

# Investigation of Multiwall Carbon Nanotube Modified Hydroxyapatite on Human Osteoblast Cell Line using iTRAQ Proteomics Technology

J.L. Xu<sup>1, a</sup>, K.A. Khor<sup>1, b</sup>, J.J. Sui<sup>2, c</sup>, W.N. Chen<sup>2, d</sup>

<sup>1</sup> School of Mechanical & Aerospace Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798

<sup>2</sup> School of Chemical & Biomedical Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798

Emails: <sup>a</sup>jlxu@ntu.edu.sg, <sup>b</sup>mkakhor@ntu.edu.sg, <sup>c</sup>suij0001@ntu.edu.sg, <sup>d</sup>wnchen@ntu.edu.sg

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**Abstract.** Hydroxyapatite (HA) is a bioactive ceramic material with a chemical composition similar to natural bone, and carbon nano tubes (CNT) is able to enhance the brittle ceramic matrix without detrimental to the bioactivity. This study reported an attempt to use a commercially multiwalled CNT strengthen brittle hydroxyapatite bioceramics. Using iTRAQ-coupled 2D LC-MS/MS analysis, we report the first study of protein profile in osteoblasts from human osteoblastic cell line incubated separately on HA with and without strengthening multiwall CNT surfaces. Sixty proteins were identified and quantified simultaneously at the initial culturing stage of 3 days. The results were validated by Western blotting for selected proteins: Fetuin-A, Elongation factor II and Peroxiredoxin VI. Fetuin-A showed up-regulation, and Peroxiredoxin VI gave down-regulation in the osteoblasts cultured on HA based ceramic surfaces. Similar regulation was expressed by the protein of Elongation factor II on the phase pure HA surfaces as compared to the control group cultured on the polystyrene substrate. Relatively high EF 2 expression was detected on the phase the surfaces of CNT strengthen HA samples.

## Introduction

Carbon nanotubes (CNT) have aroused increasing interests due to their remarkable tensile strength, high resilience, flexibility and other unique structural, mechanical, brilliant electrical and physicochemical properties [1]. Osteoblasts (bone forming cells) can attach, proliferate and differentiate on the surfaces of hydroxyapatite (HA) based materials. Genetic studies in mice have provided new insights into the transcriptional regulation of osteoblast differentiation [2]. This study was to determine the changes in proteins osteoblasts from a cell line which were cultured on HA and CNT reinforced HA biomaterials. We employed an isobaric tagging for relative and absolute quantification (iTRAQ) technology to investigate the changes in protein mixture composition under the different physiologically relevant conditions. The iTRAQ technique is based upon chemically tagging the N-terminus of peptides generated from protein digests that have been isolated from cells in, for example, two different states [3]. The two labeled samples are then combined, fractionated by nano LC and analyzed by tandem mass spectrometry.

In this study, we used iTRAQ-coupled 2D LC-MS/MS analysis to study the protein profile in osteoblasts from human osteoblastic cell line incubated separately on HA with and without strengthening multiwall CNT surfaces. Several proteins showed obvious up- and down-regulation in the cells cultured on the HA samples as compared to the polystyrene substrates. The results were validated by Western blotting for selected proteins incorporated in the osteoblasts: fetuin-A, elongation factor II (EF 2) and peroxiredoxin VI (PRX 6). In addition, we will study a novel Ca<sup>2+</sup> binding protein, calgizzarin, which regulated the cytoskeletal function through its Ca<sup>2+</sup> dependent interaction with annexin I [4].

## Materials and Methods

A spark plasma sintering system (Sumitomo Coal Mining SPS system, Dr. Sinter Modal 1050, Japan) was used to consolidate the powder feedstock at sintering temperature of 1100 °C for 3 min with a heating/cooling rate of 100°C/min. The powder feedstock used for SPS were spray dried HA and multiwall carbon nanotube (CNT) reinforced HA. A commercially CNT with outside diameters of 10-30 nm, inside diameters of 3-10 nm and the lengths from 1 to 10 µm was selected to reinforce HA. 2 vol % of CNT powder was mixed with 98 vol% spray dried HA powder in ethanol and mechanical stirred for 5 days. The powder mixture was dehydrated in an open air and lightly ground using a mortar and pestle to decrease the damage to CNT by mixing.

Osteoblasts from a cell line of hFOB 1.19 (ATCC, CRL-11372) were seeded with an initial cell density of  $5 \times 10^4$  /cm<sup>2</sup> onto the sterilized samples that were placed in a 6-well culture tray and incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere. The culture medium used for each sample was 3ml of Dulbecco's modified Eagle's medium/F12 supplemented with 10 volume % of fetal bovine serum and 5 volume % of antibiotics. After reaching to 90% confluence the cells were treated with, the cells were collected and lysed in 150 µl of 8 M urea, 4% (W/v) CHAPS and 0.05% SDS (W/v) on ice for 20 min with regular vortexing. Protein was centrifuged at  $15,000 \times g$  for 60 min at 4 °C, supernatant was removed, and protein quantified using the 2-D Quant Kit (GE Healthcare). A standard curve was made using BSA as a control. 100 µg of each sample was precipitated by the addition of 4 volumes of cold acetone at -20 °C for 2 h, dissolved in the solution buffer, denatured and cysteins blocked as described in the iTRAQ protocol (Applied Biosystems). Each sample was then digested with 20 µl of 0.25 µg/ µl sequence grade modified trypsin (Promega, USA) solution at 37 °C overnight and labeled with the iTRAQ tags as follows: control group, iTRAQ 114; HA, iTRAQ 115 ; CNT modified HA, iTRAQ 116. The labeled samples were than pooled before analysis. To verify that sample preparation techniques do not interfere with digestion and labeling procedures. The bovine serum albumin (BSA) standard solution (Pierce, USA) was performed acetone precipitation, enzymatically digested with trypsin and labeled with the iTRAQ reagents as previously stated. These differentially labeled digests were mixed at a ratio of 1: 2: 1.5: 2 and analyzed by LC-MS/MS (Applied Biosystems, USA).

The cell morphology was examined using a scanning electron microscope (SEM). The cells fixation was done according to the previous study [5]. Total protein lysates extracted from three independent experiments as described above for LC MS/MS analysis were used separately in the Western blot analysis. Specially, 30 µg of total proteins from each samples was separated on a 8% SDS-PAGE. Proteins were then transferred onto nitrocellulose membrane (GE Hybond), and the detection of the respective proteins was performed using specific antibodies. Enhanced chemiluminescence was performed using SuperSignal West Pico chemiluminescent reagent and CL-Xposure film was used for detection. The antibodies from Santa Cruz used in this study were as follows: (1) Rabbit anti-human fetuin A polyclonal antibody, (2) Rabbit anti-human EF 2 polyclonal antibody, and (3) Rabbit anti-peroxiredoxin 6 polyclonal antibody.

## Results and discussion

Figure 1 showed a typical osteoblast cells morphology after fixation. Though the adhesion on ceramic surfaces was slower than on the culture plate, no apparent differences in the cell morphology on any the surfaces were found. Cells formed bridges across the undulations and spread over them. Some elongated cells were also observed. Different protein expression levels were obtained from the various culture substrates.

As shown in Fig. 2, the highest protein expression value was achieved on the plastic culture dishes as compared to the HA based ceramic surfaces. The presence of CNT seemed to promote the protein expression level compared with the surfaces without strengthening CNT. This may be attributed to the complex structure of carbon nanotubes, which consist of two separate regions with

different physical and chemical properties. The sidewall of the tube and the end cap of the tube will influence the peptides and proteins bound to the surface of ceramics reinforced with CNTs.

To establish the protein profile in the osteoblast cells incubated on the various HA based substrates, the incubated cells were collected and lysed prior to their analysis. Use iTRAQ, we were able to identify and quantify a total of 78 unique proteins simultaneously from the various HA based biomaterials, with 95% confidence. Identification of protein with significant expression level was based on the ProtScore with the cutoff at 2.0, which gave the confidence value of 99%. According to these criteria, 60 proteins with a 99% confidence were identified. Cytoskeletal proteins including actin, tubulin, and vimentin as well as metabolic enzymes are clearly different across the groups of HA and CNT reinforced HA (Data not shown).

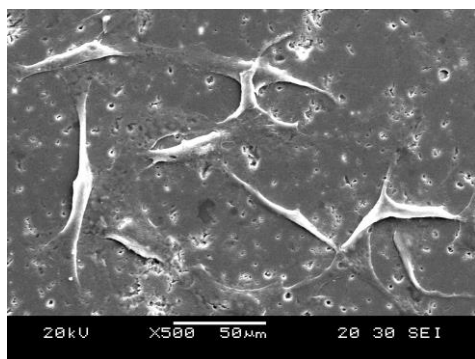


Fig. 1 SEM micrograph showing the osteoblast cell morphology on a typical HA disc after 1 day of incubation.

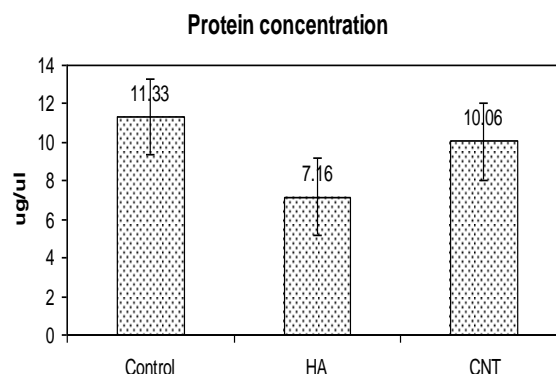


Fig. 2 Protein expression levels on various substrates.

Most proteins showed similar expression levels between the substrates. Some gave significant differences in the expression as shown in Figure 3. Fetuin-A showed significantly increased levels (~100%) in the samples cultured on the HA based substrates compared with the level in the control group. A representative spectrum was shown in Fig. 4, showing a peptide, CNLLAEK, from Fetuin-A protein. The peptide from Fetuin-A shows that the enhanced levels of 115 and 116 labels compared with control one labeled with 114. Fetuin-A is regarded as a potent inhibitor of systemic calcification to prevent the spontaneous formation of  $Ca^{2+} : PO_4^{3-}$  solid phases in extra-cellular fluids [6]. Multiple physiological roles for fetuin homologs have been suggested, including ability to bind to HA crystals and to specially inhibit the tyrosine kinase activity of the insulin receptor. The higher fetuin A concentrations are associated with the suppressed calcification.

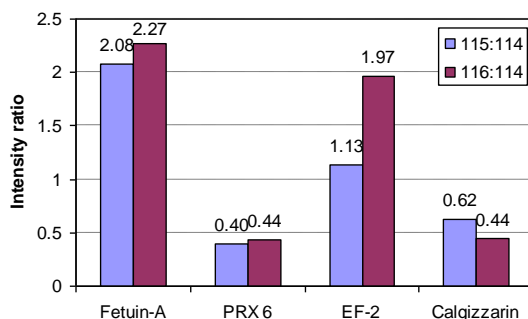


Fig. 3 Significantly expressed proteins as compared to the level observed in the control group.

Similar expression of EF 2 was observed in the sample cultured on phase pure HA surfaces and plastic culture dishes. Up-regulation of EF 2 was detected in the samples with reinforced CNT. EF 2 participates in the elongation phase during protein biosynthesis on the ribosome and supports the

translocation of tRNAs and of mRNAs on the ribosome so that a new codon can be exposed for decoding. Obvious down-regulation (~50%) was detected in the proteins of PRX 6 and calcizzarin. Peroxiredoxins are a ubiquitous family of antioxidant enzymes that control cytokine-induced peroxide levels and thereby mediate signal transduction in mammalian cells. It specifically prevents enzymes from metal-catalyzed oxidation. It can be regulated by changes to phosphorylation, redox and possibly oligomerization states [7]. Calcizzarin is a member of the large S100 family of EF-hand proteins and these proteins bind selectively to  $Ca^{2+}$  ions with very high affinity. It is an excellent candidate to function as cell specific transducer in calcium signaling pathways [4]. Yet its significance awaits for further investigation.

To assess if the changes in the protein levels were significant, Western blot analysis was carried out for fetuin-A, EF 2 and PRX 6 (no commercial antibody was available for the calcizzarin). Results shown in Fig. 5 indicated that the levels of fetuin-A were higher in cells incubated with HA based substrates compared with the level in the control group cultured on plastic dish. The level of PRX 6 is obviously higher than those levels in the samples incubated on HA based substrates. Relatively high EF 2 level was detected in the CNT modified samples as compared to the comparable results between the control group and phase pure HA surfaces.

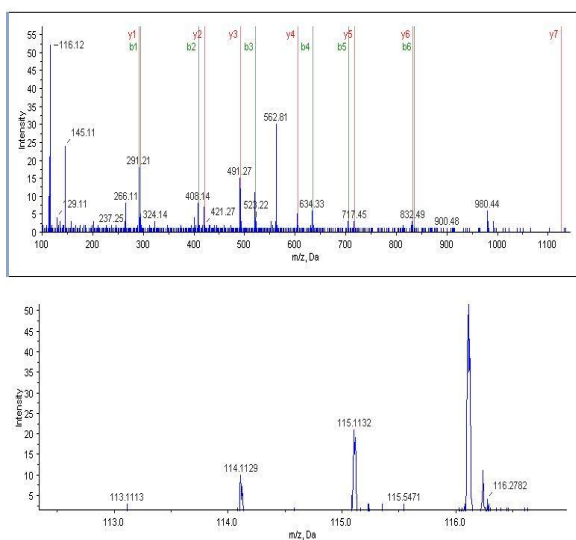


Fig. 4 A representative MS/MS spectrum showing a peptide, CNLLAEK, from Fetuin-A protein.

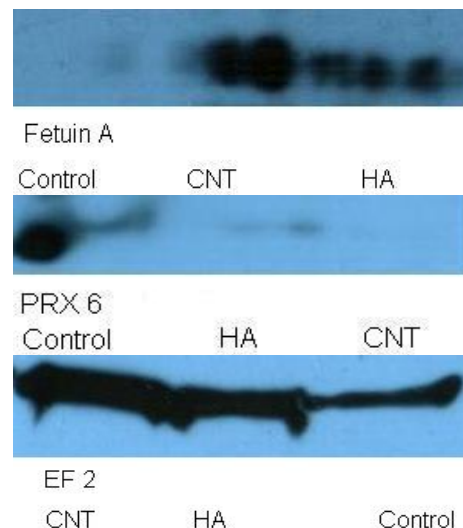


Fig. 5 Western blot analysis using commercially available antibodies.

## Conclusions

In this study, we were able to identify and quantify 60 unique proteins simultaneously from the various HA based biomaterials, with 99% confidence. The results suggest that iTRAQ could be used to identify cellular proteins associated with the osteoblasts from hFOB cell line after culturing on HA based bioceramic samples. In addition, that the presence of multiwall CNT slightly promoted the increases in protein expression and the cytoskeleton protein distribution as compared with the samples without the modification of multiwall carbon nanotubes.

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