JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 40–45

Lipid uptake in ePTFE arterial prostheses implanted in humans

D. MANTOVANI, E. BÉDARD, M. MAROIS, R. GUIDOIN, G. LAROCHE
Quebec Biomaterials Institute, Saint-François d'Assise Hospital and Laval University, Departments of Surgery and Metallurgy, Quebec City, Quebec, G1L 3L5, Canada

Lipid uptake in 104 ePTFE microporous vascular prostheses implanted in humans was investigated using Fourier transform infrared (FTIR) spectroscopy. The assignment of the infrared features observed in the spectra of explanted ePTFE microporous vascular prostheses shows unambiguously the presence of fatty acids in the structure of the arterial prosthesis wall. In addition, higher lipid concentration is found on the external side of the prostheses before 500 days of implantation, after which this behaviour is reversed. Finally, it seems that a greater amount of lipids are present on the surfaces of prostheses implanted in extra-anatomical sites as compared to those implanted in anatomical sites.

1. Introduction

Expanded polytetrafluoroethylene (ePTFE) vascular prostheses are widely acknowledged as the most reliable synthetic arterial substitutes in peripheral vascular surgery when autologous vein is not available [1–3]. The superior *in vivo* mechanical stability and the good mechanical properties of microporous Teflon® polymer constitute the principal advantage of ePTFE arterial substitutes over other prosthetic materials [4–6]. However, the absence of endothelialization [4] constitutes a serious disadvantage. This is likely to lead to prosthesis failure, especially when it is used in peripheral vascular applications. Indeed, thrombosis is known to be the most frequent complication occurring in ePTFE prostheses [4, 7, 8].

Very complex metabolic activities occur across an arterial wall [9], and as a consequence of these phenomena, lipid uptake is known to contribute to atherosclerotic changes in vessel walls [10, 11]. To date, a considerable number of studies have been carried out to address the atherosclerosis problem [12–14], and several mathematical models have been proposed to simulate arterial transmural transport [15, 16] and to model the accumulation of lipids in blood vessel walls [17–19]. These works have permitted the determination of vascular sites that have a high probability developing atherosclerosis [20].

However, in vascular substitutes, lipid accumulation in both chemically processed biological grafts and synthetic blood conduits has rarely been studied [21–25]. Lipid uptake in human umbilical graft implanted in humans as femoro-popliteal grafts [21] has been proposed as one of the reasons for subsequent graft failure. As far as autogenous grafts are concerned [22], it has been suggested that a process indistinguishable from vessel atherosclerosis affects vein grafts implanted over a long period. This process has been identified as contributing to their failure. Lipid uptake in synthetic vascular prostheses has concerned investigators since as long ago as 1958 [23], when it was reported that lipid chronic hypercholesterolemia produced changes in Nylon® and Orlon® arterial replacements in canine thoracic aorta. Furthermore, lipid atherosclerotic uptake in vascular prostheses, such as Dacron® and Teflon®, was found to increase with the duration of implantation [24]. Moreover, recent studies have shown that lipid uptake in arterial prostheses is more significant than in arteries [25]. Despite the fact that lipid uptake has also been pointed out as being one of the causes leading to the failure of synthetic arterial prostheses, little attention has been paid to the need for a better understanding of the mechanisms leading to the failure of the arterial substitutes. This lack of attention is attributable to the fact that no distinction has been made between lipids present in the arterial prosthesis wall and those observed in the surrounding tissues. In fact, the presence of lipids hydrophobically bound to ePTFE leads to reorganization of the ePTFE's polymer chains, which is observed, from a macroscopic point of view, as modifications of its material mechanical properties [26].

The aim of this study is to investigate lipid uptake on the internal and external surfaces of ePTFE arterial prostheses implanted in humans. To reach this goal, infrared spectra of 104 arterial prostheses have been recorded for both the luminal and external surfaces using the attenuated total reflectance (ATR) technique. In several instances, this technique has been shown to be a valuable tool for the characterization of biological systems [27]. The lipid concentration was determined by defining a lipid concentration index (LCI), which basically consists of the ratio between the area under the absorption peaks in the 2700 and 3100 cm^{-1} spectral region, due to lipids, and the area

0957–4530 © *1996 Chapman & Hall*

ranging from 1450 to 900 cm^{-1}, characteristically that of ePTFE. Finally, the LCI has been used successfully to determine the time dependence of lipid uptake in ePTFE arterial substitutes implanted in humans as well as the effect of the prostheses' implantation site on lipid uptake.

2. Materials and methods

2.1. The prostheses

Between 1989 and 1992, 165 microporous ePTFE vascular prostheses were harvested from hospitals across Canada, France, Italy and the USA. Among these specimens, we selected a sub-group of 104 explanted prostheses which had an adequate surface area for Fourier transform infrared (FTIR) analyses, and where the clinical history of the prostheses was sufficiently well known for further analyses. The criterion for selecting the prostheses samples was fixed by the FTIR technique that requires two square samples, with an area of 0.25 cm^2, which were available for the investigation of both luminal and external side of the specimens.

These 104 prostheses were retrieved from reoperations after periods of implantation ranging from 1 to 5040 days, with an average duration of 585 days. They were retrieved in 17 centres in Canada, France, Italy and the USA from 104 patients (56 men, 25 women and 23 not specified) whose mean age at implantation was 65 (range: 23–93 years). Forty prostheses were implanted as femoro-popliteal bypasses, eight as femoro-distal bypasses, 16 as vascular accesses for haemodialysis, ten as femoro-femoral bypasses and eight as axillo-femoral bypasses. For the remaining 22 prostheses the site of implantation was not specified in the clinical data supplied by surgeons. The sites of implantation were divided into extra-anatomical (axillo-femoral, femoro-femoral) and anatomical sites (femoro-popliteal, femoro-distal and arterio-venous shunts) in order to evaluate whether the implantation sites could affect the lipid uptake. The reason for explantation of the prostheses was surgical removal for complications. The most frequent complication requiring explantation of the grafts was thrombosis, which was observed in 69 cases, and infections (11) followed by false aneurysms (7). For the remaining 17 prostheses the reason of explantation was not specified. Of the 104 prostheses, 74 prostheses were reinforced Gore-Tex® vascular grafts, 18 were Impra® and one was a Vitagraft® (data not specified for 11 prostheses). They were mainly standard wall, with four being thin wall, three tapered and three externally supported. The microstructure of all the prostheses was a microporous one, composed of several fibrils linking nodules [28]. The internal diameter was 6 mm for 66 prostheses, 8 mm for 28, 12 mm for one, and for the remaining nine prostheses it was not indicated and could not be determined.

2.2. Graft harvesting and processing

This work is part of an ongoing co-operative retrieval programme for collecting and evaluating arterial prostheses implanted in humans. According to this programme's objectives, a standard procedure was developed for the preparation of all prostheses specimens. After excision, each prosthesis was opened longitudinally and carefully rinsed with heparinized saline. It was then fixed and stored in a buffered solution of 10% formaldehyde and shipped, with a form containing the relevant clinical data, to the Quebec Biomaterials Institute, St-François d'Assise Hospital, Quebec City, for investigations. The prostheses were first examined macroscopically and photographed. Representative areas of the internal and external capsules were selected for pathological studies and scanning electron microscopy (SEM). The specimens were then subjected to a standard washing process to remove all adherent tissues. This was accomplished by boiling the specimens for 5 min in a 5% sodium bicarbonate solution followed by immersion in a commercial bleach solution, twice for 2 h each, at room temperature. Finally, the specimens were rinsed in distilled water for 1 h. After drying at room temperature for 24 h, the specimens were stored in capped Petri dishes. The cleaned specimens were examined macroscopically and photographed. Some were prepared for SEM examination and those to be used for chemical analyses were dried in a vacuum oven at 40 $\pm$ 2 °C for 48 h and stored in tightly capped glass bottles.

2.3. Fourier transform infrared spectroscopy

FTIR-spectra were recorded using a Nicolet Magna-550 spectrometer (Nicolet, Madison, WI, USA) equipped with a Deuterated Tri-Glycine Sulphate (DTGS) detector and a germanium coated Potassium Bromide (KBr) beamsplitter. 250 scans were routinely acquired with an optical retardation of 0.25 cm to yield a 4 cm^{-1} resolution. Because we were mainly interested in surface characterization, the ATR mode was used to obtain the infrared spectra using a Split Pea attachment (Harrick Scientific Corp., Ossining, NY) equipped with a silicone hemispherical 3 mm diameter internal reflection element.

Since the Beer–Lambert law states that the absorbency due to a chemical specie is directly related to its concentration, the amount of lipid as compared to that of ePTFE has been measured by rationing the area under the curve between 2700 and 3100 cm^{-1}, due to stretching modes of methylene and methyl groups of lipids, over the area ranging from 800 to 1450 cm^{-1}, where spectral absorptions due to the stretching modes of CF_2 groups of ePTFE are observed. As stated above, this ratio (hereafter called LCI for lipid concentration index) is directly related to the lipid/ePTFE ratio, the proportionality constant being the ratio of the molar absorbtivity constant of lipids between 2700 and 3100 cm^{-1} and that of ePTFE between 800 and 1450 cm^{-1}.

3. Results

Fig. 1a shows the infrared spectra of a virgin ePTFE arterial prosthesis. As can be seen from this figure, the

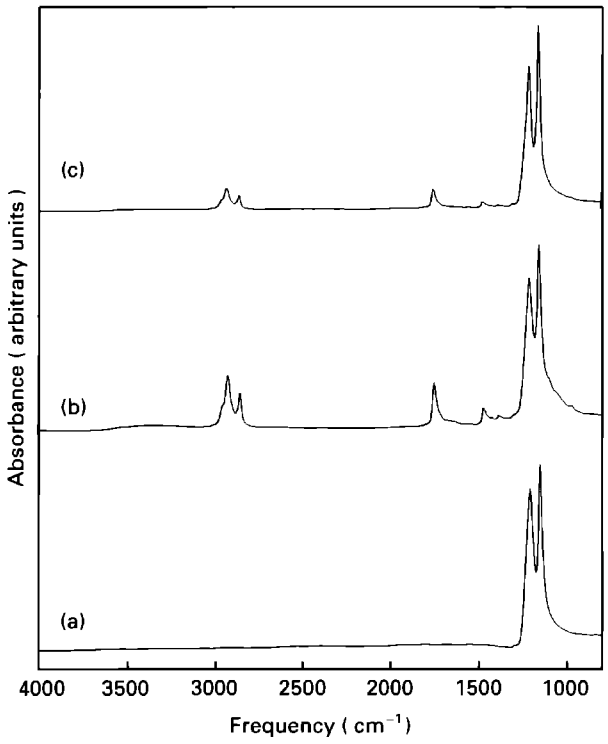

Figure 1 Fourier transform infrared spectra of ePTFE arterial prostheses: (a) virgin prosthesis; (b) internal surface of an explanted prosthesis; and (c) external surface of an explanted prosthesis retrieved after 1460 days of implantation.

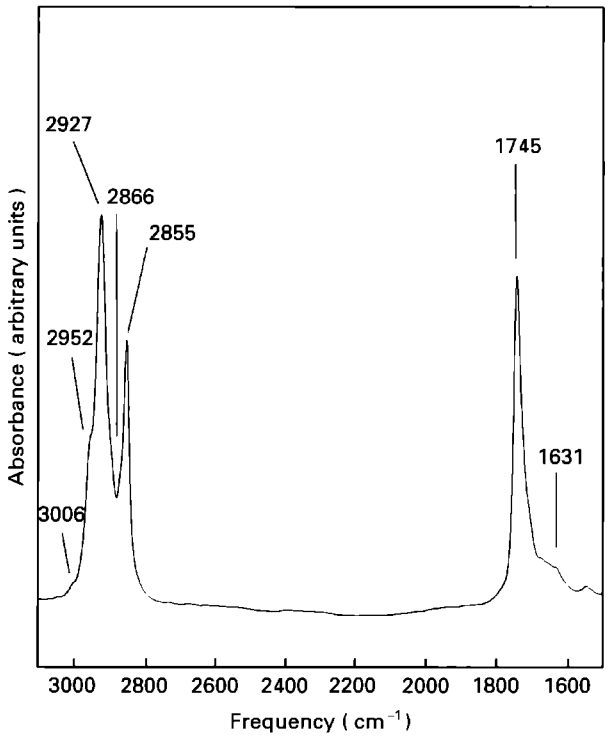

Figure 2 Details of the 1500–3100 cm^{-1} infrared spectral region highlighting the additional infrared features present in the spectra of ePTFE vascular prostheses following implantation.

infrared spectrum of expanded polytetrafluoroethylene exhibits a very simple profile due to the simplicity of its molecular structure. Indeed, the two most important absorptions are observed at 1145 and 1205 cm^{-1} due to symmetric and antisymmetric stretching mode vibration of the CF_2 groups, respectively. Following implantation in humans, the infrared spectra of both the internal and external surfaces of the prostheses (Fig. 1b and 1c, respectively) clearly show that biological molecules retention is present in the polymer structure since new infrared features are observed. As seen in Fig. 2, the assignment of these additional infrared peaks shows unambiguously that the molecules retained in the prostheses' microporous structure are unsaturated fatty acids. Indeed, the 2700–3100 cm^{-1} spectral region is dominated by two strong bands at 2855 and 2927 cm^{-1} due to the methylene symmetric and antisymmetric stretching modes, respectively. Weaker bands due to symmetric and asymmetric stretching modes of the terminal methyl groups are also present at 2866 and 2952 cm^{-1}. Unsaturations in the lipid acyl chains are also detected by the presence of the 3006 cm^{-1} infrared feature due to the olefinic C–H stretching mode and that of the 1631 cm^{-1} stretching absorption due to C=C functionality. Finally, typical infrared absorption of the ester carbonyl groups is observed at 1745 cm^{-1}.

The relationship between the LCI and the duration of implantation for both external and internal surfaces of the ePTFE arterial prostheses implanted for a period of time ranging from 1 day to 15 years is shown in Fig. 3. Data presented in Fig. 3 show that the time dependence of the lipid concentration profile on both the luminal and external side of the prostheses exhibits

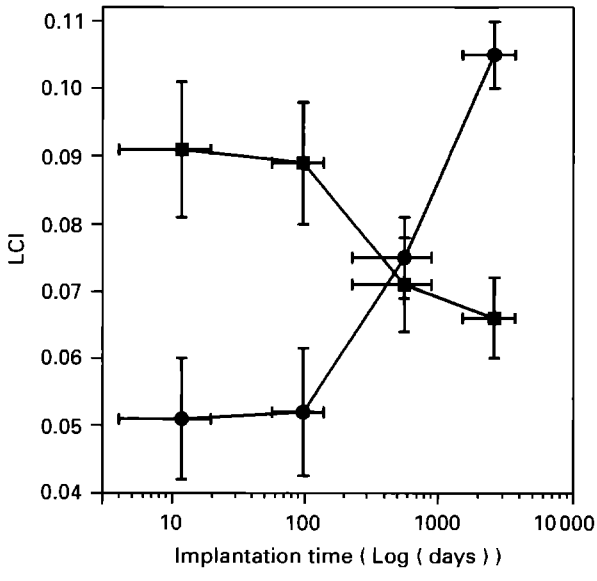

Figure 3 Time dependence of the lipid concentration index (LCI) for ePTFE arterial prostheses implanted in humans: —●— luminal surface; —■— external surface.

converse behaviour. On the internal surface, the lipid concentration, although detectable, is relatively low during the first 100 days of implantation. After this period, a marked increase of the lipid concentration is observed. Conversely, the external side of the prostheses is covered by an important amount of lipid right after the implantation, while after 100 days the lipid concentration significantly decreases. It should also be noticed that the lipid concentrations on both sides of the prosthesis are about the same at 500 days of implantation. After this period, the lipid concentration continuously increases on the luminal surface of the prostheses while it decreases on the external one.

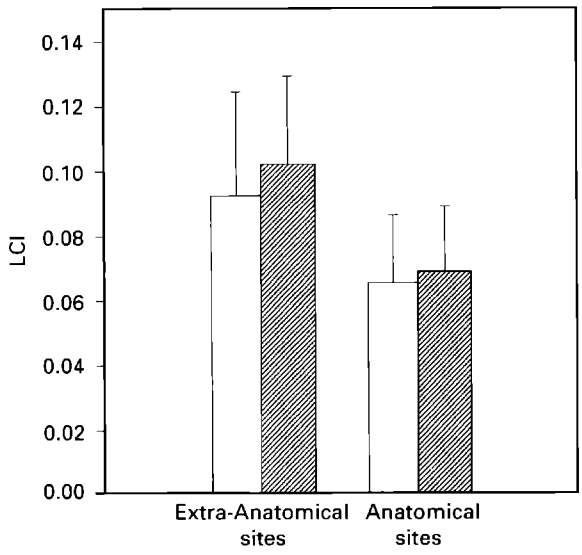

Figure 4 Lipid concentration index (LCI) as a function of the sites of implantation of the ePTFE arterial prostheses: □ luminal surface; ▨ external surface.

The importance of the site of implantation on the lipid uptake in arterial prostheses has also been investigated, as seen in Fig. 4. Although the standard deviations on these data are quite large due to human variability (such as sex, age, diabetes, dyslipidemia, smoking, alcoholism, etc.), it seems that more lipids are observed in prostheses implanted in extra-anatomical sites.

4. Discussion

The event expected when implanting a vascular prosthesis into the human body is that the luminal healing process will take place. In this context, in ePTFE arterial prostheses, the wall porosity is an important factor to take into account [29, 30]. On the one hand, to prevent bleeding or haemorrhage, porosity must not be too high; on the other hand, the porosity should be sufficiently high for tissue infiltration which is the first step for complete luminal endothelialization, which, in turn, is required for an ideal non-thrombogenic vascular prosthesis [31–33]. Unfortunately, in actual cases where ePTFE arterial prostheses are implanted in humans, infiltration is rarely observed and endothelialization is completely absent [4, 34, 35]. For example, in ePTFE prostheses implanted in baboons, a complete endothelial coverage was observed when the average internodal distance of the microporous structure of ePTFE prosthesis was twice that utilized in humans [36]. However, prostheses with such porosity could not be used clinically because of the above-mentioned haemorrhage and bleeding consequences. The approaches generally considered to stimulate endothelial coverage can be classified into two methods: modification of the biological environment [37, 38] or of the interface between the tissues and the prosthesis [39–42]. To date, no remarkable progress has been realized by these methods and the ideal vascular prosthesis has yet to be developed.

Our approach for attaining a better understanding of this problem is to consider implanted ePTFE microporous arterial prosthesis as acting similarly to a selective permeable membrane, separating the blood flow from the surrounding tissues. In this context, molecules in the blood flow and in the surrounding tissues diffuse through the microporous structure of the prosthesis [43] principally by convective and diffusive mechanisms [44–46], since pressure and concentration gradients, related to instantaneous values of blood pressure and to biological molecule concentrations, are present in both the external and luminal sides of the prosthesis. Among the molecules that diffuse through the arterial prosthesis wall, some show only low-level affinity for hydrophobic ePTFE, while others become more strongly attached to the polymer structure, thus modifying the organization of the polymer chains [26]. From the data presented herein, it is clear that lipids are among those molecules that are bound tightly and fastly to the ePTFE prosthesis structure. Indeed, lipids have been detected on both surfaces of an ePTFE arterial prosthesis implanted in a human for only one day (results not shown). In addition, FTIR data show that lipids are the only molecules remaining in the prosthesis structure after the cleaning procedure. The rapid kinetic of lipid uptake along with the high affinity of lipids for ePTFE may lead to the inhibition of fibroblast and collagen infiltration, which is a slower process [4, 34].

At this stage of the discussion, the origin of the lipids found in the prosthesis' structure has to be addressed. A part of the answer to this question is given by the assignment of the infrared features observed in the spectra of the explanted prostheses that shows the presence of unsaturated fatty acids in the chemical structure of the absorbed lipids. This lipid structure is observed in the biological membrane of some blood elements, such as red blood cells and platelets [47]. The possibility that lipids absorbed in the ePTFE prosthesis come from these blood elements [48] should not be ruled out. On the other hand, one should also consider that the external side of an ePTFE arterial prosthesis implanted in an extra-anatomical site is subjected to fatty tissues in which unsaturated fatty acids are also present, as suggested in Fig. 4.

The time dependence of the lipid concentration profile seems to be governed by complex mechanisms which are probably related to the healing process of the prostheses. Indeed, the results presented in Fig. 3, showing, before 100 days of implantation, a high lipid concentration on the external surfaces of the prostheses along with a low lipid concentration on the inner sides, probably indicates the presence of important lipid concentrations and blood pressure gradients between the luminal and the external sides of the prostheses, thereby leading to accumulation on the outer side. Upon healing, the formation of an external capsule (which is generally formed between 3 and 6 months after implantation) probably leads to a decrease of these two gradients and thus to a simultaneous diffusion of lipid molecules from both sides of the prostheses, as observed in Fig. 3 after 500 days of implantation.

One should keep in mind that the results presented above are, as mentioned previously, subject to human variability. A complete understanding of the lipid uptake in arterial prostheses thus requires *in vitro* experiments in which the experimental conditions (such as physiological fluid pressure, lipid concentration, membrane permeability, etc.) are determined with accuracy. Such experiments are under investigation in our laboratory.

5. Conclusions

FTIR techniques provide a new insight for the characterization of lipid uptake in ePTFE arterial prostheses. Indeed, our results show that lipids are attached strongly to both external and luminal surfaces of the wall of ePTFE arterial prostheses when implanted in humans. Furthermore, their concentration is related to the duration of implantation and the site of implantation. In addition, such lipid uptake should be taken into account as an explanation for the poor endothelialization coverage observed in ePTFE arterial substitutes.

Acknowledgements

This work was supported in part by the Natural Science and Engineering Research Council (NSERC-Canada), "Fonds de la Recherche en Santé du Québec" (FRSQ-Quebec), "Fonds pour la Formation de Chercheurs et l'Aide à la Recherche" (FCAR-Quebec), Medical Research Council (MRC-Canada) and Quebec Biomaterials Institute. The authors would like to extend their gratitude to Suzanne Bourassa and Marielle Corriveau for their technical assistance. Sincere thanks are owed to the surgeons and pathologists who contribute to the co-operative retrieval programme.

References

1. R. ROSS, *New England J. Med.* **314** (1986) 488.
2. D. L. FRY, *Arteriosclerosis* **7** (1987) 88.
3. W. J. QUINONES-BALDRICH, R. W. BUSUTTIL, J. D. BAKER, C. L. VESCERA, S. S. AHN, H. I. MACHLEDER and W. S. MOORE, *J. Vasc. Surg.* **16** (1992) 209.
4. R. GUIDOIN, N. CHAKFÉ, S. MAUREL, T. HOW, M. BATT, M. MAROIS and C. GOSSELIN, *Biomaterials* **14** (1993) 678.
5. S. K. GUPTA, F. J. VEITH, E. ASCER and K. R. WENGERTER, in "Vascular surgery", edited by R. B. Rutherford (W. B. Saunders, Philadelphia, 1989) p. 460.
6. M. E. McCLURKEN, J. M. McHANEY and W. M. COLONE, in "Vascular graft update: safety and performances", edited by H. E. Kambic, A. Kantrowitz and P. Sung (American Society for Testing and Materials, Philadelphia, 1986) p. 86.
7. C. D. CAMPBELL, D. GOLDFARB and R. ROE, *Ann. Surg.* **182** (1975) 138.
8. A. FLORIAN, L. H. COHN, G. J. DAMMIN and J. J. COLLINS, *Arch. Surg.* **111** (1976) 267.
9. E. B. SMITH, in "Dynamics of arterial flow", edited by S. Wolf and N. T. Werthessen (Plenum Press, New York, 1979) p. 245.
10. A. W. CLOWES, in "Atherosclerosis and arteriosclerosis: human pathology and experimental animal methods and models", edited by R. A. White (CRC Press, Boca Raton, 1989) p. 3.
11. A. AVERBOOK, S. E. WILSON and G. H. WHITE, in "Atherosclerosis and arteriosclerosis: human pathology and experimental animal methods and models", edited by R. A. White (CRC Press, Boca Raton, 1989) p. 17.
12. S. L. WILENS and R. T. McCLUSKEY, *Amer. J. Med. Sci.* **224** (1952) 540.
13. R. M. ZWOLAK, T. R. KIRKMAN and A. W. CLOWES, *Arteriosclerosis* **9** (1989) 374.
14. J. R. CROUSE, R. P. BYINGTON, M. G. BOND, M. A. ESPELAND, J. W. SPRINKLE, M. McGOVERN and C. D. FURBERG, *Control. Clin. Trials* **13** (1992) 495.
15. D. L. FRY, *Amer. J. Physiol.* **363** (1985) H240.
16. C. G. CARO, M. J. LEVER, Z. LAVER-RUDICH, F. MEYER, N. LIRON, W. EBEL, K. H. PARKER and C. P. WINLOVE, *Atherosclerosis* **37** (1980) 397.
17. X. DENG, M. W. KING and R. GUIDOIN, *ASAIO J.* **39** (1993) M489.
18. X. DENG, Y. MAROIS, M. W. KING and R. GUIDOIN, *ibid.* **40** (1994) 186.
19. X. DENG, Y. MAROIS, T. HOW, Y. MERHI, M. W. KING and R. GUIDOIN, *J. Vasc. Surg.* **21** (1995) 135.
20. W. W. MEYER, in "Dynamics of arterial flow", edited by S. Wolf and N. T. Werthessen (Plenum Press, New York, 1979) p. 353.
21. R. GUIDOIN, P. E. ROY, P. LANGERON, P. PUPPINCK, R. PAYNTER and C. GOSSELIN, *J. Mal. Vasc.* **12** (1987) 329.
22. K. W. WALTON, G. SLANEY and F. ASHTON, *Atherosclerosis* **54** (1985) 49.
23. G. L. JORDAN, M. E. DeBAKEY and B. HALPERT, in Proceedings of the Society for Experimental Biology and Medicine, New York, 1958, p. 484.
24. K. W. WALTON, G. SLANEY and F. ASHTON, *Atherosclerosis* **61** (1986) 155.
25. E. CHIGNIER, J. GUIDOLLET, C. LHOPITAL, P. LOUISOT and R. ELOY, *Amer. J. Pathol.* **137** (1990) 531.
26. L. E. NIELSEN and R. F. LANDEL, in "Mechanical properties of polymers and composites" (2nd edn) (Marcel Dekker, New York, 1994) p. 557.
27. R. M. GENDREAU, *Trends Anal. Chem.* **5** (1986) 68.
28. R. GUIDOIN, S. MAUREL, N. CHAKFÉ, T. HOW, Z. ZHANG, M. THERRIEN, M. FORMICHI and C. GOSSELIN, *Biomaterials* **14** (1993) 694.
29. M. A. GOLDEN, S. R. HANSON, T. R. KIRKMAN, P. A. SCHNEIDER and A. W. CLOWES, *J. Vasc. Surg.* **11** (1990) 838.
30. E. POLLOCK, E. J. ANDREWS, D. LENTZ and K. SHEIKH, *Trans. Amer. Soc. Artif. Int. Organs* **XXVII** (1981) 405.
31. K. BERGER, L. R. SAUVAGE, A. M. RAO and S. J. WOOD, *Ann. Surg.* **175** (1972) 118.
32. J. V. RICHARDSON, C. B. WRIGHT and L. F. HIRATZKA, *J. Surg. Res.* **28** (1980) 566.
33. E. SIROIS, M. F. CÔTÉ and C. J. DOILLON, *Int. J. Artif. Organs* **16** (1993) 609.
34. A. W. CLOWES, T. R. KIRKMAN and M. M. CLOWES, *J. Vasc. Surg.* **3** (1986) 877.
35. E. CHIGNIER, J. GUIDOLLET, Y. HEYNEN, M. SERRES, G. CLENDINNEN, P. LOUISOT and R. ELOY, *J. Biomed. Mater. Res.* **17** (1983) 623.
36. A. W. CLOWES, T. R. KIRKMAN and M. A. REIDY, *Amer. J. Pathol.* **123** (1986) 220.
37. J. M. SEEGER and N. KLINGMAN, *J. Surg. Res.* **38** (1985) 641.
38. S. P. MASSIA and J. A. HUBBEL, *J. Biomed. Mater. Res.* **25** (1991) 223.
39. B. D. RATNER, T. HORBETT, A. S. HOFFMAN and S. D. HAUSCHKA, *ibid.* **9** (1975) 407.
40. B. KASEMO and J. LAUSMAA, in "Surface characterization of biomaterials", edited by B. D. Ratner (Elsevier Science, Amsterdam, 1988) p. 1.
41. D. M. BRUNETTE, *ibid.* p. 203.
42. Y.-S. YEH, Y. IRIYAMA, Y. MATSUZAWA, S. R. HANSON and H. YASUDA, *J. Biomed. Mater. Res.* **22** (1988) 795.

43. J. R. PHILIP, in "Circulatory and respiratory mass transport", edited by G. E. W. Wolstenholme and J. Knight (J. & A. Churchill Ltd., London, 1969) p. 25.
44. F. YUAN, S. CHIEN and S. WEINBAUM, *J. Biomech. Eng.* **113** (1991) 314.
45. M. J. LEVER and M. T. JAY, *Frontiers Med. Biol. Eng.* **5** (1993) 45.
46. W.-S. KIM and J. M. TARBELL, *Trans. Amer. Soc. Mech. Eng.* **116** (1994) 156.
47. D. CHAPMAN, *Biochem. Soc. Trans.* **21** (1993) 258.
48. A. B. CHANDLER and R. A. HAND, *Science* **134** (1961) 946.

Received 29 June
and accepted 4 July 1995