Effects of baicalin-modified poly(D,L-lactic acid) surface on the behavior of osteoblasts

WEN GUANG LIU, XIAO WEI LI, YONG SHENG LI, KAI YONG CAI, KANG DE YAO*
Research Institute of Polymeric Materials, Tianjin University, Tianjin 300072, People's Republic of China
E-mail: wgliu@tju.edu.cn; wenguangliu@sina.com

ZHIMING YANG, XIUQIONG LI
The First University Hospital, West China University of Medical Science, Chengdu, 610041, People's Republic of China

In the present study, the functions of rat calvaria osteoblasts on baicalin-modified poly(D,L-lactic acid) (PDLLA) films were investigated *in vitro*. The surface characteristics of surfaces (both modified and control) were investigated by water contact angle measurement and electron spectroscopy for chemical analysis (ESCA). Cell morphologies on these surfaces were examined by scanning electron microscopy (SEM). Cell adhesion and proliferation were used to assess cell growth on the modified and control surfaces. The MTT assay was used to determine cell viability and alkaline phosphatase (ALP) activity was performed to evaluate differentiated cell function. Compared to control films, cell attachment of osteoblasts on baicalin-modified PDLLA film was significantly higher ($P < 0.05$ and $P < 0.01$) after 6 h and 8 h culture, and cell proliferation was also significantly greater ($P < 0.05$ and $P < 0.01$) at the end of 4th and 7th day, respectively. The MTT assay suggested that the cell viability of osteoblasts cultured on baicalin-modified PDLLA film was significantly higher ($P < 0.05$) than that seeded on the control. Meanwhile, the ALP activity of osteoblasts cultured on modified films was also considerably enhanced ($P < 0.01$) compared to that found on control. These results revealed that the biocompatibility PDLLA could be improved by surface modification with baicalin.

1. Introduction

The development of tissue engineering has accelerated the demand for biodegradable, and biocompatible materials [1, 2]. Such biomaterials were fabricated into 3D scaffold which served to reinforce and organize the regenerating tissue [3]. Thus, it was necessary for biomaterials to interact intimately with cells and/or hosts. Polyesters including poly(L-lactic acid) (PLA), poly(glycolic acid) (PGA) were such a kind of biomaterials, and they were widely used in tissue engineering field [4–6]. However, the main disadvantage of polyester was the lack of recognition site on its surface. Such defect also existed in other synthetic biomaterials.

Therefore, the rationale for surface modification of biomaterials was straightforward [7]. With well-engineered surface modification, the mechanical properties and functionality of the device would be unchanged, but the biocompatibility, biocognizability or biofunctionality would be enhanced. Following this idea, surface modification could be used to introduce sites on the surface for immobilization of biomolecules or recognition ligands [8–12], or to prevent surface from non-specific interaction or biomolecular adsorption [13, 14].

In the present study, baicalin was initially used to modify PDLLA film. Baicalin was a flavonoid compound purified form Chinese herbal medicinal plant *Scutellaria baicalensis Georgi* (Huang Qin). Many researchers [15–18] have reported its antioxidant activity and anti-inflammatory activity. In addition, Malorni *et al*. [19] has confirmed that antioxidant N-acetyl-cysteine increased the cell adhesion capability. We anticipated the cell affinity of PDLLA surface could be enhanced by baicalin immobilization.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic acid) (PDLLA, 98% L-lactide and 2% D-lactide) was provided by Chengdu Institute of Organic Chemistry, Chinese Academy of Science, with a weight average molecular weight (Mw) of 2.5×10^5. Baicalin was provided by Chengdu Chinese Herbal Medicine Plant, the purity was over 97%.

*Author to whom all correspondence should be addressed.

2.2. Film preparation and immobilization

A 1 g sample of PDLLA was dissolved in 20 ml chloroform, and then the solution was transferred to a 90-mm diameter glass plate to form film by evaporation. The obtained PDLLA films were used as control specimens.

Immobilization of baicalin on PDLLA was carried out according to Quirk *et al*. [20] using an entrapment method. Briefly, a mixture of acetone/water = 3 : 1 was prepared, and the PDLLA films were immersed for 1 h. Then the treated films was transferred to baicalin solution (4%) and immersed for 24 h, and followed by rinsing with distilled water and phosphate buffered saline (PBS). The resultant film was dried and stored.

2.3. Characterization of surface

As an indication of surface energy of the modified polymer surface, water contact angles were determined using the sessile drop method and an image analysis system. The water droplet was about 0.5 µl to prevent gravitational distortion of the spherical profile. Each determination was obtained by averaging the results of at least four measurements.

The surface compositions of the modified PDLLA and control films prepared as above-mentioned were determined by X-ray photoelectron spectroscopy (XPS). XPS analysis was conducted using a Perkin Elmer 5600 ESCA system (PerkinElmer Inc. CA, USA) equipped with a monochromatic AlK_{α} source. Typical pressure in the analysis chamber during spectral acquisition was 10^{-9} Torr. All samples were analyzed at a photoelectron take-off angle of 55°. Setting the hydrocarbon peak maximum in the C1s spectra to 285.0 eV referenced the binding energy scales for the samples.

2.4. Cell culture

Osteoblasts were isolated via sequential collagenase digestions of neonatal rat calvaria according to established protocol [21]. They were cultured at 37 °C in a humidified atmosphere of a 5% CO_2 in air, in 50 cm^2 flasks containing 5 ml Dulbecco's modified Eagle medium (DMEM; Gibco), 10% fetal bovine serum (FBS; Gibco). The medium was changed every three days and for sub-culture; the cell monolayer was washed twice with PBS and incubated with trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA; Gibco) for 10 min at 37 °C to detach the cells. Osteoblasts at the fourth passage were used in the experiments.

2.5. Cell morphology

For morphological observation, osteoblasts were seeded on baicalin-modified PDLLA and control films at a density of 40 000 cells/cm^2 (equivalent to approx. 100% confluency). Cells cultured on the baicalin-modified and control films were examined by scanning electron microscopy (SEM, AMRAY, USA). The cells adhered on the films were washed with PBS after 12 h of incubation, and then fixed with 2.5% glutaraldehyde in PBS for 1 h at 4 °C. After washing with PBS, the cells were dehydrated through a series of graded alcohol and then dried. The films were then gold sputtered in vacuum and examined by SEM observation with 300 × magnification.

2.6. Cell attachment

To determine the attachment kinetics of cell adhesion, osteoblasts were seeded onto the modified PDLLA and control films with a density of 40 000 cell/cm^2 (equivalent to approx. 100% confluence) in 24-well plate. The cells were allowed to attach on the films undisturbedly in a humidified incubator (37 °C and 5% CO_2) for 2, 4, 6 and 8 h, respectively. At each time point, plates were rinsed twice with PBS and then stained *in situ* with Commassie Brilliant Blue staining solution; adherent cells (on each of five random fields per substrate) were thus visualized and counted using a light microscope.

2.7. Proliferating assay

Osteoblasts were seeded in 24-well plate, at a density of 30 000 cell/cm^2 (equivalent to approx. 75% confluency) onto the modified and control films. The cells were maintained in complete medium being refreshed every 2 days. Assay was performed at 1, 4 and 7 days, respectively. At each time interval, plates were rinsed twice with PBS and then stained *in situ* with Commassie Brilliant Blue staining solution; adherent cells (on each of five random fields per substrate) were thus visualized and counted using a light microscope.

2.8. MTT assay

The MTT assay was used as a measurement of relative cell viability. After the osteoblasts were cultured in 24 well for 7 days, the cell viability was evaluated using the MTT assay (MTT; Sigma), in which 100 µl of MTT (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. After the culture, the blue formazan reaction product was dissolved by adding 0.5 ml DMSO and transferred to a 96 well plate. The absorbance was measured at 570 nm using a Bio-Rad 550 spectrophotometric microplate reader (Bio-Rad Laboratories, USA).

2.9. Total intracellular protein content

This procedure was performed according to Webster [22] in a six well plate. Osteoblasts (30 000 cell/cm^2) were seeded onto the modified PDLLA and control films, and then cultured in DMEM with 10% fetal bovine serum under cell culture conditions for 7 days. At the end of the prescribed time interval, osteoblasts were washed twice with PBS and then harvested in 1.0 ml double-distilled water with a disposable cell scrapper (Costar), sonicated in ice bath for 10 min, and then centrifuged for 10 min at 2000 rpm. The supernatant was used for determining the total protein content. Total protein content in the cell lysates was determined spectrophotometrically using a commercially available kit. Light absorbance of these samples was measured at 570 nm on a Bio-Rad 550 spectrophotometric microplate reader. Total intracellular

protein (expressed as mg) synthesized by osteoblasts cultured on the films of interest was determined from a standard curve of absorbance vs. known concentration of albumin run in parallel with experimental samples.

2.10. Alkaline phosphatase assay

In this section, osteoblasts were cultured in a six well plate which was covered with modified PDLLA and control films. After removal of the culture medium, the cell layer was washed twice with PBS, harvested in 1.0 ml double-distilled water with a disposable cell scrapper (Costar), sonicated in ice bath for 10 min, and then centrifuged for 10 min at 2000 rpm. The supernatant was used for determining the ALP activity, according to Otto [23] with paranitrophenyl phosphate (Sigma) as a substrate. The absorbance at 405 nm was measured via a spectrophotometer in a 96-well microplate reader (Bio-Rad 550). The ALP activity (expressed as μmols of converted p-nitrophenol/min) was normalized by total intracellular protein synthesis (determined as described in the total intracellular protein content section) and thus expressed as μmols p-nitrophenol/min/mg protein. ALP activity of osteoblasts cultured on the untreated PDLLA films served as controls.

2.11. Statistical analysis

Experiments were run in quadruplicate per sample. All data were expressed as means $\pm$ standard deviation (SD) for $n = 4$. Single factor analysis of variance (ANOVA) technique was used to assess statistical significance of results. Scheffe's method was employed for multiple comparison tests at level of 95% and 99%.

3. Results and discussion

3.1. Characterization of films

Water contact angle measurement can evaluate the hydrophobicity and hydrophilicity of films. Measurement of contact angles on the baicalin-modified surfaces gave an indication of the relative hydrophilicity of these surfaces after the modification procedure. The water contact angle of baicalin-modified PDLLA and control film is listed in Table I. It indicated that the contact angle of modified PDLLA film was significantly lower (more hydrophilic) than that of control ($P < 0.01$). This might be related to the water-soluble nature of baicalin onto the modified film surfaces.

Table II lists the atomic concentration of surfaces modified with baicalin. The main surface components were C and O. The oxygen content of PDLLA surface modified with baicalin decreased slightly. From the data, it was possible to estimate the ratio of baicalin units to lactic acid units at the polymer surface. Using the C/O ratio of pure baicalin (1.91), compared to that of baicalin-modified PDDLA surfaces (C/O = 1.37), the baicalin surface coverage was estimated as 71.7%.

TABLE I Water contact angles on PDLLA surfaces modified with baicalin[a]

Sample	Contact angles (degree)
PDLLA	69 $\pm$ 3
Baicalin-modified PDLLA film	50 $\pm$ 3

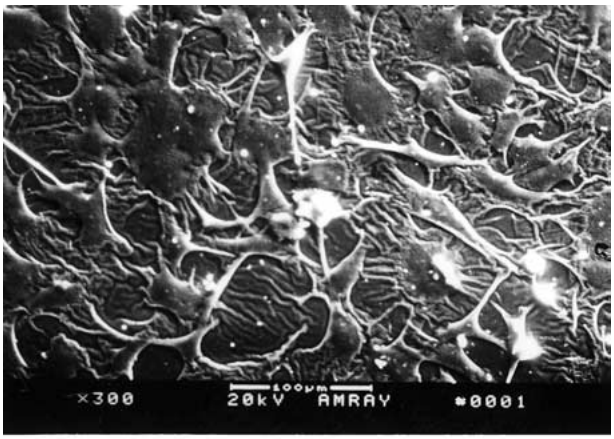
[a] Average $\pm$ standard deviation ($n = 4$).

TABLE II Elemental composition of PDLLA film modified with baicalin

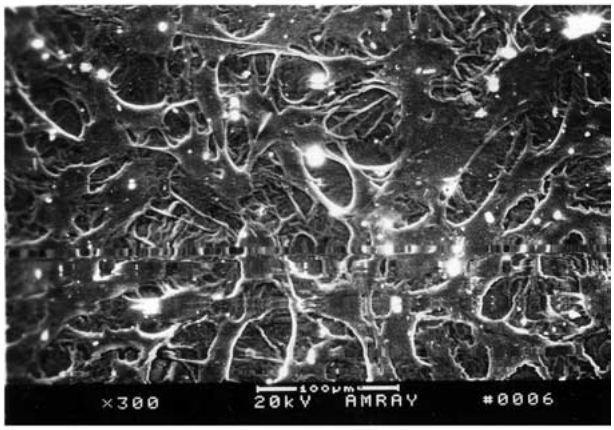
Sample	Composition (%)		
	C	O	C/O
PDLLA film	55.8	44.2	1.26
Baicalin-modified PDLLA film	57.8	42.2	1.37

3.2. Cell morphology

Fig. 1 showed the morphology of osteoblasts cultured on baicalin-modified PDLLA and control film. No obvious differences in morphology were noted between the cells grown on the baicalin-modified PDLLA and control film, even though the cells adhered on baicalin-modified films were even more flattened than those on control. However, the number of cells adhering on baicalin-modified film was slightly increased than that on control.



(a)



(b)

Figure 1 SEMs of osteoblasts cultured directly on (a) PDLLA film; and (b) baicalin-modified PDLLA film after 12 h incubation (initial seeding density was 40 000cells/cm^2).

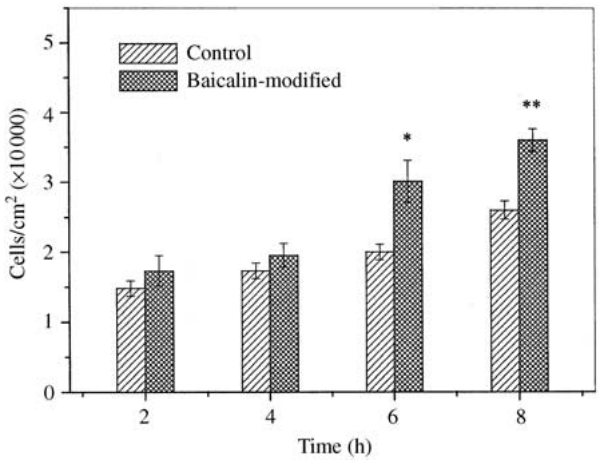


Figure 2 Attachment of osteoblasts to baicalin-modified PDLLA and control films increased over an 8 h time frame. Initial seeding density was 40 000 cells/cm^2. Error bars represent means $\pm$ SD for $n = 4$. *$P < 0.05$; **$P < 0.01$ (compared to the control).

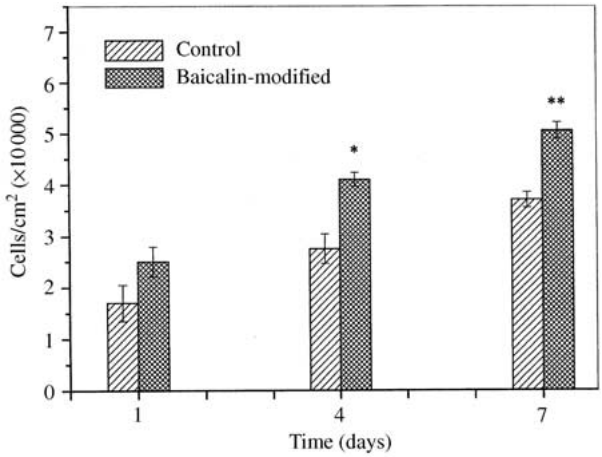


Figure 3 Proliferation kinetics of osteoblasts culture on baicalin-modified PDLLA and control films. Initial seeding density was 30 000 cells/cm^2. Error bars represent means $\pm$ SD for $n = 4$. *$P < 0.05$; **$P < 0.01$ (compared to the control).

3.3. Cell attachment

To assess cellular adhesion, films were seeded with a confluency number of cells. More of rat osteoblasts were attached on baicalin-modified PDLLA films as compared to PDLLA controls (Fig. 2) in a period of 8 h. At the time intervals of 2 and 4 h, there were no significant differences between baicalin-modified PDLLA and control films. However, at the time points of 6 and 8 h, the number of cells attached on baicalin-modified PDLLA sample showed statistical difference ($P < 0.05$ and $P < 0.01$, respectively) as compared to control film.

During cell culture, a critical aspect of cell behavior is the adhesive interactions of cells with other cells or with the extracellular matrix [24]. The adhesion pathways were associated with numerous cell alterations, such as the focal adhesions, and specific changes in cytoskeleton network organization. However, cytoskeleton changes were strongly related to the redox status of the cell. Consequently, during inflammation and the chemical-induced alternation in redox status of cells, i.e. the intracellular balance between pro-oxidants and anti-oxidants, a change of their adhesive behavior via cytoskeleton modification could occur. Fiorentini [25] *et al*. confirmed that N-acetylcysteine could protect epithelial cells against the oxidative imblance, thus promoting their growth. The results of our study are in agreement with it.

3.4. Cell proliferation

Osteoblast proliferation on baicalin-modified PDLLA and control films was tested after 1, 4 and 7 days culture (Fig. 3). The cell number attached on the modified PDLLA film after 4 and 7 days culture was significantly greater ($P < 0.05$ and $P < 0.01$) than that on control. Although the cell number of osteoblasts attached on baicalin-modified films was slightly higher than that on control after 1 day culture, no significant difference was observed ($P > 0.05$).

It was generally agreed that the hydrophobicity/hydrophilicity of a film surface could affect the degree of cell adhesion [26, 27]. Lampin *et al*. reported that the hydrophiliclty of a film was believed to be a factor affecting the surface energy (surface tension). Surface energy (tension) of materials might influence the adsorption of serum proteins, and in turn governing the biological response, such as cell adhesion and proliferation. Our study implied that the hydrophilicity of film surface was correlated to the degree of osteoblast proliferation to some extent.

3.5. Cell viability

In the present study, MTT assay was used to evaluate the relative cell viability. MTT was a pale yellow substrate (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide), which would be reduced by living cells to a dark blue formazan. This process required active mitochondria and it was an accurate measure of the viability of cells in culture. As shown in Fig. 4, the present study provided the evidence of cell viability using MTT assay after 7 days culture at an initial cell density of 30 000 cells/cm^2. The result demonstrated that the cell viability of osteoblasts cultured on baicalin-

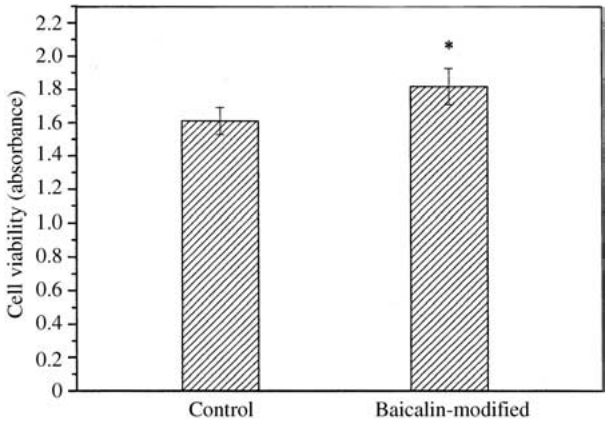


Figure 4 MTT assay. Formosan absorbance expressed as a measure of cell viability from osteoblasts seeded onto baicalin-modified PDLLA and control films. Error bars represent means $\pm$ SD for $n = 4$. (Initial seeding density was 30 000cells/cm^2.) *$P < 0.05$ (compared to the control).

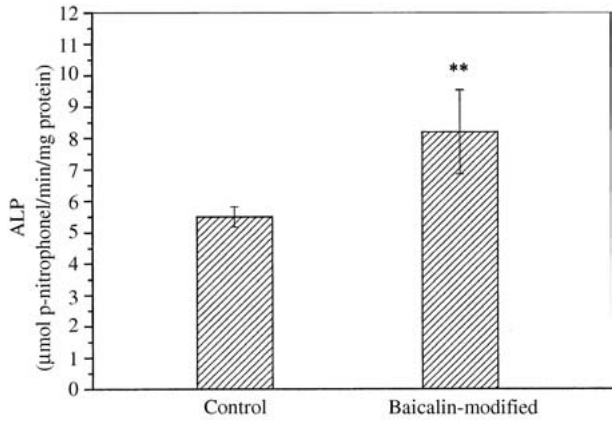


Figure 5 Alkaline phosphatase activity of osteoblasts seeded on baicalin-modified PDLLA and control films for 7 days. Error bars represent means ± SD for $n = 4$. (Initial seeding density was 30 000 cells/cm^2.) $^{**}P < 0.01$ (compared to the control).

modified PDLLA films was remarkably increased $(P < 0.005)$ than on the control.

3.6. Alkaline phosphatase (ALP) activity

The alkaline phosphatase activity was tested after osteoblast proliferated for 7 days in 6-well plate. Fig. 5 exhibited the total alkaline phosphatase activity of osteoblasts cultured on both baicalin-modified PDLLA and control films. It revealed that the ALP activity of osteoblasts seeded on baicalin-modified PDLLA surface was significantly greater $(P < 0.001)$ than that on control. The result indicated that baicalin had an effect on osteoblast differentiation function. From the above data, it was reasonable to consider that baicalin was a promising material for surface modification of either 2D surface or 3D bone reconstruction scaffolds and fixation devices, and warranted further long-term *in vivo* studies.

4. Conclusion

In this study, baicalin was successfully introduced to modify PDLLA films with an entrapment method. The water contact angle measurement suggested that it had increased the hydrophilicity of PDLLA surfaces, and ESCA analysis confirmed that baicalin-modified surface had changed the chemical composition of PDLLA surface. The cellular *in vitro* model exhibited the effects of viability. More importantly, baicalin-modified PDLLA surface could enhance osteoblast differentiation function, as demonstrated by ALP activity assay. Our study suggested that baicalin could be useful for surface modification of biomaterials as a bioactive moiety. It was reported that the oxidative state of actin is critical for its organization [25]. While the antioxidative activity of baicalin might buffer those effects strictly based on cytoskeletal oxidation. Thus, baicalin could inhibit the side-by-side linking of actin filament that was typical of cell injury processes leading to retraction, rounding and blebbing. In summary, antioxidative baicalin could protect osteoblasts against the oxidative imbalance, thus promoting their cellular behavior.

Acknowledgment

This work was financially supported by the National Basic Science Research and Development Grants (973) via grant G199954035.

References

1. J. A. HUBBELL, *Biotechnology (NY)* **13** (1995) 565.
2. S. J. PETER, M. J. MILLER, A. W. YASKO, M. J. YASZEMSKI and A. G. MIKOS, *J. Biomed. Mater. Res.* **43** (1998) 422.
3. S. W. MADIHALLY and H. W. T. MATTHEW, *Biomaterials* **20** (1999) 1113.
4. Y. S. NAM and T. G. PARK, *ibid.* **20** (1999) 1783.
5. D. J. MOONEY, D. R. BALDWIN, N. P. SUH, J. P. VACANTI and R. LANGER, *ibid.* **17** (1996) 1417.
6. H. LO. S. KADIYALA, S. E. GUGGINA and K. W. LEONG, *J. Biomed. Mater. Res.* **30** (1996) 475.
7. B. D. RATNER, *Biosensors Bioelectron.* **10** (1995) 797.
8. J. M. BRYNAER, E. DETRAIT, O. NOISER, T. BOXUS and Y. SCHNEIDER, *Biomaterials* **20** (1999) 1773.
9. A. REZANNIA and K. E. HEALY, *Biotechnol. Prog.* **15** (1999) 19.
10. J. A. NEFF, P. A. TRESCO and K. D. S. CALDWELL, *Biomaterials* **20** (1999) 2377.
11. R. A. QUIRK, W. C. CHAN, M. C. DAVIES, S. J. TENDLER and K. M. SHAKESHEFF, *ibid.* **12** (2000) 865.
12. H. SOH, Y. S. HWANG, J. E LEE, C. D. HAN and J. C. PARK, *ibid.* **22** (2001) 219.
13. Y. J. KIM, I. K. KANG, M. W. HUB and S. C. YOON, *ibid.* **21** (2000) 121.
14. S. B. JO and K. PARK, *ibid.* **21** (2002) 605.
15. T. B. NG, F. LIU and Z. T. WANG, *Life Science* **66** (2000) 709.
16. Z. H. GAO, K. X. HUANG, X. L. YANG and H. B. XU, *Biochim. Biophys. Acta* **1472** (1999) 643.
17. B. Q. LI, T. FU, W. H. GONG, N. DUNLOP, H. F. KUNG, Y. D. YAN, J. KANG and J. M. WANG, *Immunopharmacology* **49** (2000) 295.
18. T. KRAKAUER, B. Q. LI and H. A. YOUNG, *FEES Lett.* **500** (2001) 52.
19. W. MALORNI, P. MATARRESE, R. RIVABENE, S. PARADISI and G. DONELLI, *Biomaterials* **17** (1996) 921.
20. R. A. QUIRK, M. C. DAVIS, S. J. B. TENDLER and K. M. SHAKESHEFF, *Macromolecules* **33** (2000) 258.
21. D. A. PULEO, L. A. HOLLERAN, R. H. DOREMUS and R. BIZIOS, *J. Biomed. Mater. Res.* **25** (1991) 711.
22. T. J. WEBSTER, C. ERGUN, R. H. DOREMUS and R. W. SIEGEL, *Biomaterials* **21** (2000) 1803.
23. D. E. OTTO, J. K. NULEND, P. PATKA, E. H. BORGER and H. J. TH. M. HAARMAN, *J. Biomed. Mater. Res.* **32** (1996) 513.
24. R. L. JULIANO and J. A. VARNER, *Curr. Opin. Cell Biol.* **5** (1993) 812.
25. C. FIORENTINI, L. FALZANO, R. RIVABENE, A. FABBRI and W. MOLORNI, *FEBS Lett.* **453** (1999) 124.
26. M. LAMPIN, L. C. WAROPUIER-CLEROUT, M. DEGRANGE and M. F. SIGOT-LUIZARD, *J. Biomed. Mater. Res.* **36** (1997) 99.
27. G. CHANG, D. R. ABSOLOM, A. B. STRONG, G. D. STUBLEY and W. ZINGG, *ibid.* **22** (1998) 13.

Received 11 September 2002
and accepted 9 July 2003