Journal of Chromatography, 572 (1991) 41–49
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6072

Determination of cholesterol and cortisone absorption in polyurethane

I. Methodology using size-exclusion chromatography and dual detection

J. G. DILLON* and M. K. HUGHES

Food and Drug Administration, Center for Devices and Radiological Health, Office of Science and Technology, Division of Mechanics and Materials Science, 12200 Wilkins Avenue, Rockville, MD 20852 (USA)

(First received April 24th, 1991; revised manuscript received June 26th, 1991)

ABSTRACT

A size-exclusion chromatographic method is described for measuring the absorption of the steroid-based lipids cholesterol and cortisone into Pellethane 2363, a polyurethane used in biomedical implants. The method uses refractometry and ultraviolet diode-array detection, with tetrahydrofuran as the mobile phase. Using an injection volume of 150 μl, the lower limit of accurate measurement for cholesterol (refractive index detection) was 6 μg/ml with a lower limit of detection, based on a 2:1 signal-to-noise ratio, of 0.15 μg (1 μg/ml). For cortisone (ultraviolet detection), the lower accurate limit was 0.6 μg/ml with a lower limit of 0.015 μg (0.1 μg/ml). The results show that after 44 h, 2037 μg/g cholesterol and 3131 μg/g cortisone were absorbed by the polyurethane. The method eliminates extensive sample manipulation and is sensitive to low levels of lipid in the presence of a high-molecular-mass synthetic polymer.

INTRODUCTION

The use of many different plastic materials in implantable devices is now widespread. Despite this, the influence of the biological environment on the long-term properties of the plastic has received little attention. Early use of the synthetic polymer silicone in the heart valve poppet revealed absorption of fatty acids, neutral fats, steroids, steroid esters, phospholipids, monoglycerides, glycolipids, and lipoproteins [1]. The absorption of these biological components resulted in discoloration, weight gain, swelling, wear, and splitting of the poppet leading to serious valve malfunction [2]. The authors of a more recent report suggest that these naturally occurring biological components may influence the mechanical properties of polyurethane, another type of elastomeric polymer intended for blood pump applications [3]. Some investigators also believe that the thrombosis and hemostasis mechanism at the blood–polymer interface can be affected by these components [4].

0378-4347/91/$03.50

To better understand the chemical interaction between these diverse biological components and blood-contacting implants, we have undertaken the first of several studies to measure the absorption of two steroid-based lipids, cholesterol and cortisone, into a polyurethane, Pellethane 2363.

Cholesterol and cortisone were chosen because they are naturally occurring and represent a range in chemical polarity, a factor probably important in predicting biological component–polymer interaction. Although serum cholesterol is part of a much larger and more complex lipoprotein carrier, its singular presence in the carrier micelle may be relevant to the process of degradation. Work by Tonyali *et al.* [5] indicates that an enhancement of polymer degradation occurs if a low-molecular mass molecule capable of plasticizing the polymer is first solubilized by surrounding molecules thus forming a micelle. In serum, cholesterol may serve as the low-molecular-mass plasticizer while low-density (LDL) and high-density (HDL) lipoproteins serve as the solubilizing agent transporting the cholesterol into the polymer. Hence knowing the amount and rate of cholesterol (and cortisone) absorption may provide one aspect of the experimental verification needed for theoretical models being developed in our laboratory. It is hoped that these models could predict the resistance or lack of resistance of a polymer to components in the biological system.

Additional incentive for this choice has been provided recently by Sharma and Chandy [6] who concluded that the prolonged use of steroids (or the estrogen-containing oral contraceptive agents) may not be advisable for patients having an artificial implant in contact with blood.

Pellethane 2363 was selected as representative of a wide class of biomedical polymers known as polyurethanes. Specifically, Pellethane 2363 is a polyether-urethane whose compositional variation, represented by the designations 80A and 55D, also allows the study of varying phase composition on chemical absorption.

Several factors were considered in picking the method for measuring the amount of cholesterol and cortisone absorbed. Because of the many analyses required to measure the absorption over an extended time, it was necessary to keep sample manipulation before and after lipid uptake to a minimum. These restrictions made pre- or post-derivatization and solvent extraction procedures less desirable although both have been used to isolate cholesterol and cholesterol esters from serum [7–9]. The presence of the high-molecular-mass polyurethane component (3000–1 600 000 daltons) eliminated thin-layer and gas chromatography as alternatives. Additionally, the simultaneous absorption of water, the small sample size (4 mg), and the estimated minimum lipid absorption (0.1%) eliminated the use of weight difference to measure the amount absorbed. Nominal estimates of 0.1% absorption led to the need for a minimum detection level of 0.6 μg for cortisone or cholesterol in the presence of a high-molecular-mass component one thousand times more concentrated.

Size-exclusion chromatography (SEC) was selected as the best method to sep-

arate the high-molecular-mass polyurethane from the absorbed lipid. Early work by Nystrom and Sjovall [10] showed that low-molecular-mass hydrophilic polymers could be separated from lipid-soluble compounds by gel permeation using mixed solvent systems. Gas flow counter detection of ^{14}C-labeled compounds, titrimetric, or gravimetric analysis was used depending on the component of interest. In the present study, dual detection was chosen coupling refractive index (RI) and photodiode-array detectors to measure quantitatively the uptake of the low UV-absorbing cholesterol and UV-absorbing cortisone, respectively. All components were soluble in the UV-transparent solvent tetrahydrofuran (THF).

This paper describes, to our knowledge, the first quantitative assessment of steroid-based lipid absorption into a biomedical plastic using SEC separation and dual detection. The method eliminates extensive sample manipulation and is sensitive to low levels of lipid in the presence of a high-molecular-mass component.

EXPERIMENTAL[a]

Materials and reagents

Dimensionally uniform, custom-extruded Pellethane 2363-80A and 2363-55D tubing (Medical Profiles, Livona, MI, USA) were used to minimize sample variability. The tube was thin-walled (0.2 mm) to decrease the time to achieve chemical equilibrium with the surrounding lipid media. The use of the small sample size would also insure that the solution concentration of relatively insoluble lipid was always in excess and would not limit absorption. A 3 mm tube length resulted in a sample size of approximately 4 mg and a geometrical surface area of 0.22 cm^2.

Cholesterol (Aldrich, Milwaukee, WI, USA) was purified by recrystallization from ethanol [11]. Cortisone (Alltech Assoc., Deerfield, IL, USA), sodium cholate (Aldrich) and pH 7.6 phosphate-buffered saline (Sigma, St. Louis, MO, USA) were used as received. THF (Burdick and Jackson, Baxter Scientific Products, McGaw Park, IL, USA) was filtered, vacuum-degassed and continuously sparged with helium. To minimize competitive absorption, the number of ancillary components in the lipid solution was kept to a minimum. However, due to the near insolubility of cholesterol in water, sodium cholate was used as a solubilizing agent. The cholesterol solubilizing solution was prepared according to the method of Sugihara *et al.* [12] with substitution of phosphate buffer for the borate buffer described. Blank runs of the solution and buffer alone showed no absorbance at the monitoring wavelength chosen (see below). The concentration of cholesterol in the resulting emulsion was 1.65 mg/ml. A saturated solution of cortisone in physiologic saline contained 0.25 mg/ml cortisone. All solutions were kept refrigerated following preparation.

---

[a] The opinions or assertions about specific products identified by brand name contained herein are the private views of the authors and are not to be construed as conveying either an official endorsement or criticism by the U.S. Department of Health and Human Services or the Food and Drug Administration.

Instrumentation

A Waters Model 510 pump was used with a WISP automatic sample injector, Model 410 refractometer (sensitivity 64, scale factor 20), and Model 990+ UV photodiode-array detector (acquisition duration 28 min, data acquisition rate 0.2 pts/s) (all Waters, Milford, MA, USA). The columns (300 mm × 7.8 mm) were one Waters Ultrastyragel linear "mixed bed", one 50 nm pore size and one 10 nm pore size hooked in series. The mobile phase was THF at a flow-rate of 1.0 ml/min. The column oven and refractometer temperatures were 40°C and the injection volume was 150 μl. Based on examination of the individual contour plots of UV absorption, a monitoring wavelength of 212 nm for cholesterol or 222 nm for cortisone was selected. The UV signal was sent, concurrent with the RI signal, to a Maxima 820 (Waters) data collection system (data acquisition rate 0.45 pts/s) for post-run peak integration.

Sample preparation

Individual Pellethane rings were cut with a scalpel and weighed. The rings were placed into 4-ml glass vials and covered with approximately 2 ml of either the cholesterol or cortisone solution. The vials were rotated (Lab-Line Orbit shaker) at 37°C for the desired time. Upon removal, each ring was placed in a fine-meshed basket, washed in 300 ml of stirred fresh deionized water for 2 min, transferred to a 4-ml WISP vial and dried under vacuum overnight. A 1-ml aliquot of THF was then added to each vial to dissolve the ring and absorbed lipid. Following dissolution, the solution was filtered through a 0.5-μm Millex-SR (Millipore) PTFE filter. Approximately 0.3 ml of the filtered solution was placed into a 0.3-ml glass insert for analysis. A single injection of each sample was carried out with a minimum of three samples per time period. The high concentration of polyurethane (0.4%, w/w), although off scale during UV analysis, was chosen to maximize the cortisone or cholesterol signal.

Instrument calibration

Molecular mass calibration of the column set was carried out using polystyrene narrow-molecular-mass standards (Waters and Supelco, Bellefonte, PA, USA) in THF. Standard solutions containing cholesterol or cortisone at concentrations equivalent to 400, 200, 100, 25, 6.25, and 3.125 μg/ml were prepared in THF by dilution from stock solutions in THF. The stock solutions contained 800, 800, 0.8, and 0.8 mg/ml cholesterol, cortisone, Pellethane 80A and 55D respectively. Volumes were adjusted to a constant concentration of polyurethane of 0.4 mg/ml. Three injections (150 μl) of each concentration were analyzed.

RESULTS

The column calibration curve obtained with polystyrene standards is shown in Fig. 1. The chromatograms for cholesterol and cortisone in the presence of Pel-

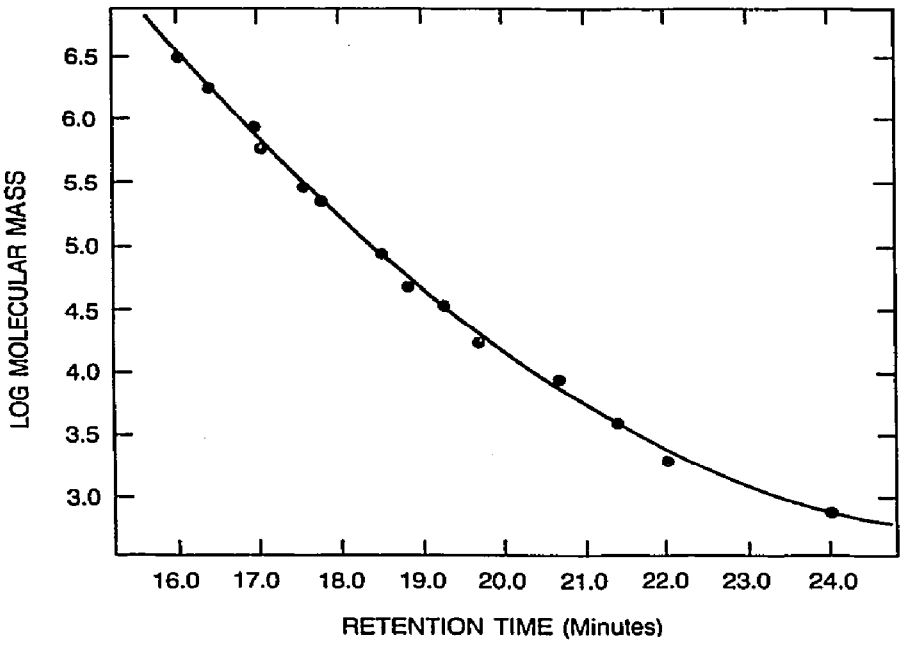

Fig. 1. Molecular mass calibration curve.

lethane 2363-80A are shown in Fig. 2. The cholesterol RI calibration curves were linear between 3.125 and 400 μg/ml with correlation coefficients of 0.99989 for cholesterol (n = 18) in the presence of Pellethane 2363-80A and 0.99959 (n = 18)

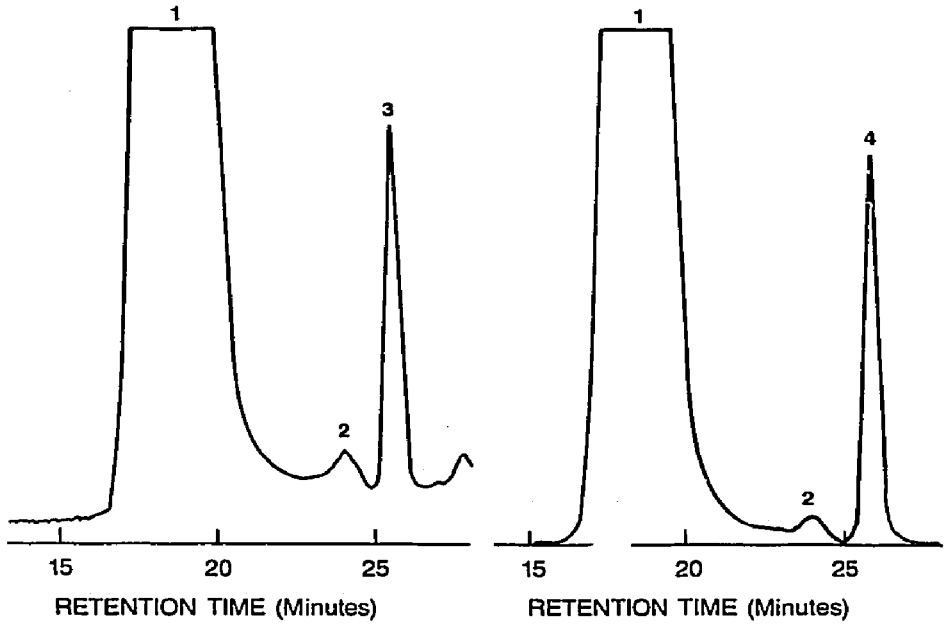

Fig. 2. Separation of cholesterol (left, 400 μg/ml, peak 3) and cortisone (right, 400 μg/ml, peak 4) in the presence of Pellethane 2363-80A (0.4%, w/v, peak 1) and an unidentified additive (peak 2). Chromatographic conditions: columns, 300 mm × 7.8 mm Ultrastyragel linear, one 50 nm pore size and one 10 nm pore size in series; mobile phase, THF; flow-rate, 1.0 ml/min; detector, refractometer (left), UV photodiode-array (right), 222 nm; temperature, 40°C.

TABLE I

ABSORPTION OF CHOLESTEROL AND CORTISONE IN PELLETHANE 2362-80A

Values in parentheses are relative standard deviations (%).

Time (h)	Cholesterol		Cortisone	
	Concentration (mean ± S.D.) (μg/g)	Uptake (%)	Concentration (mean ± S.D.) (μg/g)	Uptake (%)
1	995 ± 110 (11)	0.10	303 ± 46 (15)	0.03
1.5	1492 ± 149 (10)	0.15	737 ± 55 (7.4)	0.07
3	1690 ± 142 (8.4)	0.17	1244 ± 54 (4.4)	0.12
9	2010 ± 101 (5.1)	0.20	1542 ± 70 (4.6)	0.15
21	2043 ± 184 (9.0)	0.20	2220 ± 105 (4.7)	0.22
32	2151 ± 238 (11)	0.22	2496 ± 322 (13)	0.25
44	2037 ± 164 (8.1)	0.20	3131 ± 244 (7.8)	0.31

with Pellethane 2363-55D. Similarly, the cortisone UV curves were linear between 3.125 and 200 μg/ml with correlation coefficients of 0.99941 in the presence of 80A and 0.99996 with 55D. The relative standard deviation (R.S.D., $n = 3$) for each calibration concentration ranged from 0.3 to 3.3%, increasing with decreasing concentration. Using an injection volume of 150 μl, the lower limit of accurate measurement for cholesterol (RI detection) was 6 μg/ml with a lower limit of detection (sensitivity), based on a 2:1 signal-to-noise ratio, of 0.15 μg (1 μg/ml). For cortisone (UV detection), the lower accurate limit was 0.6 μg/ml with a lower limit of 0.015 μg (0.1 μg/ml).

A summary of the absorption of cholesterol and cortisone with time is shown in Tables I and II. After 44 h, 2037 μg/g cholesterol and 3131 μg/g cortisone were

TABLE II

ABSORPTION OF CHOLESTEROL AND CORTISONE IN PELLETHANE 2363-55A

Values in parentheses are relative standard deviations (%).

Time (h)	Cholesterol		Cortisone	
	Concentration (mean ± S.D.) (μg/g)	Uptake (%)	Concentration (mean ± S.D.) (μg/g)	Uptake (%)
1	1018 ± 18 (1.8)	0.10	263 ± 29 (11)	0.03
1.5	1167 ± 40 (3.4)	0.12	366 ± 40 (10.8)	0.04
3	1277 ± 100 (7.8)	0.13	435 ± 33 (7.7)	0.04
9	1662 ± 195 (12)	0.17	550 ± 69 (13)	0.06
21	1873 ± 211 (11)	0.19	744 ± 47 (6.3)	0.07
32	1864 ± 192 (10)	0.19	957 ± 103 (11)	0.10
44	1805 ± 202 (11)	0.18	922 ± 54 (5.8)	0.09

found in Pellethane 2363-80A. Pellethane 2363-55D, following 44 h in cholesterol and cortisone solution, was found to contain 1805 and 922 μg/g, respectively.

DISCUSSION

SEC was effective in separating the absorbed lipid component from the main Pellethane peak and from what appears to be an additive at 24.26 min (RI detector) (R = 1.3 and 1.6 for cholesterol and cortisone, respectively) thus making peak integration easier. A comparison of the retention times for cholesterol (25.48 min) and cortisone (25.97 min) (both RI detector) indicate partial separation (R = 0.6) based on a molecular mass difference of 26 although non-size-exclusion effects, such as adsorption, cannot be excluded.

The use of diode-array detection with contour plotting capability proved very beneficial allowing the selection of the optimum wavelength for cortisone detection thus minimizing interference from the low-molecular mass tail of the main polyurethane peak and coeluting polymer additives.

The minimum detection level for cholesterol obtained in this study (0.15 μg) is comparable to or significantly better than those previously reported using reversed-phase liquid chromatography (RPLC) without a macromolecular component. Duncan *et al.* [7] reported a value of 0.25 μg for cholesterol monitored at 200 nm in an isopropanol–acetonitrile mobile phase. Carroll and Rudel [13] reported their best results were obtained at a concentration level greater than 10 μg for cholesterol esters using UV detection at 213 nm. The use of a refractometer by Perkins *et al.* [14] allowed detection down to 5 μg (signal-to-noise ratio = 10) for cholesterol palmitate. Rees *et al.* [15] using coupled RI and UV (254 nm) detectors, RPLC, and a methanol–chloroform–water mobile phase found a useful lower limit of detection for cholesterol acetate of 50 μg for the RI detector.

As expected, the minimum detection value for cortisone (0.1 μg/ml) was less than for cholesterol (1 μg/ml). This value is similar to other reports where both normal-phase [16,17] and reversed-phase chromatography [16,18] have been used to measure the concentration of cortisone in extracts of complex mixtures. Ballerini *et al.* [16] using UV detection (242 nm) described detection of 150 μg/ml cortisone in adrenocortical extracts. Walters and Dunbar [17] reported detection of 0.1 μg/ml cortisone (UV, 254 nm) while Jenner and Richards [18] using UV (260 nm) reported the limit of detection of 2.5 nmol/l (0.9 ng/ml) cortisone.

Comparative values for absorption of cholesterol or cortisone *per se* into any biomedical implant material could not be found. Most investigators [1–5,19–22] do not provide absolute values for lipid uptake. However, Dong *et al.* [23] have reported values of 0.32 μg/cm^2 for the adsorption of radiolabeled LDL, a much larger, more complex molecule, in another type of biomedical polyurethane, Biomer, after 2 h. Assuming only surface adsorption, our investigation shows, for cholesterol alone, approximately 35 and 30 μg/cm^2 adsorption onto 80A and 55D, respectively, after 2 h. For cortisone, 13 and 9 μg/cm^2 were adsorbed onto 80A and 55D, respectively, in 2 h.

Surprisingly, equilibrium absorption levels had not been reached by 44 h, especially for cortisone. Preliminary measurements at 96 h indicate an apparent continued increase in cortisone absorption and a leveling off of cholesterol absorption. Additional work is underway to verify this. The extended time needed for reaching equilibrium is one indicator that surface adsorption is a very minor contributor to the overall uptake of lipid. A theoretical estimate of surface adsorption indicates that approximately 0.1 $\mu g/cm^2$ lipid could occur, far less than what was measured.

Occasionally, measurements of cholesterol and cortisone at each time period varied considerably between ring samples. In these instances, the R.S.D.s ranged up to 42% for 80A and up to 18% for 55D. In contrast, the R.S.D.s for multiple injections of the same ring sample (system precision) were considerable smaller, ranging from 0.3 to 3.3% even at very low concentrations of cholesterol or cortisone. One explanation may be non-reproducible wetting of the ring surface by the cholesterol or cortisone solutions. Another may be chemical inhomogeneity at the surface of the ring. Both would lead to fluctuations in the amount absorbed with the latter possibly influencing the reliability of the polyurethane.

To conclude, the separation and detection of two lipids, cholesterol and cortisone, using SEC and dual detection was successful. The methodology described has several advantages including the elimination of pre- and post-run sample derivatization, incomplete extraction procedures, and sample clean-up. The limit of detection for the two analytes was very good, expecially for cholesterol. The technique also preserves the capability to analyze any molecular mass change in the high-molecular-mass component. If this analysis is not needed, the chromatography run time (approximately 35 min) could be considerably reduced by adjustment of the column set. However, two limitations must be considered if adopting the method: first, all components must be soluble in the solvent; and second, if UV detection is needed, a UV-transparent solvent must be used.

REFERENCES

1 R. Carmen and P. Kahn, *J. Biomed. Mater. Res.*, 2 (1968) 457.
2 W.C. Roberts and A.G. Morrow, *Am. J. Cardiol.*, 22 (1968) 614.
3 K. Hayashi, T. Matsuda, H. Takano and M. Umezu, *J. Biomed. Mater. Res.*, 18 (1984) 939.
4 T. Chandy and C. P. Sharma, *J. Colloid Interfac. Sci.*, 108 (1985) 164.
5 K. Tonyali, C. E. Rogers and H. R. Brown, *Polymer*, 28 (1987) 1472.
6 C. P. Sharma and T. Chandy, *Biomaterials*, 10 (1989) 609.
7 I. W. Duncan, P. H. Culbreth and C. A. Burtis, *J. Chromatogr.*, 162 (1979) 281.
8 J. C. Kuo and E. S. Yeung, *J. Chromatogr.*, 229 (1982) 293.
9 D. R. Newkirk and A. J. Sheppard, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 54.
10 E. Nystrom and J. Sjovall, *Anal. Biochem.*, 12 (1965) 235.
11 D. D. Perrin, W. L. F. Armarego and D. R. Perrin, *Purification of Laboratory Chemicals*, Pergamon Press, New York, 2nd ed., 1980, p. 174.
12 G. Sugihara, K. Yamakawa, Y. Murata and M. Tanaka, *J. Phys. Chem.*, 86 (1982) 2784.
13 R.M Carroll and L.L. Rudel, *J. Lipid Res.*, 22 (1981) 359.

14 E. G. Perkins, D. J. Hendren, J. E. Bauer and A. H. El-Hamdy, *Lipids*, 16 (1981) 609.
15 H. H. Rees, P. L. Donnahey and T. W. Goodwin, *J. Chromatogr.*, 116 (1976) 281.
16 R. Ballerini, M. Chinol and M. Ghelardoni, *J. Chromatogr.*, 193 (1980) 413.
17 M. J. Walters and W. E. Dunbar, *J. Pharm. Sci.*, 71 (1982) 446.
18 D. A. Jenner and J. Richards, *J. Pharm. Biomed. Anal.*, 3 (1985) 251.
19 H. P. Chin, E. C. Harrison, D. H. Blankenhorn and J. Moacanin, *Circulation*, 43/44 (Suppl. 1) (1971) 51.
20 R. Carmen and S. C. Mutha, *J. Biomed. Mater. Res.*, 6 (1972) 327.
21 A. Takahara, J. Tashita, T. Kajiyama and M. Takayanagi, *J. Biomed. Mater. Res.*, 19 (1985) 13.
22 N. Jayakumari, M. Chitra and K. S. Iyer, *J. Biomed. Mater. Res.*, 23 (1989) 1261.
23 D. E. Dong, J. D. Andrade and D. L. Coleman, *J. Biomed. Mater. Res.*, 21 (1987) 683.