In vitro assessment of the biological response to nano-sized hydroxyapatite

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Nano-sized, rod-like hydroxyapatite (nHA) crystals were produced and shown to be phase-pure by X-ray diffraction analysis, as no secondary phases were observed. The nHA suspension was electrosprayed onto glass substrates using a novel processing routine to maintain nanocrystals of hydroxyapatite. The biocompatibility of nHA was determined using human monocyte-derived macrophages and human osteoblast-like (HOB) cell models. The release of lactate dehydrogenase (LDH) from human monocyte-derived macrophages was measured as an indicator of cytotoxicity. The release of the inflammatory cytokine, tumour necrosis factor alpha (TNF-α) from cells in the presence of nHA crystallites was used as a measure of the inflammatory response. Although there was some evidence of LDH release from human monocyte-derived macrophages when in contact with high concentrations of nHA crystals, there was no significant release of TNF-α. Moreover, nHA-sprayed substrates were able to support the attachment and the growth of HOB cells. These results indicate that nHA crystals may be suitable for intraosseous implantation and offers the potential to formulate enhanced composites for biomedical applications.

1. Introduction

Designing a biomaterial, which mimics the structure and properties of natural tissue offers the potential of extending the lifetime of implants. Bone can be considered as an apatite reinforced collagen composite at the ultramicrostructural level [1]. Hydroxyapatite (HA), a major inorganic component of bone, has been used extensively and successfully in biomedical implants for bone regeneration. To mimic the structure of bone, many efforts have been made to incorporate HA in a tough polymer matrix, such as polyethylene, polylactic acid and collagen [2–4].

Nanometre-scale hydroxyapatite (nHA), more closely mimics the size of HA crystals in natural bone and can be produced in our laboratory [5]. This process offers the possibility of producing new bone analogue composites by incorporating nHA in the microstructure of polymers to improve further their mechanical and biological properties. In the development of nHA for biomedical applications, an evaluation of the biological response of nHA is required to provide useful information on the design and application of nHA to enable it to fulfill its full potential.

It has been shown that the size, morphology and structure of HA particles can influence the biological response [6–8]. As-dried HA particles could stimulate greater inflammatory cytokine release compared to well-sintered HA. Nano-sized HA precipitates may have higher solubility and therefore affect the biological responses. After the initial response to an implant from leukocytes, bone-forming osteoblast cells start to become involved. Hence, to monitor closely the consequent events when a system incorporating these nano-sized crystals is in contact or in reaction with the biological environment, the cytotoxicity and inflammatory response to nHA crystals should be investigated. This evaluation was carried out in the present study using a primary human monocyte-derived macrophage model and a human osteoblast-like (HOB) cell model was used to test the effects of nHA on the potential for bone formation.

The osteoconductive ability of bone replacement

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materials is closely related to HOB cell behaviour, particularly cell adhesion on contact with the surface. Attachment, adhesion and spreading represent the first phase of cell/material interactions and the quality of this interaction will influence subsequent cell proliferation and differentiation. An evaluation of the adhesion of osteoblasts on nHA facilitates understanding and optimisation of the bone/biomaterials interface, and this characteristic was also investigated in this study.

2. Materials and methods

2.1. Production and characterisation of nHA

nHA with a Ca/P ratio of 1.67 was synthesised by a precipitation reaction between calcium hydroxide ($Ca(OH)_2$) and orthophosphoric acid (H_3PO_4) (both AnalaR grade, BDH, UK). H_3PO_4 solution (0.3 M) was added drop-wise to 0.5 M $Ca(OH)_2$ solution under continuous stirring at room temperature, while the pH was kept above 10.5 by the addition of ammonia solution. Stirring was maintained for a further 16 h after complete addition of the reactants. The precipitate obtained was further aged for a week. The morphology of nHA precipitates was examined using a JEOL 200CX transmission electron microscope (TEM) with an accelerating voltage of 200 keV. The crystal structure was studied using a Philips PW1730 X-ray diffractometer using CuK_α radiation. The data were collected from 25° to 50° with a step size of 0.05° and a scan time of 6 s was used.

2.2. Cytotoxicity

A primary human monocyte-derived macrophage model [9] was used to test the cytotoxicity of nHA crystals. The concentration of nHA in culture medium was estimated by using a haemocytometer under a Leica microscope with frame grabber after staining nHA with 1 wt % silver nitrate to increase the contrast with the understanding that a proportion of the particles would be below the limit of optical resolution. nHA crystallites with minimum concentrations of 1–100 million particles were added to each test well containing 0.5 million cells. After 24 h of culturing, the release of lactate dehydrogenase (LDH, Cytotox 96; Promega, Southampton, UK) from damaged cells, an indicator of cytotoxicity, was measured in serum and serum-free medium, as the amount of enzyme activity correlates to the number of damaged cells. Culture medium (ctr1) and macrophages not in contact with nHA particles (ctr2) were used as the test controls. The release of the inflammatory cytokine tumour necrosis factor alpha (TNF-α) from cells in the presence of nHA crystallites was determined by an enzyme-linked immunosorbent assay (ELISA, Endogen, Woburn, USA) as a measure of the potential inflammatory response.

2.3. Electrospraying of nHA

To study the biological response of HOB cells when directly cultured on nHA, suspensions containing nHA was electrosprayed onto glass substrates [5]. Thus the ability of nHA droplet deposits (relics) to support the growth of HOB cells as a function of time can be determined. The processing of nHA by electrospraying is detailed elsewhere [5]. Briefly, nHA particles were suspended in ethanol to give a slurry with a concentration of 3 vol % nHA. Electrospraying was carried out using the equipment previously reported [10, 11] but with a ring-shaped ground electrode, which produces a diverging spray rather than a focused stream of droplets. Freshly prepared nHA suspension was expelled from the needle at $1.7 \times 10^{-9}\ m^3\ s^{-1}$ with the applied voltage set at 6 kV, and stable electrospraying was achieved. Droplets of nHA were sprayed on a glass substrate for 30 s. The structure of nHA relics was examined using a JEOL 6340 field emission scanning electron microscope (SEM) at an accelerating voltage of 5 keV after coating with a thin layer of Pt. The surface roughness of nHA relics on the glass substrate was measured by atomic force microscopy (Nanoscope III, Digital Instrument).

2.4. AlamarBlue™ assay

The ability of nHA to support the growth of bone-forming HOB cells (Promocell, UK) was determined by alamarBlue™ assay. Glass substrates (10 mm × 10 mm) containing electrosprayed nHA relics were sterilised by dry heat at 160 °C for 4 h. HOB cells (2×10^4 cells) were then seeded directly on the surface of these substrates and incubated at 37 °C in a humidified air atmosphere of 5% CO_2. HOB cells cultured on tissue culture plastic were used as the test control. The growth of HOB cells on the nHA-sprayed specimen was monitored regularly by examining under a phase contrast microscope. The metabolic activity of HOB cells on the substrates after intervals of four and seven days of culturing was determined using the alamarBlue™ assay (Serotec, Oxford, UK). The absorbance was measured on a plate reader at a wavelength of 570 nm with a reference wavelength of 600 nm.

2.5. Immunofluorescence and cytoskeletal observations

After 20 h and two days of culture, the cells on the substrates were fixed in 4 wt % paraformaldehyde in phosphate buffered saline (PBS) with 1 wt % sucrose for 15 min, washed with PBS, and made permeable at 4 °C for 5 min [12]. The samples were incubated with 1 wt % bovine serum albumin (BSA) in PBS at 37 °C for 5 min to block the non-specific binding. This was followed by adding FITC-conjugated phalloidin (Sigma, Poole, UK) at 37 °C for 1 h; or by adding anti-vinculin monoclonal antibody (Sigma, Poole, UK; 1 : 100 in 1% BSA/PBS) at 37 °C for 1 h. After thorough washing with 0.5 wt % Tween 20 in PBS, a secondary biotin-conjugated antibody (Vector Laboratories, Peterborough, UK; 1 : 50 in 1% BSA/PBS) was added at 37 °C for 1 h. After washing with 0.5 wt % Tween 20/PBS (5 min × 3), Texas red-conjugated streptavidin (Vector Laboratories, Peterborough, UK) was added at 4 °C for 30 min. The samples were mounted in Vectorshield fluorescent mountant (Vector Laboratories, UK) and viewed using a Leica SP2 confocal laser scanning microscope (CLSM).

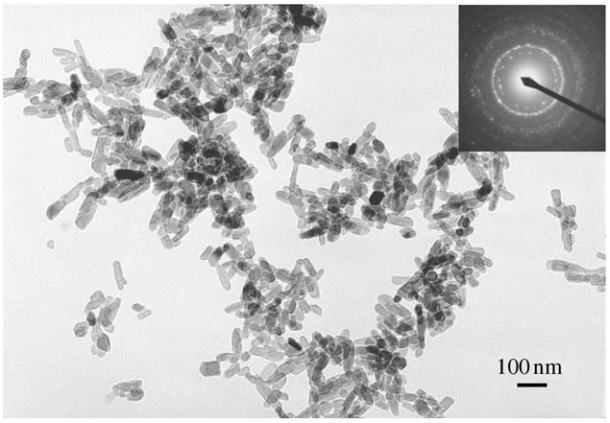


Figure 1 Transmission electron micrograph and SAD of nHA particles.

2.6. Scanning electron microscopy

After seven days of culturing HOB cells on the nHA substrate, the cultures were fixed, stained with 1 wt % osmium tetroxide and dehydrated in a graduated series of alcohols and finally critical point dried (Polaron E3000 CPD). The sample surface was coated with a thin layer of carbon before it was examined using a JEOL 6340 field emission SEM.

3. Results and discussion

3.1. Characterisation of nHA

The size of rod-like nHA particles was about 50–80 nm in length, revealed during TEM examination. Selected area diffraction (SAD) showed a spotted pattern, indicating a polycrystalline material (Fig. 1). The XRD analysis of nHA powder revealed no secondary phases (Fig. 2).

The vast majority of relics produced by electrospraying the nHA suspension were $< 1\,\mu m$ and atomic force microscopy revealed that the mean surface roughness of the deposit was about 33 nm. Whether the spraying process is electrostatic atomisation [13] or an electrically forced jet [14] is being investigated at present.

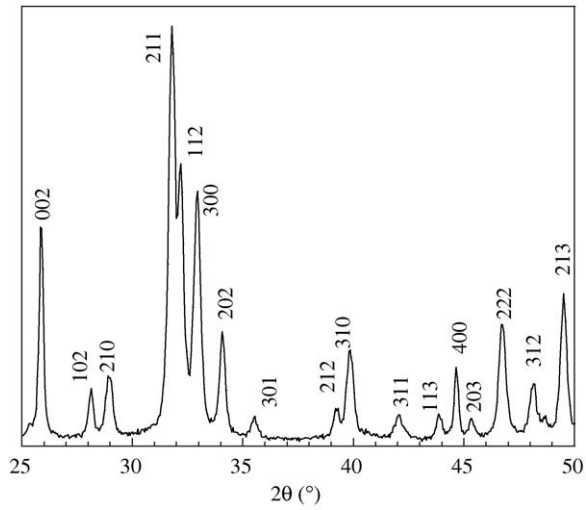


Figure 2 X-ray diffraction pattern of nHA particles.

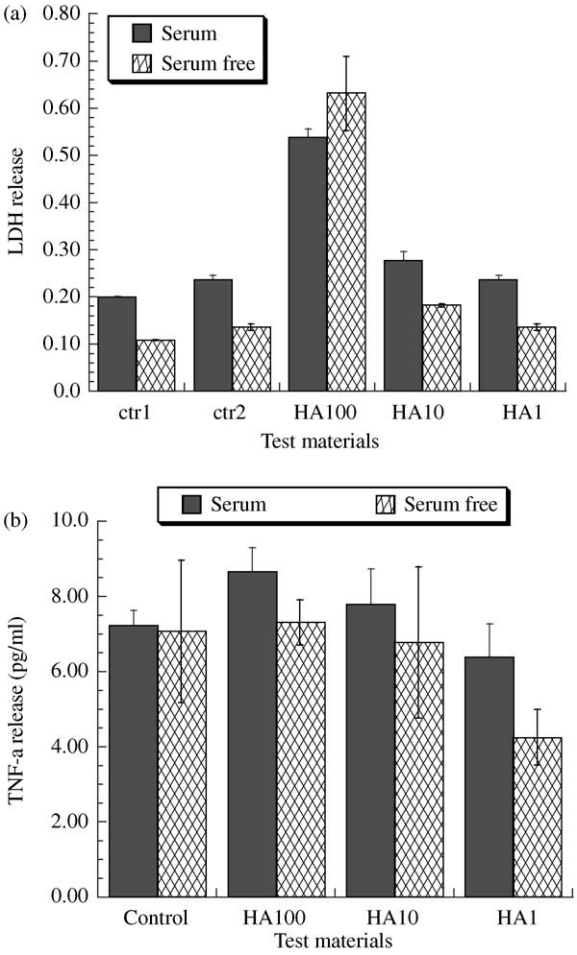


Figure 3 The release of (a) LDH and (b) TNF-α from macrophages after 24 h treated with nHA crystallites with concentration from 100 (HA100) to 1 (HA1) million particles per test.

3.2. LDH and TNF-α release

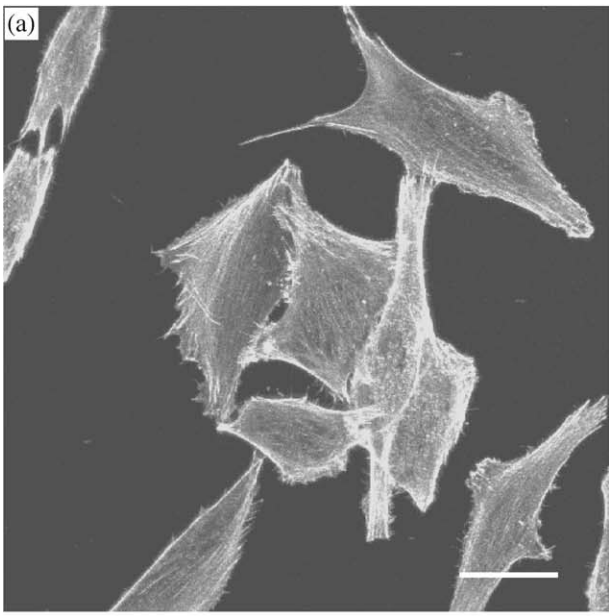
Except for the response to high concentration of nHA (100 million particles), the level of LDH release was generally higher in serum-containing medium. The LDH release from human monocyte-derived macrophages increased when cells were in contact with nHA crystallites, indicating damage to the cell membrane, but the levels of LDH release decreased significantly when the nHA concentration was reduced from 100 to 1 million (Fig. 3(a)).

There was some evidence of LDH release from macrophages when in contact with high concentrations of nHA crystallites, but it is unlikely that such a high concentration of nHA could be released into the surrounding environment when using it as the filler in a composite system.

No significant differences were seen between responses in serum-free and serum-containing medium for the TNF-α release. The level of release of TNF-α was not significantly greater than the control at all concentrations of nHA (Fig. 3(b)), indicating that nHA crystals may not trigger a severe inflammatory response.

3.3. Cytoskeletal observation

The development of the cell cytoskeleton on nHA substrates was studied by immuno-fluorescent staining of cytoskeletal proteins (actin and vinculin) involved in



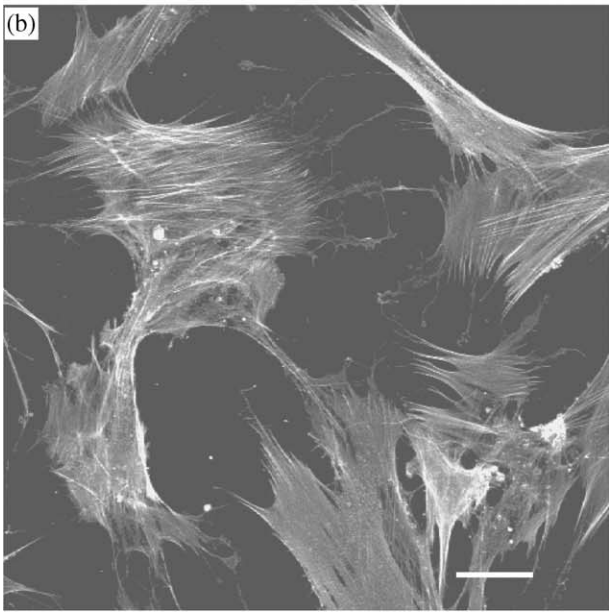


Figure 4 CLSM images of actin cytoskeleton for HOB cells on (a) control and (b) nHA-sprayed substrate after 20 h of culture. Scale bar = 40 μm.

focal contacts to understand the quality of cell adhesion. Immunofluorescent staining at 20 h revealed initial interactions between HOB cells and the nHA and control substrates. Cells could be seen to have contactile, actin stress fibres throughout the cells on the nHA-sprayed substrate (Fig. 4) while actin stress fibres were more apparent around the periphery of the cells on the control substrate. Vinculin immunostaining showed there were plenty of the focal contacts on the substrates (Fig. 5).

3.4. The attachment and growth of HOB cells

The metabolic activity of HOB cells on the nHA-sprayed substrates was higher than that on standard tissue culture control after four days of culture from alamarBlue™ assay, indicating that more cells attached to the nHA-sprayed substrate. The cell activity increased from day four to day seven, which demonstrates that the nHA-

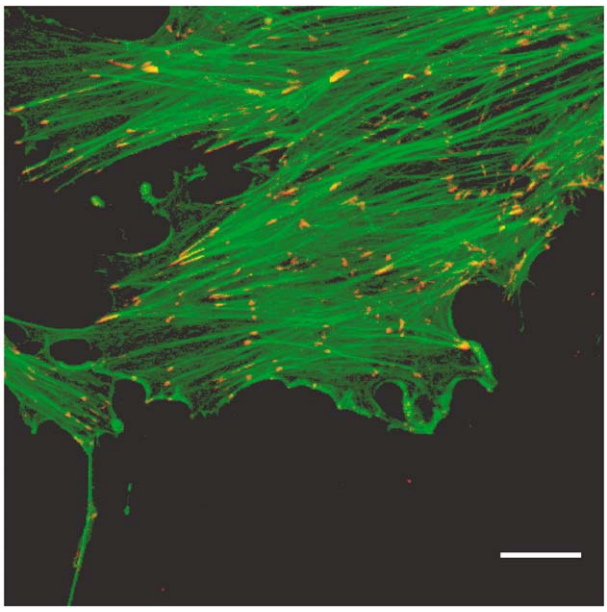


Figure 5 CLSM images of actin (green) and vinculin (red) cytoskeleton for HOB cells on a nHA-sprayed glass substrate after two days of culture. Scale bar = 20 μm.

sprayed substrates were able to support the growth of HOB cells *in vitro* during seven days of culture (Fig. 6).

The ability of nHA to promote the attachment of HOB cells was further studied by SEM. HOB cells were able to attach to the nHA-sprayed substrates and maintained their osteoblastic morphology with visible filapodia attached to nHA particles (Fig. 7(a)). There were large areas of confluent cells after seven days of culture and fibre-like extracellular matrix was produced (Fig. 7(b)).

4. Conclusions

There was some evidence of LDH release from human monocyte-derived macrophages when in contact with high concentrations of nHA crystals, therefore great care should be taken in designing the application of nHA. However, there was no significant release of the inflammatory cytokine TNF-α, indicating that nHA

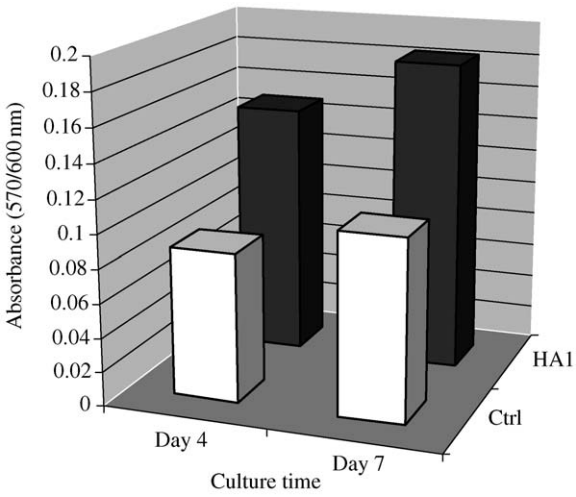


Figure 6 The growth of HOB cells on nHA relics during seven days of culture measured by the alamarBlue™ assay.

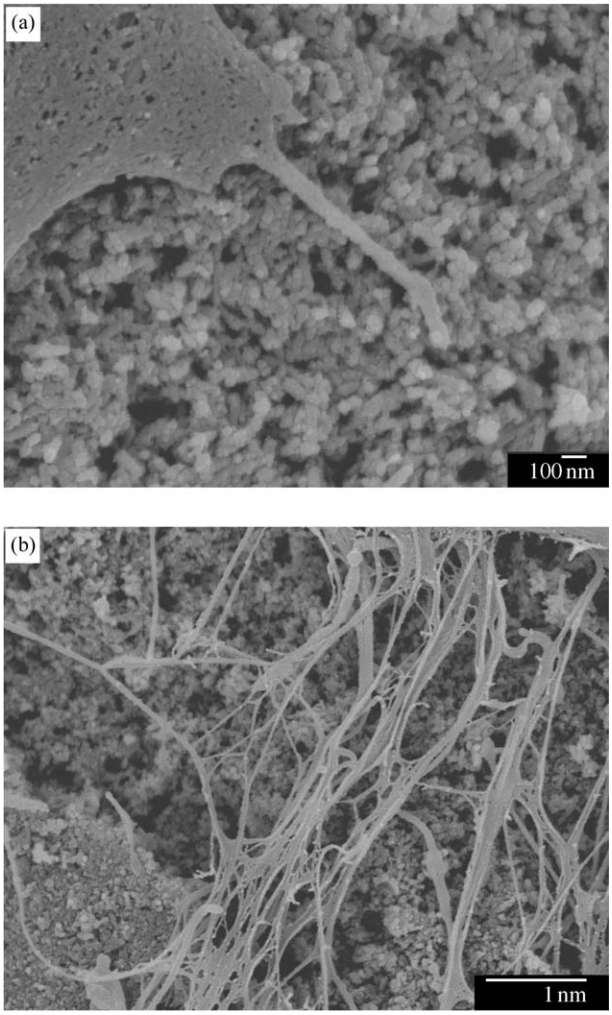


Figure 7 Scanning electron micrographs showing (a) the attachment of HOB cells on nHA particles and (b) extracellular matrix produced by HOB cells on the nHA-sprayed glass substrate.

crystallites may not stimulate an inflammatory response. Moreover, nHA-electrosprayed substrates were able to support the attachment and the growth of HOB cells. These results indicate that the nHA crystals are likely to produce favourable biological responses *in vivo*, and this offers the potential to formulate composites for biomedical applications.

Acknowledgments

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References

1. W. BONFIELD, *Metals Mater.* **3** (1987) 712.
2. W. BONFIELD, M. WANG and K. E. TANNER, *Acta. Mater.* **46** (1998) 2509.
3. C. DURUCAN and P. W. BROWN, *J. Biomed. Mater. Res.* **51** (2000) 717.
4. W. ZHANG, S. S. LIAO and F. Z. CUI, *Chem. Mater.* **15** (2003) 3221.
5. J. HUANG, S. N. JAYASINGHE, S. M. BEST, M. J. EDIRISINGHE, R. A. BROOKS and W. BONFIELD, *J. Mater. Sci. Lett.* **39** (2004) 1029.
6. J. SUN, Y. TSUANG, W. H. CHANG, J. LI, H. LIU and F. LIN, *Biomaterials* **18** (1997) 683.
7. J. T. NINOMIYA, J. A. STRUVE, C. T. STELLOH, J. M. TOTH and K. E. CROSBY, *J. Ortho. Res.* **19** (2001) 621.
8. Y. HARADA, J. WANG, V. DOPPALAPUDI, A. WILLIS, M. JASTY, W. HARRIS, M. NAGASE and S. GOLDRING, *J. Biomed. Mater. Res.* **32** (1996) 19.
9. J. A. WIMHURST, R. A. BROOKS and N. RUSHTON, *J. Bone Joint Surg.* **83-B** (2001) 278.
10. S. N. JAYASINGHE and M. J. EDIRISINGHE, *J. Mater. Res. Innovat.* **7** (2003) 62.
11. S. N. JAYASINGHE, M. J. EDIRISINGHE and T. DEWILDE, *ibid.* **6** (2002) 92.
12. M. J. DALBY, L. DISILVIO, E. J. HARPER and W. BONFIELD, *Biomaterials* **23** (2002) 569.
13. S. N. JAYASINGHE and M. J. EDIRISINGHE, *J. Aerosol. Sci.* **33** (2002) 1379.
14. S. N. JAYASINGHE and M. J. EDIRISINGHE, *ibid.* **35** (2004) 233.

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