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The cytotoxicity evaluation of Kevlar and silicon carbide by MTT assay

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The MTT test has been widely used as a rapid and sensitive method for screening anticancer drugs. In this paper, we used this method to assess the cytocompatibility of three materials: Kevlar 29, silicon carbide and polyvinyl chloride (PVC) in both a quantitative and a qualitative manner. The materials were prepared by cleaning in 70% ethanol, autoclaved or gamma-sterilized. Extracts were prepared at four time periods (1, 2, 3 and 4 weeks) and two temperatures (37 °C and 80 °C). The extracts were used in the MTT assay and the data were collected and analysed with ONEWAY and DUNCAN procedures using the statistical computer package SPSS^x. The MTT staining procedure was also used in direct contact with the materials. The result from the MTT assay demonstrated that Kevlar, SiC and PVC extracted at 37 °C were not cytotoxic while PVC extracted at 80 °C did show some cytotoxicity, especially the material that had been gamma-sterilized. In the direct contact test the Kevlar showed no cytotoxicity. The SiC did show some localized toxicity when the material had been autoclaved, however, SiC subject to prior cleaning with ethanol showed no cytotoxicity. The PVC that had been autoclaved caused a cytotoxic response whereas the material that had been gamma-sterilized or cleaned in ethanol showed good cytocompatibility. This paper demonstrates that the MTT staining procedure is a useful technique to study the cytocompatibility of materials in both a quantitative and a qualitative manner. It is also shown that the cellular response to the materials tested is dependent on the method of preparation.

1. Introduction

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide)-based colorimetric assay was originally described by Mosmann [1] as a useful method for the measurement of cytotoxicity and cell proliferation. It is reported that the mitochondrial enzyme succinate-dehydrogenase within viable cells is able to cleave the tetrazolium salt MTT into a blue-coloured product (formazan) [2] which is soluble in iso-propanol and can be read by an automatic micro-plate scanning spectrophotometer, which offers major advantages in speed, simplicity, cost and safety [2]. It is proposed that the amount of the formazan product is proportional to the number of viable cell present [3, 4], that is, the higher the optical density the greater the number of viable cells. It has been widely used in the assessment of anticancer drugs [5, 6] and recently, this method has been used as a screening test for the cytotoxicity evaluation of materials [7]. In this laboratory the reproducibility of the MTT assay has been systematically studied and improved by using a procedure in which the columns of the microtitre plate are randomly assigned to test groups which increases the validity and accuracy of the test results [8]. In this paper we present data on the cytocompatibility of three materials using the improved procedure for the MTT assay. We also demonstrate that the MTT staining technique can be used in the direct contact test to study qualitatively cell proliferation and activity.

2. Materials and method

2.1. MTT assay

2.1.1. Materials

Three materials were studied, two fibre materials, Kevlar 29 (K) and silicon carbide (SiC) (Goodfellow Metal Ltd., England), and one sheet, PVC (VT Plastics Ltd., UK). The PVC was used as the positive control in the study. The materials were washed for 5 min in double distilled water in an ultrasonic bath twice, placed in 70% ethanol for 30 min and dried in a fume hood. The PVC was prepared either by placing it in 70% ethanol (PVC1) or by gamma-radiation sterilization (PVC2). The surface areas of the materials were measured and standardized to approximately 40 cm^2. The extracts of the materials were prepared in sterilized double distilled water. The extracts were prepared over four time periods at two temperatures; 1 week, 2 weeks, 3 weeks and 4 weeks at both 37 °C and 80 °C. The PVC2 extracts were prepared only for 1 week, 2 weeks and 4 weeks.

2.2. Cell line

The established cell line L929 (ICN Flow, Irvine, Scotland) was used in this study and maintained in

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growth medium 199 supplemented with 5% foetal calf serum, 50 Iu ml^{-1} penicillin and 50 ug ml^{-1} streptomycin. The cells came to confluence after one week's incubation (5% CO_2, 37 °C) and the monolayer was then harvested by trypsinization. The cell suspension was counted by Coulter Counter (Model "7", Coulter Electronics LTD, England) before it was diluted 1 + 7 with culture medium.

Two groups of MTT assays were carried out. For the first group, eight 96-well microtitre flat-bottomed plates were numbered and used to study Kevlar, SiC and PVC1. For the second group, six plates were used for SiC, PVC1 and PVC2. All the cells were thoroughly mixed with the medium in a large container to ensure an even distribution and to minimize cell clumping, then 200 µl of the cell suspension was seeded into each well of the microtiter plates in order from left to right within a plate and from plate 1 to plate 8 or plate 6 using a multipipette. The cells were maintained in the incubator immediately after cell seeding for 48 h.

2.3. MTT colorimetric assay

After this time the medium was aspirated and replaced with fresh medium and the previously prepared extracts. Two controls were used in the test, one with pure medium (Cont1) and another with 50% medium plus 50% D.D. water (Cont2) as the negative control since the sterilized D.D. water was used for the material extracts. Each plate had both controls. The material extracts were diluted 1 + 1 with fresh medium. Each group contained 12 wells. The groups were allocated randomly to the columns of the microtitre plates. The cells were maintained in the incubator for another 24 h after the material extracts were added.

MTT was dissolved at a concentration of 1 mg ml^{-1} in a solution containing 50% PBS and 50% growth medium without phenol red. The growth medium in the microtitre plates was removed and 50 µl of MTT solution was added to each well. The plates were incubated for 4 h at 37 °C, then the MTT solution was removed; 100 µl of iso-propanol was added to each well and the plates gently shaken by hand to ensure dissolution of the blue formazan. They were returned to the incubator for a further 5–10 min before examination in the spectrophotometer. The optical density of each well was measured using an automatic microplate reader (MR700, Dynatech Lab Inc, Europe) with a 670 nm reference wavelength and 570 nm test wavelength. The plates were stored at 4 °C in a refrigerator while waiting to be read to prevent any further colour change.

All the data were collected and examined by the analysis of variance with ONEWAY and DUNCAN statistical procedures using the computer package SPSS^x.

2.4. Qualitative study by MTT assay

Before the isopropanol is added the blue formazan stain is present in those cells that are metabolically active. Examination of these cells by light microscopy allows a qualitative assessment of the cellular response to the extracts or, more importantly, allows examination of the spatial distribution of the cellular response in a direct contact test. These analyses can be compared directly with the data obtained from the spectrophotometer, which is the average value from the entire well.

The same materials were used for this group: Kevlar 29, SiC and PVC. The materials were prepared either by sterilization in the autoclave or by placement in 70% ethanol, and the PVC was sterilized by gamma-radiation as above. The samples were adhered to 35 mm culture dishes with Silastic adhesive (Dow Corning Co, USA). One dish was used as a negative control and contained the adhesive alone. The cells were seeded into the dishes and maintained in the incubator for 4 days. They were observed under the light microscope each day to check the cell growth. On the fourth day, the medium was replaced by 1 ml MTT solution. After a further incubation of 1 h photographs were taken under the light microscope.

3. Results

3.1. MTT assay

The number of cells in suspension after diluting (1 + 7) was approximately 1.1×10^5 per ml and therefore approximately $5.5 \times 10^4/0.2$ ml cells were seeded in each well. The effect of the three factors, the material, the temperature and the extraction time periods were analysed using ONEWAY and DUNCAN statistical packages in SPSS^x.

Fig. 1 shows the comparison of the material extracts of Kevlar, SiC and PVC1 at 37° and 80 °C with two controls. Significant differences are observed between controls 1 and 2 indicating that the distilled water affected the cell growth and therefore all further comparisons of the material extracts will be with the water control. For the extracts prepared at 37 °C, although statistically significant differences were measured between a few of the optical density readings using the Duncan analysis, these materials did not show any significant cytotoxicity in comparison with the distilled water control. At 80 °C no cytotoxicity was

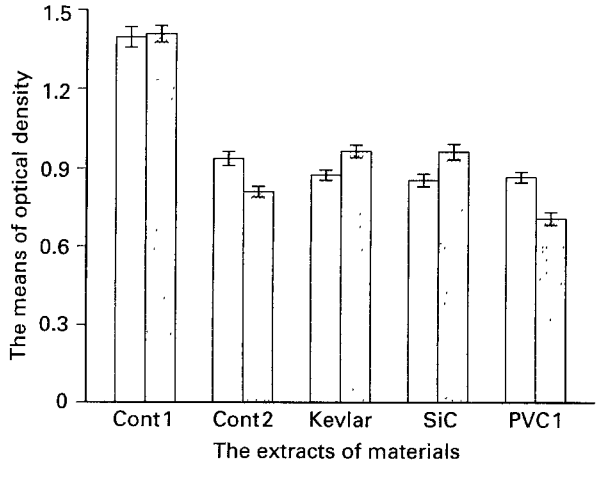

Figure 1 Comparison among three material extracts for the first group of the MTT assay.

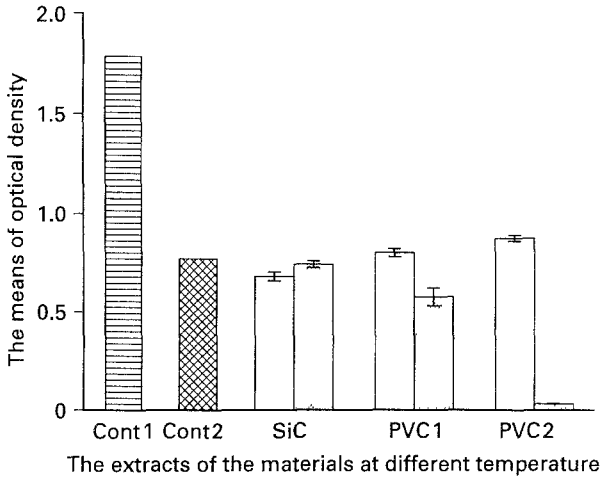

Figure 2 Comparison among three material extracts for the second group of the MTT assay.

observed for the extracts of Kevlar or SiC, however, a slight cytotoxicity is shown due to the extract from PVC1.

Fig. 2 shows the data for the second group of extracts comparing SiC, PVC1 and PVC2, SiC did not show any toxicity as in the first test ($p > 0.05$). PVC1 still gave rise to some toxicity at 80 °C ($p < 0.05$) and a high level of toxicity was observed for the extract of PVC2 at 80 °C ($p < 0.05$). It was observed during test that the extraction of PVC at 80 °C caused the culture medium to become acidic. The 4-week extract for PVC2 was measured to be pH 3.5. These data presented in Figs 1 and 2 are the cumulative data from all four weekly extractions. Figs 3, 4 and 5 present the data for SiC, PVC1 and PVC2, respectively, for each week of extraction.

Fig. 3 demonstrates that SiC did not produce any significant cytotoxicity at any time period or either temperature. Fig. 4 shows that PVC1 only caused a cytotoxic response after 4 weeks extraction at 80 °C. From Fig. 5, however, it is clear that PVC2 causes a significant cytotoxic response after only 1 week at 80 °C.

3.2. Direct contact test

Fig. 6a presents a representative view of the control formazan staining on the cell culture dish showing

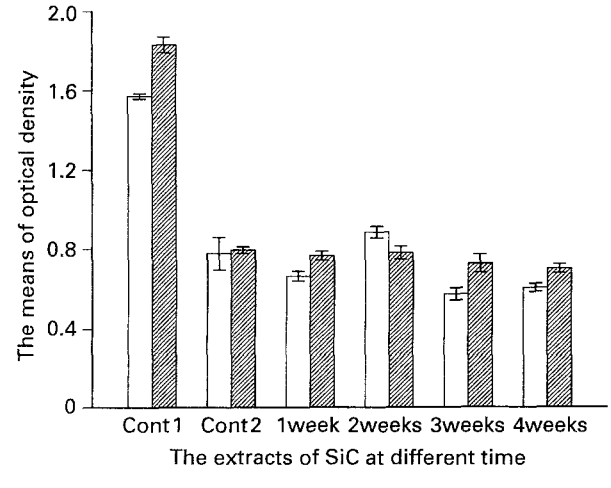

Figure 3 Comparison among four time periods of SiC extracts.

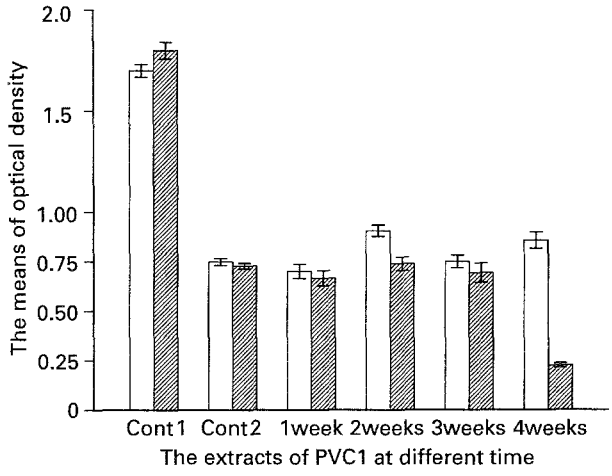

Figure 4 Comparison among four time periods of PVC1 extracts.

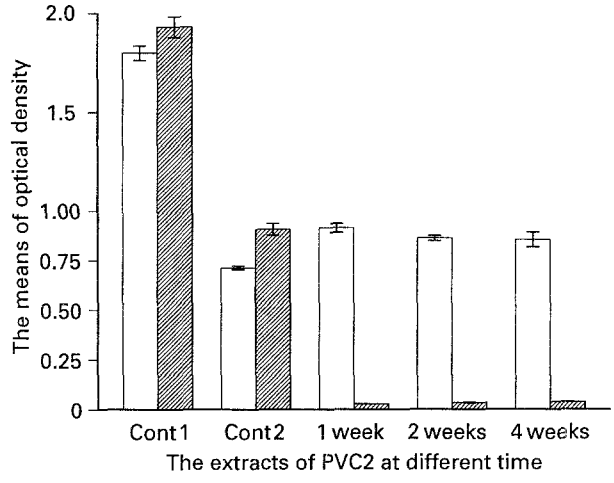

Figure 5 Comparison among four time periods of PVC2 extracts.

many well-stained cells. The cells in Fig. 6b appear very similar to the control and are representative of cells on PVC which had been prepared in 70% ethanol for 30 min. Fewer cells are observed in Fig. 6c, which is representative of the cellular response on the gamma-sterilized PVC. Fig. 6d demonstrates the cytotoxicity of the PVC sterilized in the autoclave with few cells on the materials and those near the edge of the material showing little staining.

Fig. 7 presents representative examples of the direct contact response to the fibre materials. Fig. 7a demonstrates the good cytocompatibility of the Kevlar fibres, with well-stained cells growing all around the fibres. Fig. 7b demonstrates the good cellular response to the SiC after washing in ethanol, and Fig. 7c shows the cytotoxic response to the autoclaved SiC.

4. Discussion

The MTT assay has been proved to be a very sensitive, precise, convenient and rapid test method for the quantitative cytotoxicity evaluation of biomaterials and is now used routinely in our laboratory.

The result for Kevlar in this study proved to be consistent with those reported by Wening and Zimmerman [9, 10] which showed Kevlar to be very stable in the biological environment and to demonstrate good biocompatibility *in vitro*. Wening *et al.* [9]

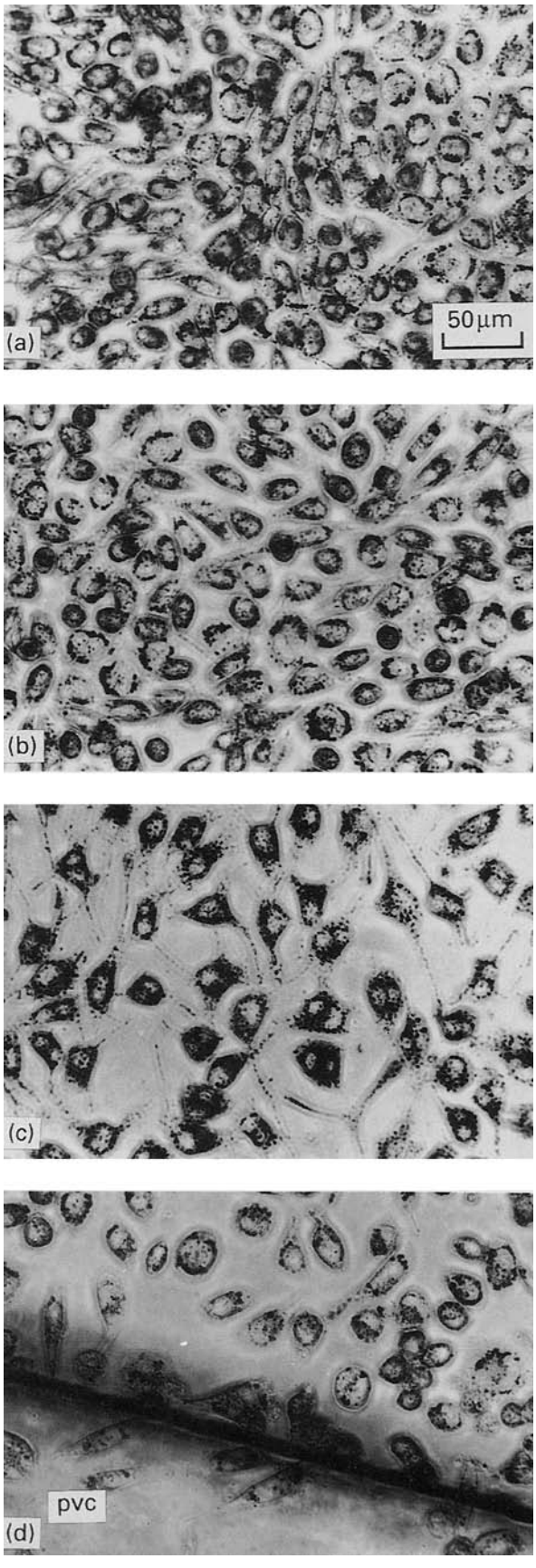

Figure 6 A representative view of PVC prepared in different ways by the MTT assay: (a) control, which shows that the cells are well stained by formazan product; (b) on the surface of PVC prepared in 70% ethanol, which is similar to the control; (c) fewer cells are observed on the surface of gamma prepared PVC; and (d) cytotoxic response to the autoclaved PVC.

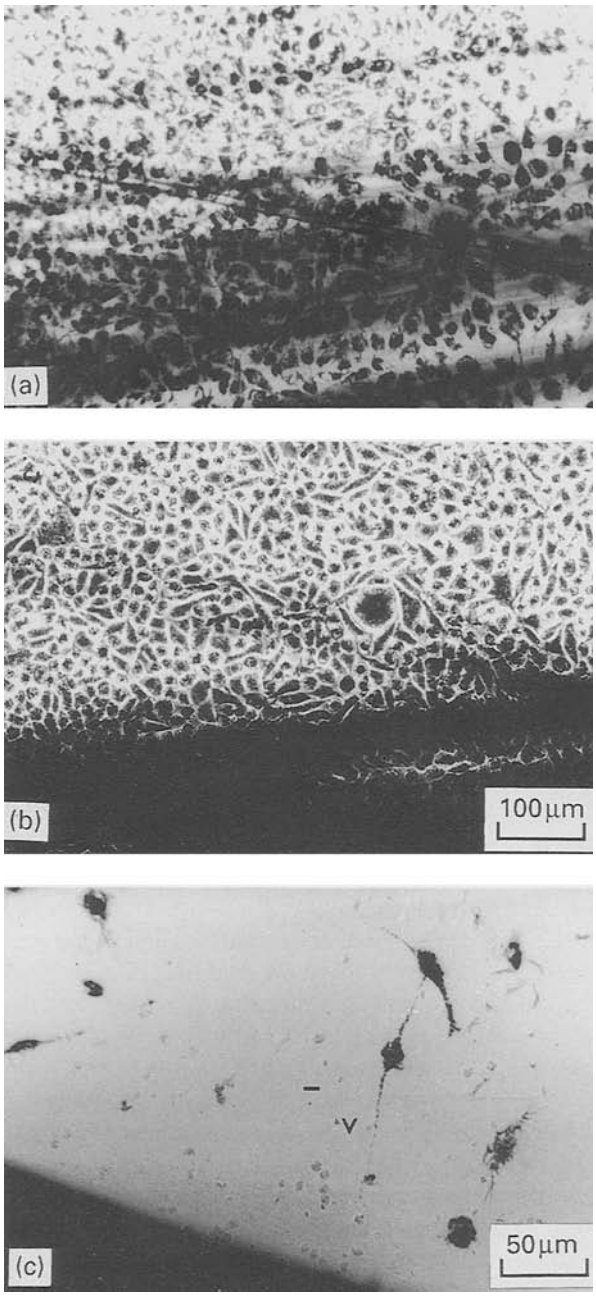

Figure 7 A representative view of the fibre materials by the MTT assay: (a) Kevlar fibres and (b) ethanol-prepared SiC show good cytocompatibility, while (c) SiC sterilized by autoclave gives cytotoxic response.

claimed that incubation in human plasma (blood groups A and O) for up to 26 weeks had no effect upon the surface characteristics of Kevlar fibres. The diameter of the fibres remained unchanged and their surface showed no sign of erosion. Using the rat tendon cell model, Zimmerman *et al.* [10] found that Kevlar was comparable to carbon AS4 and nylon sutures in terms of the cellular response and cell outgrowth rates.

Silicon carbide was found, from the MTT assay results, to show good cytocompatibility. In the direct contact test the SiC also showed good cytocompatibility for the samples which had been washed in 70% ethanol for 30 min, however, considerable cytotoxicity was observed when the material was autoclaved without prior washing in ethanol. This suggests that there

was a substance on the fibre, maybe a processing aid, that was cytotoxic and that it was soluble in ethanol and therefore removed in the washing step. This demonstrates the great importance of material preparation for biomedical use.

Various cellular responses have been observed with the PVC depending on the method of preparation. When the PVC was placed in ethanol prior to extraction or incubation directly with the cells a slightly toxic response was observed only for the samples extracted for 4 weeks at 80 °C. For the PVC samples that were gamma-sterilized it was shown that the 80 °C extractions were all highly toxic although the extractions at 37 °C did not show any toxicity. The PVC treated with ethanol and the gamma-sterilized PVC did not show any significant toxicity in the direct contact test. The PVC that had been autoclaved, however, was shown to be cytotoxic in the direct contact test. PVC is known to show cytotoxicity due to leaching of plasticiser. These data would suggest that the gamma-sterilized (and to a greater extent the autoclaved) PVC were influenced by these processes such as to allow the plasticiser to leach from the materials more easily. It is possible that the ethanol washing step could remove the plasticiser from the surface layer of the PVC and thus if the leaching was diffusion controlled it would take longer for a sufficient concentration of plasticiser to be toxic to be present in the extract. This could explain why there was no toxicity observed until the samples were extracted for 4 weeks.

The question of sterilization of PVC is an important one due to its widespread use in the clinical environment. Although the PVC and SiC that were used in this study was not medical grade it demonstrates the sensitivity of the materials to the method of preparation. This study has demonstrated that Kevlar and SiC which have been cleaned correctly show good cytocompatibility with L929 fibroblasts. It has been shown that with this staining technique the cellular response can be examined both quantitatively and qualitatively, which can give a good overall picture of the events occurring in this *in vitro* situation.

References

1. T. MOSMANN, *J. Immun. Meth.* **65** (1983) 55.
2. F. DENIZOT and R. LANG, *ibid.* **89** (1986) 271.
3. D. GERLIER and N. THOMASSET, *ibid.* **94** (1986) 57.
4. M. FERRARI, M. C. FORNASIERO and A M. ISETTA, *ibid.* **131** (1990) 165.
5. M. ISELT, W HOLTEI and P HILGARD, *Drug Res.* **39 (II)** (1989) 747.
6. T. HONGO, Y. FUJII and Y. IGARASHI, *Cancer* **65** (1990) 1263.
7. D. SGOURAS and R DUNCAN, *J. Mater. Sci. Mater. Med.* **1** (1990) 61.
8. H WAN, R. WILLIAMS, P DOHERTY and D. F. WILLIAMS, *J. Mater. Sci. Mater. Med.* **5** (1994) 154.
9. J. V. WENING and D. E. LORKE, *Clin. Mater* **9** (1992) 1.
10. M ZIMMERMAN and K. E. GORDON, *J. Biomed. Mater. Res.: Appl. Biomater.* **2** (1988) 339.