

Three-Dimensional Macroporous Carbon Nanotube Scaffolds for Stem Cell Expansion and Maintenance

Gaurav Lalwani, Anu Gopalan, Michael D'Agati, Sunny Patel, Yahfi Talukdar and Balaji Sitharaman

Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794-5281

1. Introduction

Human mesenchymal stem cells (MSCs) show promise for several therapeutic applications including regenerative medicine, drug discovery, cellular therapy and disease modeling. Expansion of stem cells and maintenance of their self-renewal capacity *in vitro* requires specialized robust cell culture systems. Conventional approaches consisting of animal derived matrices and a cocktail of growth factors have several limitations such as consistency, scalability, and pathogenicity (risk of infection). Furthermore, to achieve high cell densities for practical therapeutic applications, 3D culture systems have been recommended over conventional 2D substrates. To overcome the above limitations, multifunctional 3D porous scaffold, fabricated using synthetic materials that permit stem cell expansion and maintenance *in vitro* would be a significant advancement.

Carbon nanotubes (CNTs) exhibit excellent physiochemical properties such as high mechanical strength, electrical conductivity as well as unique opto-acoustic and electromagnetic response. Therefore, they have been investigated for several therapeutic and diagnostic biomedical applications. Indeed, there has been a growing interest in assembling carbon nanomaterials into various two- and three-dimensional architectures for the fabrication of next-generation of energy storage, electronic, super-capacitor, photovoltaic and biomedical devices and implants.[1] For

biomedical applications, carbon nanotubes and graphene have been assembled into two-dimensional films (using vacuum filtration and chemical vapor deposition (CVD) methods) and 3D foams (using CVD and sacrificial template-transfer methods) and reported as cytocompatible substrates for cellular function (proliferation and differentiation of stem cells for applications in bone, neuron and cardiac tissue engineering).[2-5] These methods have several limitations. CVD method requires very specific substrates capable of withstanding high temperatures and pressure. Vacuum filtration and spray coating methods can produce 2D substrates that may not be suitable for tissue engineering of larger organs that demand three-dimensional scaffolds. Furthermore, a general limitation of these methods is that in the absence of strong chemical bonds between the individual nanomaterials, the structural integrity of architectures assembled relies mainly on weak Van der Waal forces or on physical entanglement of the nanoparticles, leaving them prone to dissociation under physiological shear forces experienced by *in vivo* biomedical devices and implants. Furthermore, methods such as sacrificial template transfer do not allow control over the porosity of the assembled 3D scaffold, which depends on the template architecture. Therefore, the assembly of carbon nanomaterials into three-dimensional (especially with >1 mm in all three dimensions) macroporous tissue engineering scaffolds with tunable porosity across various length scales (macro, micro and

nanoscopic) would constitute a significant advancement. We have recently reported a novel, cheap and easy method to fabricate macroscopic, 3D, free standing, all-carbon scaffolds (macroporous architectures) by radical initiated thermal crosslinking and annealing of CNTs.[1] This method can fabricate macroporous (20 nm – 300 µm) all-carbon structures with high porosities (~80-85%). Furthermore, porosity of scaffolds can be controlled by the amount of radical initiator used in the crosslinking process, thereby allowing the fabrication of porous all-carbon scaffolds tailored towards specific applications.

Towards the development as multifunctional 3D CNT scaffolds for tissue engineering applications, we report the cell-material interactions of two type of all-carbon scaffolds – prepared using single- and multi- walled carbon nanotubes (SWCNTs and MWCNTs). In this study, we characterize the cytocompatibility of 3D SWCNT and MWCNT scaffolds using human adipose derived mesenchymal stem cells (ADSCs). We study the cell viability, adhesion, proliferation and infiltration of ADSCs on 3D MWCNT and SWCNT scaffolds. Furthermore, we assess the plasticity of ADSCs after long-term expansion on 3D SWCNT and MWCNT scaffolds by inducing tri-lineage (osteogenic, adipogenic and chondrogenic) differentiation according to International Society for Cellular Therapy guidelines.[6]

2. Materials and Methods:

Preparation of SWCNT and MWCNT scaffolds and ADSCs cell cultured has been described previously.[1, 7] 3D porous poly(lactic-co-glycolic) acid (PLGA) scaffolds were used as positive controls. Cell death of ADSCs after 1, 3, and 5 days of culture on SWCNT and MWCNT

scaffolds was analyzed by lactate dehydrogenase (LDH) assay. Calcein-AM was used for LIVE cell imaging after day 5. Immunofluorescence imaging was used to determine the presence of vinculin and Ki-67. To determine MSC phenotype, surface markers were analyzed as described previously. Furthermore, to confirm the plasticity of MSCs after 15 days of culture on SWCNT and MWCNT scaffolds, cells were harvested from SWCNT and MWCNT scaffolds and cultured in adipogenic, osteogenic and chondrogenic media (Lonza, MD, USA) in 24 well-plates. Cells were then assessed for tri-lineage differentiation (adipogenic: Oil-red-O staining and elution; osteogenic: Alizarin red staining, ALP assay, calcium deposition; and chondrogenic: Alcian Blue staining and collagen-II immunohistochemistry).

3. Results and Discussions:

Detailed chemical, physical, mechanical and electrical characterization of MWCNT scaffolds has been reported previously.[1] The scaffolds used in this study were macroscopic (>1 mm in all three directions) architectures. The crosslinking of SWCNTs or MWCNTs to form 3D SWCNT or MWCNT scaffolds was confirmed by high-resolution SEM and TEM imaging (results not shown). The porosity values of MWCNT and SWCNT scaffolds were $45.82 \pm 3.59\%$ and $38.62 \pm 2.91\%$, respectively, quantified by microCT and SEM image processing. The presence of macro-, micro-, and nano-porosity is critical for the exchange of nutrients and waste metabolites during cell culture. ADSCs cultured on SWCNT and MWCNT scaffolds show negligible cell death quantified by LDH assay after 1, 3, and 5 days of culture compared to poly(lactic-co-glycolic) acid (PLGA) scaffolds (control group). Calcein-

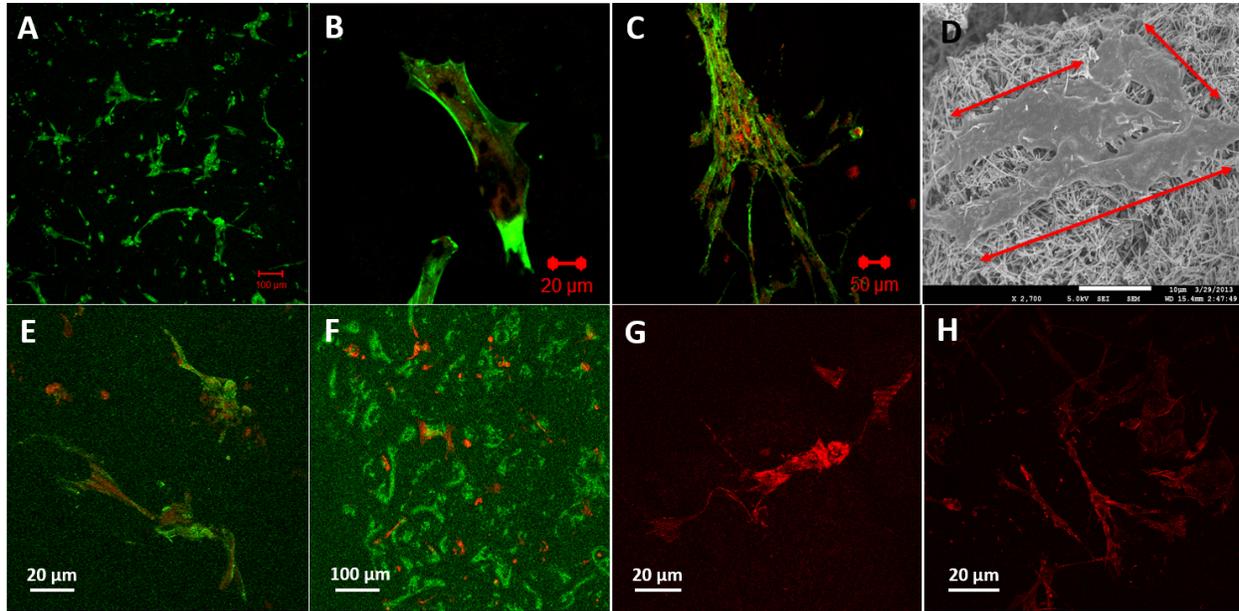


Figure 1: (A) Calcein-AM stained image showing live cells. Scale bar is 100 μ m. (B and C) Immunofluorescence images showing expression of Ki-67 and vinculin (both in red). Cytoplasm is stained green. (D) SEM image showing cell spreading and attachment. Scale bar is 10 μ m (E-H) immunofluorescence images showing the expression of CD105, CD90, and absence of CD45 and CD19, respectively. Surface antigens are green in color and cytoplasm is stained red for actin.

AM staining show the presence of live ADSCs on SWCNT and MWCNT scaffolds, corroborating LDH results (Figure 1A). Immunofluorescence results confirm the expression of cell attachment vinculin and proliferation marker Ki-67 and indicating that ADSCs attach and proliferate on SWCNT and MWCNT scaffolds (Figure 1B and C). Furthermore, SEM of ADSCs showed the cytoplasmic extensions attach to the underlying nanotube network (Figure 1D). ADSCs cultured on SWCNT and MWCNT scaffolds express stem cell phenotypic surface markers (ADSCs were positive for CD105, CD73 and CD90, and negative for CD45, CD34, CD14 and CD19) after 15 days (Figure 1 E-H). Additionally, SWCNT and MWCNT scaffolds can be de-assembled into smaller components to allow the harvest of ADSCs throughout the scaffold; hitherto not possible with polymeric scaffolds such as PLGA. The harvested ADSCs are capable of *in-vitro* expansion. Tri-lineage differentiation studies (results not shown) confirm that the ADSCs differentiate towards adipocytes (Oil-red-O

staining and elution), osteoblasts (Alizarin red staining, ALP assay, calcium deposition) and chondrocytes (Alcian Blue staining) and indicate that the scaffolds do not affect differentiation capabilities of the ADSCs.

To the best of our knowledge, this is the first study showing that 3D, macroscopic, porous SWCNT and MWCNT scaffolds with tunable porosities can be used for the expansion and maintenance of human ADSC. The ADSCs cultured on these 3D CNT scaffolds retain plasticity and can be harvested for lineage specific differentiation depending on patient-specific demand. The results of this study open avenues towards future *in vivo* safety and efficacy studies on the biocompatibility of 3D SWCNT and MWCNT scaffolds. In addition to stem cell expansion and maintenance, due to multifunctional properties of CNTs, one can envision the development these scaffolds to induce/guide specific cellular processes (such as differentiation, specific protein expression), support non-invasive longitudinal diagnosis/monitoring of tissue

regeneration, and permit stimulus based drug and gene delivery, a significant advancement towards next-generation tissue engineering and regenerative medicine applications.

4.References

- [1] Lalwani G et. al., Carbon. 2013;53:90-100.
- [2] Li N et. al., Scientific reports. 2013;3:1604.
- [3] Crowder SW et. al., Nanoscale. 2013;5:4171-6.
- [4] Nayak TR et. al. ,ACS nano. 2011;5:4670-8.
- [5] Mooney E et. al., Biomaterials. 2012;33:6132-9.
- [6] Dominici M et. al., Cytotherapy. 2006;8:315-7.
- [7] Talukdar Y et. al., Biomaterials. 2014;35:4863-77.