attached

to a surface.¹⁶⁾ Wang et al. reported that pure bulk carbon nanotubes implants were surrounded by newly formed bone in the femur without strong inflammatory response.¹⁷⁾ Inoue et al. reported that bone contact ratio on the titanium (Ti) coated CNTs was significantly higher than on the anodized Ti alone.¹⁸⁾

Long term influence of CNTs in vivo was also investigated. CNTs located inside and outside of macrophages in rat subcutaneous tissues were examined for 2 years post-implantation. The majority of the large agglomerates were present in the intercellular space, maintained a layered structure, and did not undergo degradation. Meanwhile, small agglomerates were found inside macrophages, and they were gradually degraded in lysosomes.¹⁹⁾ The binder-free MWCNT blocks possess good biocompatibility with a slight inflammatory response, which were covered by thin granulation tissue, 40- 70 μm in thickness, comprising a few lymphocytes, cell with large cytoplasmic spaces like fibroblasts and foreign-body giant cells.²⁰⁾ Graphene is an emerging CNM and is becoming remarkably popular, although its original form, i.e. graphite, which can be denoted as “vast number of layers of graphenes”, is well-known and has been used for long time in human history. Unique sheet structure of graphene and variety of its surface functionalization approach makes graphene attractive materials. Much efforts have been made to develop this CNM as novel biomaterial. One example is a unique property of graphene to target and successfully induce the necrosis of monocytoid cancer cells which cause acute myeloid leukemia and chronic myelomonocytic leukemia patients.²¹⁾ Comparison between graphene and etoposide, which is a common chemotherapeutic drug, indicated the higher specificity and toxicity of graphene on the cancer cells, confirming the negligible toxicity on other immune cells.

It should be mentioned here that several reports have pointed out cytotoxicity caused by CNTs and the other CNMs. However, in many cases the toxicity is not caused by the carbon materials themselves but from possible metal impurities which may be used and contained in the production step. Thus, it is becoming common sense that the CNMs which have been properly purified and isolated from the metal contaminants possess high biocompatibility.

3. CNTs functionalized for bone regeneration

CNTs can be easily modified with proteins in order to not only make possible an enhancement of the biocompatibility but also induce new characteristics through chemical functionalization.²²⁻²⁴⁾ Meta et al reported that proliferation and osteogenic differentiation of human osteoblastic cells were maximized on CNT membranes functionalized combining a Diels-Alder cycloaddition reaction to generate cyclohexene ($-\text{C}_6\text{H}_{10}$) followed by a mild oxidation to yield carboxylic acid groups($-\text{COOH}$).²⁵⁾ CNTs can further accelerate new bone formation in response to fibroblast growth factor (FGF).²⁶⁾ COOH-MWCNT substrate acted as a better cue, accelerating the osteogenic differentiation process.⁶⁾ CNTs which were chemically functionalized with FGF demonstrated the proliferation of rat bone marrow stromal cells. CNTs conjugated with FGF as a coating substance of a collagen sponge were implanted between the parietal bone and the periosteum of rats. At day 14 after implantation, a larger amount of newly formed bone was observed around the sponges coated with functionalized carbon nanotubes. The FGF on the CNTs-scaffold promoted bone formation because CNTs might retain the pore morphology. In addition, gradual degradation of the CNTs-coated collagen sponge could release FGF for a long period and lead to the favorable prolongation of vascularization.

4. The mechanism of bone formation by CNMs

CNTs in synthetic bone substrates may allow tissue engineers to take advantage of the fibrillar/ECM-protein-mimicking morphology of CNTs ; their affinity to adsorb a corona composed of proteins that stimulate cell-adhesion and preferential adhesion and strengthening of integrin mediated adhesions between CNTs and cells.²⁷⁾ CNHs is also capable to guide bone regeneration.^{28, 29)} Tissue reconstruction was evaluated in a rat calvarial model by covering CNHs fixed on a porous polytetrafluoroethylene membrane. CNHs promoted bone formation in the early stage, with only a slight inflammatory response (Fig. 4a-c). Detailed study on the mechanism of the bone formation revealed that alkaline phosphatase activity is dramatically increased by coculture of human monocyte derived macrophages (hMDMs) and human mesenchymal stem cells (hMSCs) in the presence of CNHs.³⁰⁾ During the cocultivation, CNHs were dominantly localized in

the lysosome of the macrophages than in hMSCs, and the amount of Oncostatin M (OSM) in the supernatant was being increased in the presence of CNHs. These results suggest that macrophages incorporated CNHs and accelerated the differentiation of hMSCs into the osteoblast triggered by the OSM release (Fig. 4d).

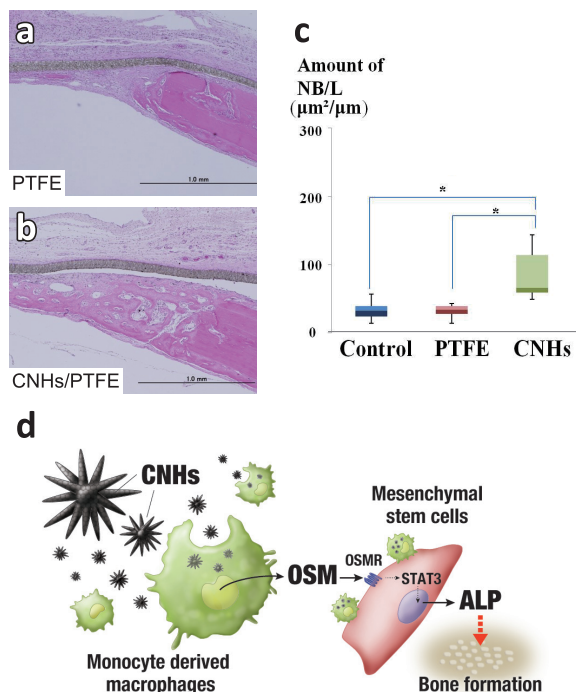


Fig. 4 Histology at 14 days after implantation of the PTFE membrane (a) and CNT/PTFE membrane (b) in the carivariar bone defects. At two weeks, the amounts of newly formed bone were larger in the CNHs/PTFE groups than in PTFE groups. ($p < 0.05$) (c). modified from Nanotechnology, 2011, 22, 65102. The macrophages engulfed CNHs and accelerated the differentiation of mesenchymal stem cells into the osteoblast via OSM release (d). modified from Nanoscale, 2015, 7.

Conclusion

As previewed, CNMs have been extensively studied for promising biomaterials for bone bioengineering as novel scaffolds. Choice of their size, length, surface modification, and induced functionalities therein will achieve desired characteristic and biocompatibility to ideal therapeutic materials for bone engeeniring.

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