Pure Appl. Chem., Vol. 83, No. 11, pp. 2063–2069, 2011.
doi:10.1351/PAC-CON-11-04-06
© 2011 IUPAC, Publication date (Web): 5 September 2011

Automated high-throughput screening of carbon nanotube-based bio-nanocomposites for bone cement applications*

Paula P. Gonçalves[1,†], Manoj K. Singh[2,†], Virgília S. Silva[1], Filipa Marques[1], Ana Marques[1], Philip R. LeDuc[3], José Grácio[3,‡], Paula A. A. P. Marques[3], Gil Gonçalves[3], and António C. M. Sousa[3]

[1]Departamento de Biologia and CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal; [2]Nanotechnology Research Division, Center for Mechanical Technology and Automation, University of Aveiro, 3810-193 Aveiro, Portugal; [3]Department of Mechanical Engineering, Carnegie Mellon University, 415 Scaife Hall, 5000 Forbes Avenue, Pittsburgh, PA 15213, USA

Abstract: In this work we demonstrate the potential of using an automated cell viability analyzer for developing high-throughput screening of orthopedic bioactive materials. We used a biomaterial of carbon nanotubes (CNTs)-based composite integrated with hydroxyapatite/polymethyl methacrylate (HA/PMMA) with controlled physical and chemical properties to evaluate the usefulness of morphometric analysis in conjunction with trypan blue dye exclusion assays in MG63 cell cultures. The MG63 cell line, derived from human bone osteosarcoma, is often used as a model for studying osteoblast-like cellular response to bioactive materials for orthopedic surgery. The viability analyzer, Vi-CELL^{TM} XR, Beckman Coulter, was used with trypan blue dye exclusion method in cell suspensions obtained after trypsinization along with determining the distribution plots of cell diameter and circularity, which are critical cellular characteristics. In addition, the activity of alkaline phosphatase (ALP), a typical representation of osteogenic activity of osteoblasts, was also measured spectrophotometrically using *p*-nitrophenol phosphate as the substrate. Comparative analysis of the frequency histogram of average cell diameter and circularity allowed for the analyses of significant alterations in cell morphology not only over time in control cultures (spherical vs. a flat morphology) but also with respect to PMMA and HA nanocomposites. After cell exposure to HA/PMMA/CNTs, a shift toward loss of cell circularity was observed. The appearances of more differentiated morphologic features were well correlated with the increase of secreted ALP activity. In conclusion, the evaluation of material-induced changes of cell morphology could represent a valuable prescreening test for bioactive properties.

Keywords: bone cement; carbon nanotubes; high-throughput methodology; nanocomposites; nanotechnology; orthopedic materials.

---

*Paper based on a presentation made at the International Conference on Nanomaterials and Nanotechnology (NANO-2010), Tiruchengode, India, 13–16 December 2010. Other presentations are published in this issue, pp. 1971–2113.
†E-mail: mksingh@ua.pt; pgoncalves@ua.pt
‡Corresponding author

INTRODUCTION

Nanotechnology introduces new carbon-based material, for example, carbon nanotubes (CNTs) and graphene. CNTs, because of their small dimensions and high aspect ratio, exhibit exceptional physical and chemical properties [1–12]. No other material can compete with their outstanding combination of mechanical, thermal, and electronic properties, which make them an outstanding reinforcement material for composites [4–7]. The ideal reinforcement material would impart mechanical integrity to the composite at high loadings, without diminishing its bioactivity. Moreover, CNTs have already been employed in the mechanics of materials domain for decades because of their high strength, which makes them ideal for a variety of applications including polymeric composite systems. Their successes in vivo though have been inhibited by physiological challenges as the promise of introducing pristine nonfunctionalized CNTs in polymer matrixes has been limited because this type of nanomaterial is practically insoluble and can accumulate in cells, organs, and tissues with dangerous effects. This problem has been overcome, however, by chemically modifying the surfaces of the CNTs, which addresses the solubility challenges in most solvents and polymers.

However, the development of bioactive materials for orthopedic surgery has created a bottleneck in testing in vivo biocompatibility where in vitro examination of cellular proliferation and differentiation could provide a valuable first-step screening method. Many materials that meet the safety criterion do not often meet the efficacy criteria in terms of cellular response [13], which often portends to poor results in compatibility later in testing. Sequential rounds of optimization of the material properties are required before a recommendation for human use is made owing to the significant cost for in vivo studies. The goal of in vitro assays is fast and low-cost screening of materials early in the discovery process. Toxicity evaluation of materials represents one of the first steps of optimization procedures. One direction for this approach is to use trypan blue dye exclusion, which is one of the most common early-round tests for use in toxicity tests. Automatic cell viability analyzers, like the Beckman Coulter Vi-CELL series, not only automate cell viability testing but also provide cell morphometric analysis of millions of individual cells in a few minutes. In this work we demonstrate the potential utility of cell viability determination using automatic cell viability analyzers [14] for developing high-throughput screening of orthopedic bioactive materials.

EXPERIMENTAL METHODS

Preparation of the bone cement composite biomaterial

Multi-walled carbon nanotubes (MWCNTs) (purity >95 %, Nanocyl-3150)-based composite integrated with hydroxyapatite-polymethyl methacrylate (HA/PMMA) with controlled physical and chemical properties has been prepared as previously described [15]. MWCNTs, with lengths of 1–5 μm and diameters of 5–10 nm, were functionalized by acid treatment and dispersed by ultra-sonication in an aqueous suspension containing the bone cement powder and HA. The suspension was granulated by the freeze-granulation technique and dried by lyophilization. Bone cement samples were prepared by mixing the composite powder with the polymerizing liquid [16]. This procedure allows the preparation of homogeneous mixtures of MWCNTs and HA in the PMMA matrix [15,17]. The final concentration of MWCNTs in the composite matrix was 0.1 % (w/w). Figure 1A discusses the two-step procedure for the preparation of bone cement by *Freeze-granulation technique*. Figure 1B shows a field emission-scanning electron microscopy (FE-SEM) image of a 0.1 % MWCNT-reinforced PMMA/HA nanocomposite material. The inset shows typical load–displacement curves of nanoindentations made at a peak indentation load of 10 mN on PMMA/HA nanocomposites with various amounts of MWCNT. From the indentation plot it is clear that 0.1 % MWCNTs reinforced PMMA/HA nanocomposite performs best mechanical properties.

© 2011, IUPAC

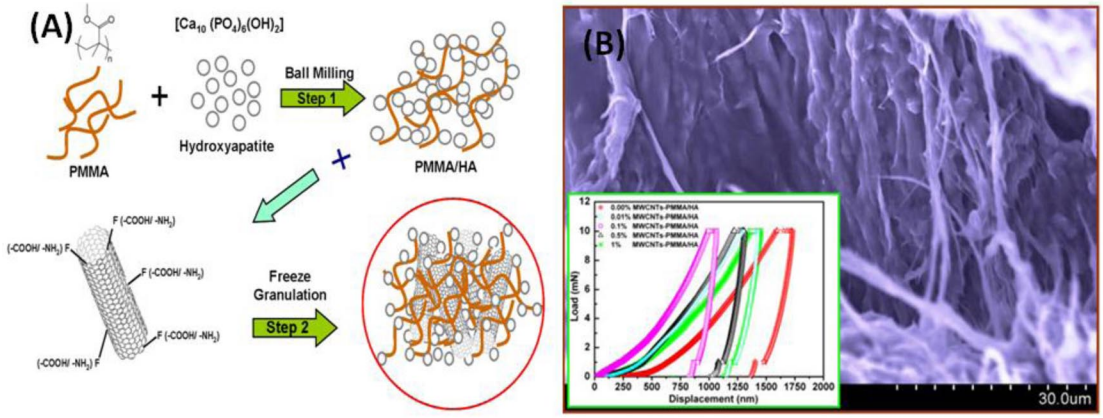

Fig. 1 Preparation of bio-nanocomposite-based bone cement. (A) The two-step procedure for the preparation of bone cement by *Freeze-granulation technique.* (B) FE-SEM image of a 0.1 % MWCNT-reinforced PMMA/HA nanocomposite material. Inset shows typical load–displacement curves of nanoindentations made at a peak indentation load of 10 mN on PMMA/HA nanocomposites with various amounts of MWCNT.

Cell culture

MG63 cells were seeded at 1.5×10^5 cells ml^{-1} on polystyrene culture plates or on disk-shaped composites and grown in Alpha Minimum Essential Medium containing 10 % fetal bovine serum, 2.5 μg/ml fungizone, 100 U/ml penicillin-streptomycin, and 85 μg/ml gentamicin [18]. Media were changed every 2–3 days, and the cultures were maintained at 37 °C in humidified atmosphere containing 5 % CO_2.

Analysis of cell cultures

MG63 cell monolayers were detached from the culture plates and dissociated by applying 0.25 % trypsin with EDTA·4Na. The cell suspensions were collected and analyzed using a viability analyzer, Vi-CELL^{TM} XR, Beckman Coulter [17]. Cellular morphometric parameters (cell diameter and circularity), cell concentration, and viability were determined after staining with trypan blue. In addition, the activity of alkaline phosphatase (ALP) was evaluated in the supernatant medium collected from individual wells of the culture plates prior to trypsinization of cell monolayers [19]. The activity of the secreted enzyme was measured spectrophotometrically at 410 nm, using 15 mM *p*-nitrophenol phosphate as the substrate in alkaline reaction medium (pH 10.3) containing 2.5 mM $MgCl_2$, at 37 °C.

PRACTICAL SIGNIFICANCE AND USEFULNESS

The dynamic process of bone remodeling consists mainly of resorption and formation that involves the activity of osteoclasts and osteoblasts. This activity is accompanied by functional and structural alterations at the cellular level that gives bone its mature structure [20]. The MG63 cell line, derived from human bone osteosarcoma [18], is often used as model for studying osteoblast-like cellular response to bioactive materials for orthopedic surgery. CNT-based composite integrated with HA/PMMA-CNTs with controlled physical and chemical properties were used to investigate the response of osteoblast to biomaterials and evaluate the potential of morphometric analysis in conjunction with trypan blue dye exclusion assays in MG63 cell cultures. These results would assist in understanding the utility of this approach in bone biocompatibility.

The trypan blue dye exclusion assays showed that cell viability remained almost unchanged during 18 days when culturing under control conditions (95.0 ± 0.1 %; n = 2875 images) as well as when

© 2011, IUPAC

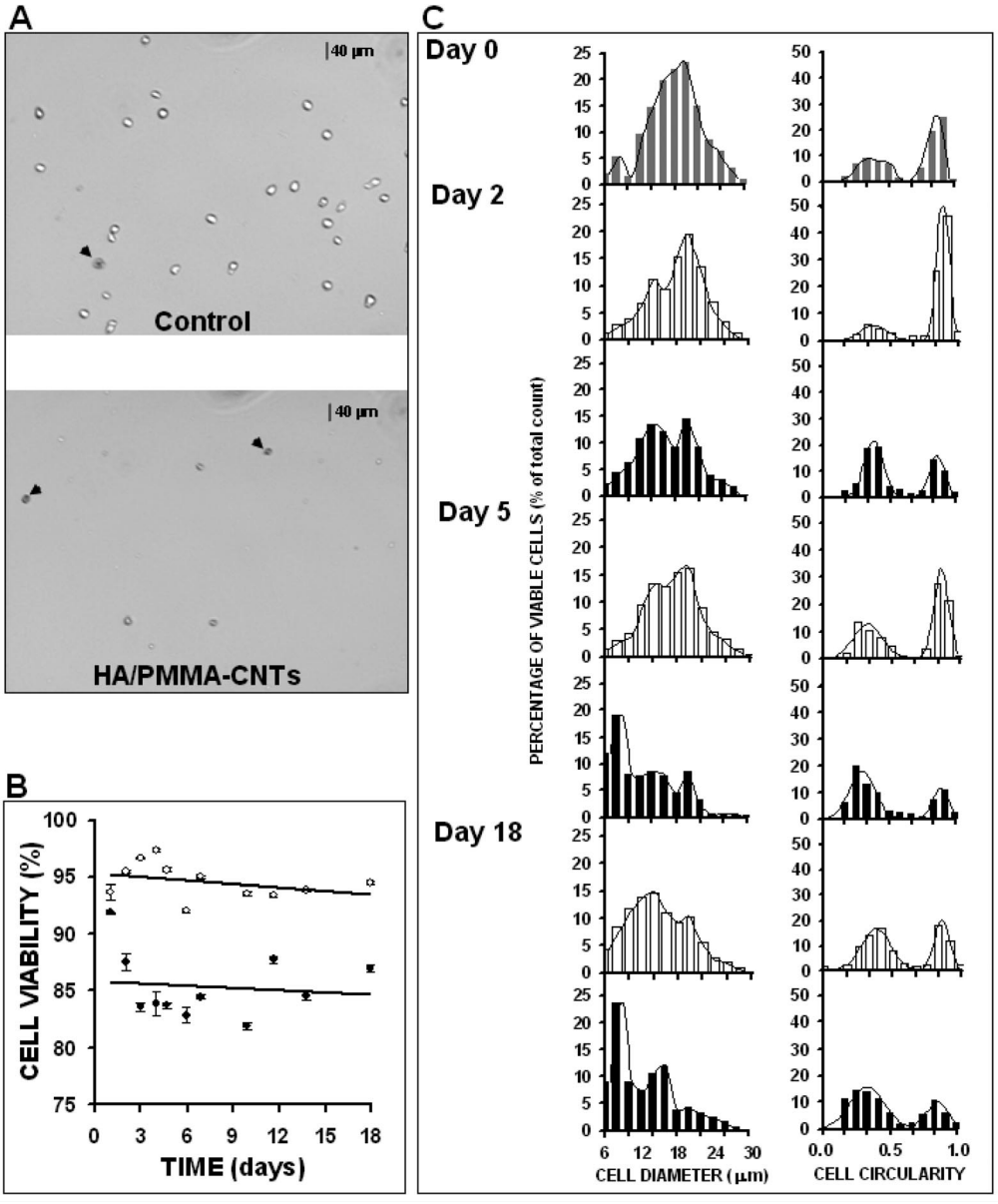

Fig. 2 Characterization of MG63 cell cultures over time using a viability analyzer Vi-CELL XR, Beckman Coulter. (A) Representative real-time images of cell populations under control conditions (polystyrene) and when cultured with HA/PMMA-CNTs. Viable cells excluded uptake of trypan blue while nonviable cells appeared darker, as indicated by the black arrow. (B) Cell viability of MG63 cell cultures remained very high during an 18-day incubation period. Experimental data are expressed as the percentage of viable cells with respect to the total number of cells in each image; the data points represent the cell viability values (mean ± S.E.) for each experimental condition (○, polystyrene; ●, HA/PMMA-CNTs). (C) Morphometric characteristics of MG63 cells during culture as revealed by the relative frequencies (measured as a percentage) of the average viable cell diameter and circularity observed in days 0 (gray bars), 2, 5, and 18 after cell seeding in polystyrene (white bars) and in HA/PMMA-CNTs (black bars). The experiments were conducted in quadruplicate, with each experimental condition evaluated for at least 200 images. The images were captured using a Vi-CELL XR video imaging system for analyzing cells in suspension, and the data correspond to a total number of analyzed cells of 65381 (control) and 7102 (HA/PMMA-CNTs).

© 2011, IUPAC

MG63 cells were cultured on HA/PMMA-CNTs (84.6 ± 0.2 %; n = 2101 images) (Figs. 2A,B). Simultaneously, an analysis of the frequency histogram of average cell diameter and circularity ($\sqrt{4A/\pi}/P/\pi$, where A and P represent the pixel cell area and perimeter, respectively) was also conducted. The results over the duration of the culturing conditions revealed significant alterations in cell morphology in control cultures when compared to the HA/PMMA-CNT material (Fig. 2C). The MG63 cell cultures were mainly viable with an average cell diameter concentrated in two major size groups of 12.93 ± 0.09 and 19.93 ± 0.04 μm (n = 65 381 cells). Over time, however, during the cell culture, there was a shift in the two size group populations as the proportion shifted significantly toward smaller cells. Concomitantly, a loss of cell circularity was also observed, suggesting an early turn-on of the morphometric profile toward smaller cells when cell cultures were in contact with HA/PMMA-CNTs. In addition, since secreted ALP is often used to monitor osteoblast maturation [21], we used this to investigate the behavior of the MG63 cells. Figure 3 clearly shows that the appearance of more differentiated morphologic features in the presence of HA/PMMA-CNTs (Figs. 3A,B), which appeared to support the results, showing that the HA/PMMA-CNTs induced an increase in secreted ALP activity (Fig. 3C).

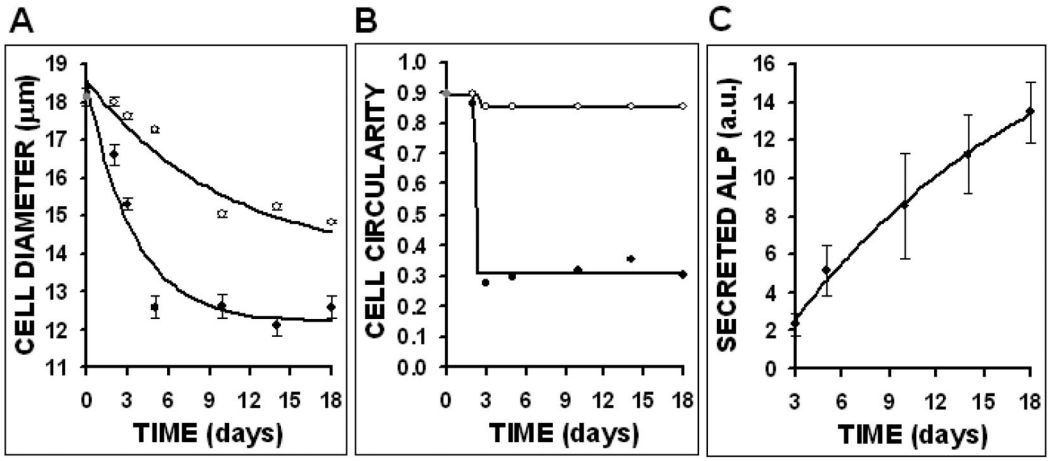

Fig. 3 Temporal evolution of morphometric characteristics of MG63 cells and secreted ALP activity. Time course data analysis of the average viable cell diameter (A), the mode of cell circularity (B), and secreted ALP (C) of MG63 cells seeded with polystyrene (○) and HA/PMMA-CNTs (●). The HA/PMMA-CNTs induced an increase of secreted ALP activity, which appeared correlated with the alterations of cell morphometric characteristics. A faster decrease of the average viable cell diameter and a sharp decline of the circularity values in response to contact with HA/PMMA-CNTs while the ratio between the values of ALP on HA/PMMA-CNTs vs. the polystyrene surface was observed to increase over time. Experimental data are expressed as mean ± S.E. of 4 independent assays performed at least in duplicate.

CONCLUSION

This paper highlights the issues relating to the in vitro evaluation of toxicity and bioactivity of CNTs-based composite integrated with HA/PMMA with controlled physical and chemical properties as a test case. There is a growing consensus that the complexity of these issues requires a multidisciplinary approach to increase the tailoring efficiency of new materials for orthopedic applications that includes physical scientists and biomedical and toxicologists among other specialists. Interestingly, in the design of biomaterials, optimization of the mechanical behavior usually comes first and concerns about its biocompatibility and cellular activity come afterwards. This approach unfortunately often leads to unde-

sirable results and can be linked to the lack of information embedded in cellular responses early in the discovery process.

Over the past decade, research and development of novel materials for orthopedic applications has turned from nontoxic, relatively bio-inert, and mechanically appropriate materials to bioactive materials that actively interact and integrate with bone tissue. Thereby, current evaluation of the cellular response to new tailored biomaterials represents an expensive and very time-consuming process, which involves performing many biological endpoint assays. In the context of orthopedic applications along with evaluation of the safety of materials for biomedical devices, evaluation of their osteoinductivity, osteogenicity, and osteoconductivity is mandatory. Briefly, it is essential to determine the ability of biomaterials to induce cell differentiation and the cell capability to produce a collagen matrix when in contact with biomaterials, in addition to demonstrate the biomaterial capability to support cell attachment and to provide an interconnected structure through which new cells can migrate. To monitor osteoinductivity, osteogenicity, and osteoconductivity, gene expression profiling technologies for analyzing panels of genes and quantification of phenotype markers by immunocytochemistry combined with sophisticated microscopy techniques are required.

From a biological perspective, broadening the evaluation of tissue/material interactions at the first steps of optimization procedures will permit the improvement of design criteria significantly.

ACKNOWLEDGMENTS

This work was supported by the International Iberian Laboratory of Nanotechnology (NANO/NMed-AT/0115/2007), the Portuguese Foundation of Science and Technology (Program Compromise with Science (to M.K.S. and P.M.) and SFRH/BPD/14677/2003 (to V.S.S.)) and the research and development agreement between the University of Aveiro and Carnegie Mellon University.

REFERENCES

1. S. Iijima. *Nature* **56**, 354 (1991).
2. R. H. Baughman, A. A. Zakhidov, W. A. de Heer. *Science* **787**, 297 (2002).
3. H. Dai. *Surf. Sci.* **218**, 500 (2002).
4. G. Goller, H. Demirkiran, F. N. Oktar, E. Demirkesen. *Ceram. Int.* **29**, 721 (2003).
5. J. Chlopek, B. Czajkowska, B. Szaraniec, F. Beguin. *Carbon* **44**, 1106 (2006).
6. R. L. Price, M. C. Waid, K. M. Haberstroh, T. J. Webster. *Biomaterials* **24**, 1877 (2003).
7. A. Peigney. *Nat. Mater.* **2**, 15 (2003).
8. W. A. Curtin, B. W. Sheldon. *Mater. Today* **7**, 44 (2004).
9. A. A. White, S. M. Best. *Int. J. Appl. Ceram. Technol.* **4**, 1 (2007).
10. A. Kis, G. Csanyi, J.-P. Salvetat, T.-N. Lee, E. Couteau, A. J. Kulik, W. Benoit, J. Brugger, L. Forro. *Nat. Mater.* **3**, 153 (2004).
11. E. Liden, E. Carlstrom, L. Eklund, B. Nyberg, R. Carlsson. *J. Am. Ceram. Soc.* **78**, 1761 (1995).
12. S. Haque, I. Rehman, J. A. Darr. *Langmuir* **23**, 6671 (2007).
13. C. J. Kirkpatrick, F. Bittinger, M. Wagner, H. Köhler, T. G. van Kooten, C. L. Klein, M. Otto. *Proc. Inst. Mech. Eng.* **212**, 75 (1998).
14. S. E. Szabo, S. L. Monroe, S. Fiorino, J. Bitzan, K. Loper. *Lab. Hematol.* **10**, 109 (2004).
15. M. K. Singh, T. Shokuhfar, J. J. A. Gracio, A. C. M. Sousa, J. M. Fereira, H. Garmestani, S. Ahzi. *Adv. Funct. Mater.* **18**, 694 (2008).
16. M. H. Pelletier, A. C. B. Lau, P. J. Smithan, G. Nielsen, W. R. Walsh. *Acta Biomater.* **6**, 886 (2010).
17. M. K. Singh, J. Gracio, P. LeDuc, P. P. Gonçalves, P. A. A. P. Marques, G. Gonçalves, F. Marques, V. S. Silva, F. Capela e Silva, J. Reis, J. Potes, A. Sousa. *Nanoscale* **2**, 2855 (2010).

© 2011, IUPAC

18. A. Billiau, V. G. Edy, Hubertine Heremans, J. Van Damme, J. Desmyter, J. A. Georgiades, P. De Somer. *Antimicrob. Agents Chemother.* **12**, 11 (1977).
19. A. Tampieri, M. Sandri, E. Landi, G. Celotti, N. Roveri, M. Mattioli-Belmonte, L. Virgili, F. Gabbanelli, G. Biagini. *Acta Biomater.* **1**, 343 (2005).
20. K. Henriksen, A. V. Neutzsky-Wulff, L. F. Bonewald, M. A. Karsdal. *Bone* **44**, 1026 (2009).
21. H. Orimo. *J. Nippon Med. Sch.* **77**, 4 (2010).

© 2011, IUPAC