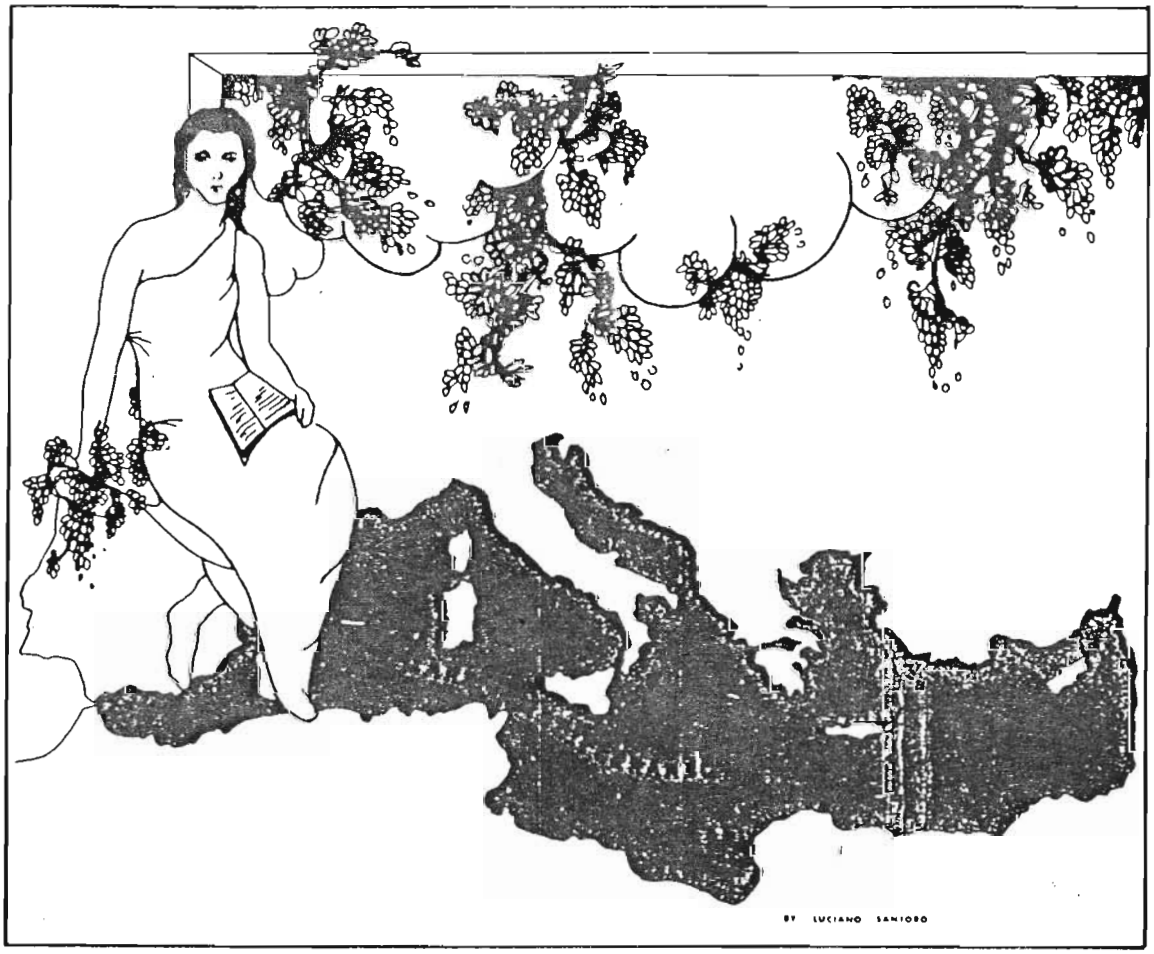
FIRST MEDITERRANEAN SCHOOL ON SCIENCE AND TECHNOLOGY OF ADVANCED POLYMER - BASED MATERIALS



Vico Equense (Naples) - Italy

11-22 September 1989

APPLICATIONS OF ADVANCED POLYMERS IN MEDICINE: BIOARTIFICIAL POLYMERIC MATERIALS

P.Giusti*, G.Soldani**, R.Mercogliano#, M.Pracella**, N.Barbani*
* Department of Chemical Engineering, University of Pisa, Pisa
** Center of Studies on Polymerization Processes, C.N.R., Pisa
# Scuola Normale Superiore/E.N.I.,Pisa,Italy

ABSTRACT

A new class of biomaterials, called "bioartificial polymeric materials", was prepared blending fibrinogen (FBNG) with a segmented polyurethane (PU), and collagen (CLG) with poly(acrylic acid) (PAA), poly(acrylamide) (PAAM), poly(vinyl alcohol) (PVAL) respectively. The PU-FBNG material was processed through a phase-inversion, spraying technique to fabricate porous vascular grafts. FBNG was subsequently converted into covalently cross-linked fibrin (FBN) through the action of thrombin (Th), fibrin-stabilizing factor (FSF), and calcium ions. Cross-linked blend resulted in a higher thermal stability with respect to native cross-linked FBN. Tensile behaviors of PU-FBN materials closely matched those of a natural artery by varying the ratio PU/FBN. In vivo experiments showed a better neointima formation and tissue ingrowth for PU-FBN(50%) grafts in comparison of PU grafts. However, 50% of FBN did not assure adequate mechanical resistence, and aneurysmal changes were seen in some grafts. CLG-based materials, processed by casting, showed a consistent protective effect of the synthetic component with respect to CLG denaturation temperature, particularly noticeable for CLG-PAA and CLG-PVAL blends. Materials advantages and drawbacks are discussed.

INTRODUCTION

Polymeric materials to be used for biomedical applications should possess both physico-chemical and mechanical properties, and in addition opportune biocompatibility suitable for being fabricated into: 1) biomedical devices, such as heart-assist pumps, chambers for artificial hearts, intra-aortic balloons, etc.; 2) parts of assisting devices, such as membranes or hollow fibers for artificial organs

(e.g. artificial kidney); and 3) implantable devices, such as macrocapsules for cells immuno-protection (e.g. bioartificial pancreas) and tubular conduits (e.g. vascular prostheses). Many synthetic polymers already commercially available show physico-chemical and mechanical properties comparable, if not better, than those of biological polymers they have to substitute, but they do not show sufficient biocompatibility. On the other hand native biological polymer, such as FBN (the cross-linked form of FBNG), and CLG possess good biocompatibility *per se*. However, their mechanical properties are often inadequate and, furthermore, they show an high production cost in comparison with synthetic polymers. Therefore, these biological polymers are, in a way or an another, unsuitable to fabricate the most part of the medical devices we listed above.

Thus, we had the idea to prepare materials based on blends of biological and synthetic polymers with the final purpose to realize processable new polymeric materials which hopefully posses simultaneously good mechanical and biocompatibility properties. We propose to call this new class of materials as "**bioartificial polymeric materials**", which are different from the so called "biosynthetic polymeric materials", synthesized by a large number of bacteria [1]. The general idea to produce these new biomaterials is schematized on Figure 1.

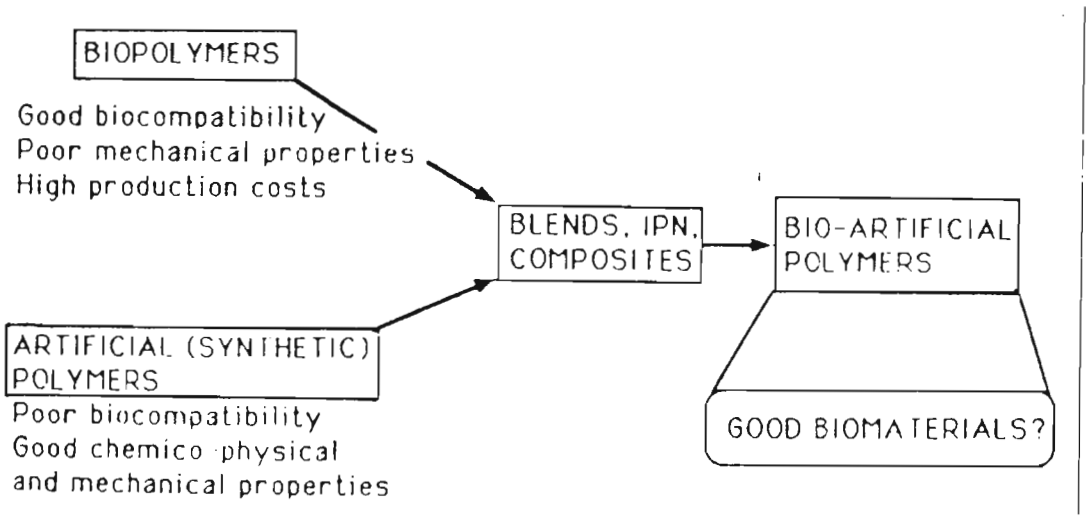


FIGURE 1. Schematic diagram to produce "bioartificial polymeric materials"

This paper describe the results we obtained from the study of: 1) a

material produced blending a segmented polyurethane with the protein FBNG, and subsequently converting FBNG into covalently cross-linked FBN through the action of Th, FSF (factor XIII), and calcium ions (Figure 2); 2) materials produced blending various commercial water-soluble polymers, such as PAA, PAAM, PVAL with the protein CLG.

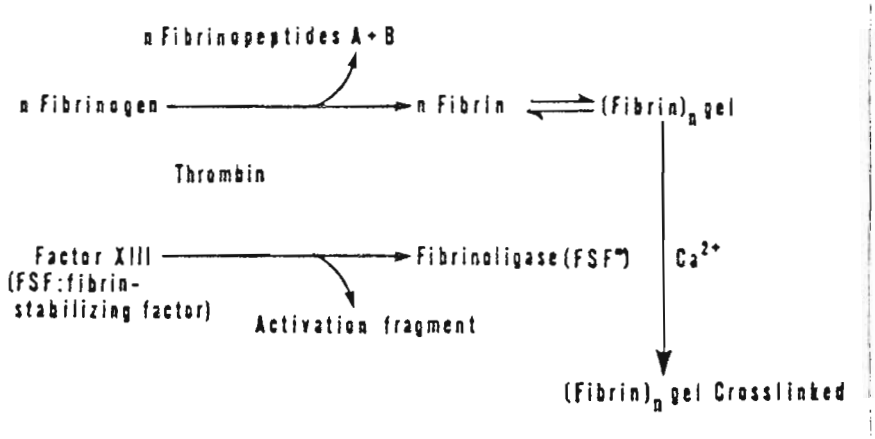


FIGURE 2. Schematic diagram of the FBN formation mechanism.

MATERIALS AND METHODS

1) FBN-Based Bioartificial Polymer.

Cardiothane® 51 a blend of poly (etherurethane) (90%) and poly (dimethylsiloxane) (10%) [2,3] (hereinafter referred as PU), was obtained from Kontron Cardiovascular, Inc., Everett, Massachusetts, U.S.A., as a 16% w/v solution in 2:1 THF-1,4 dioxane. Cross-linked FBN was produced using the following substances: FBNG, derived from human plasma (freeze-dried, 95-96% clottability), was obtained from BIAGINI FARMACEUTICI, Lucca, Italy; Th, derived from bovine plasma (freeze-dried, activity 600 NHI units/mg), was obtained from SIGMA, St. Louis, MO, U.S.A.; FSF, derived from human placentas (Fibrogammin 250®, freeze-dried, activity of 250 units/4 ml), was obtained from ISTITUTO BERING S.p.a., Aquila, Italy.

A PU solution and PU-FBN blends were used to fabricate highly porous small-diameter vascular prostheses according to the following combined phase-inversion [4], spraying process:

a) The original PU solution in 2:1 THF-1,4 dioxane was diluted, with the same solvent mixture to reach a polymer concentration of 0.4 %. The nonsolvent tolerance of the polymer solution was estimated by placing 100 ml of it in a vessel kept at a temperature of 25° C under

gentle stirring, and then slowly adding it, with a titration burette, distilled water as a nonsolvent. This was continued until incompatibility was visually detected by the appearance of turbidity or actual precipitation ("cloud point"), which occurred at approximately 20 ml of distilled water addition. Following the "cloud point" determination we added 16.5 ml of distilled water to each 100 ml of dilute PU solution to achieve a clear 0.34 % w/v polymer-solvent-nonsolvent system which would precipitate with the addition of only a small amount of water.

b) FBNG powder was ground with a mortar and pestle to give fine particles. A weighed amount of powder (sufficient to make 2:1 and 1:1 PU-FBNG suspension) was added to 100 ml of the PU solution and the mixture was vigorously stirred to maintain a homogeneous suspension to use in the spraying process.

c) To fabricate the prostheses, we designed and built a small precision lathe in which mandrels of different diameters could be rotated at different speeds. Parallel to the axis of the lathe we positioned a carriage which could move bidirectionally along the axis of the rotating mandrel, under the control of a motor which could be automatically reversed by the action of electromechanical relays controlled by micro-switches. Two modified spray guns were mounted on the carriage and positioned vertically so that their nozzles were aligned with the longitudinal axis of the mandrel, at a distance and an angle from the mandrel such that the intersection of the jets occurred at the surface of the mandrel (Figure 3).

To fabricate a prosthesis, the two glass reservoirs were filled, one with the PU solution or, alternatively, with the PU suspension, and the other with distilled water, and the two fluids were simultaneously but separately sprayed at the same flow rate onto a Teflon (Small Parts Inc., MIAMI, FLA, U.S.A.) rotating mandrel. The mandrel rotation speed and carriage movement speed were set at predetermined values, and the two fluids were sprayed at predetermined pressures, angles, and distances between nozzles and mandrel. Wall thickness was closely related to the volume of PU solution sprayed. When a desired end point was reached, the process was stopped and the Teflon mandrel with the deposited material was submerged overnight in a bath of deionized water, to allow exchange

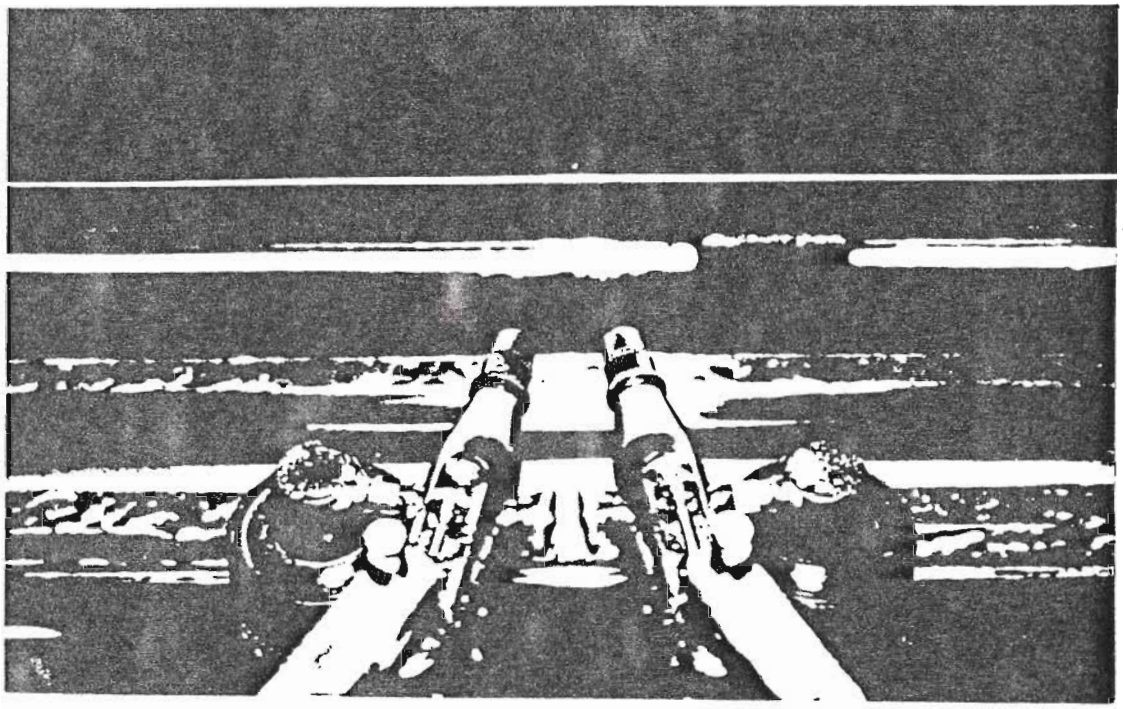


FIGURE 3. Detail of the two spray-guns, showing the intersection of the jets occurring at the surface of the mandrel.

of the solvent system for the nonsolvent. The finished prosthesis was removed from the mandrel by reducing the Teflon sleeve diameter through axial stretching and was then incubated for 3 hours at 37° C in distilled water containing 500 NIH unit/ml of Th, 10 U.I./ml of FSF, and 50 μmol/ml of calcium chloride. The prostheses were left in distilled water at room temperature until they were either tested or implanted.

2) CLG-Based Bioartificial Polymers

Mixtures were prepared using bovine achilles tendon type I collagen (M.W. 3.15×10^5), supplied by ISTITUTO GENTILI-Pisa, Italy as a 1% w/v water suspension of protein, and PAA (M.W. 9.0×10^4), PAAM (M.W. 5.0×10^6), PVAL (M.W. 1.15×10^5), supplied by ALDRICH, Milwaukee, WIS, U.S.A..

CLG-based blends were prepared at room temperature according to the following procedure: separate dilute aqueous solutions were prepared for CLG and each of the synthetic component under constant stirring; mixtures with different composition ratios (10-80% by weight) were

obtained by a dropwise addition of a solution of the desired synthetic polymer to the collagen solution. After 24 hours of vigorous stirring, samples were vacuum dried. After drying their moisture content was 10-15% by weight.

3) Thermal, Mechanical, and Morphological Characterization.

3a.Thermal Characterization.

FBN-Based Bioartifical Polymer. The apparatus used was a Perkin-Elmer DSC 4 Scanning Calorimeter. Scanning was performed at a heating rate of 20°C per min.

FBNG powder; cross-linked FBN (from a 1% FBNG solution); a PU tube; a 2:1 PU-FBNG tube (not cross-linked); and a 2:1 PU-FBN tube (cross-linked) were dried under vacuum for 5 hours at 37°C and then quickly transferred to DSC pans.

CLG-Based Bioartificial Polymers. The apparatus used was a Perkin-Elmer DSC-2C Scanning Calorimeter, calibrated with indium. Both standard aluminum pans and stainless steel high pressure capsules were used in order to optimize the thermal behavior of the samples. Scanning rate was of 5 K/min.

Dry samples of CLG and of CLG-based blends of various compositions (20-90% by weight of CLG) were accurately weighed and sealed in the DSC capsules. Sample weights were in the range of 3-5 mg.

Both bioartificial polymers data are presented as a plot of endothermic heat rate vs. temperature with all traces normalized to 1mg of substance.

3b.Mechanical Characterization

Tensile tests were performed on FBN-based tubes with a floor Instron Machine at the deformation rate of 5 mm/min. Stresses were referred to the initial cross section. All specimens, except the # 5, were small tubes of 5 mm ID, with a wall thickness of 0.35 mm. Specimen # 5 was cut from a thin sheet of cross-linked FBN.

3c.Morphological Characterization

Scanning electron microscopy (SEM) (Jeol,T300) provided information on the morphology of the CLG-based cast materials, and on the pore size, shape, surface characteristics of the luminal side, outside, and

cross-section of the FBN-based vascular prostheses. In addition, because vacuum drying significantly distort the tubular material by collapsing its spongy structure, samples were frozen and then freeze-dried before being sputter coated with gold-platinum (Edwards).

4) In Vivo Evaluation of FBN-Based Vascular Prostheses

A pilot study was designed to evaluate the materials and the fabrication technique described above at what was judged to be the two extremes of FBN content that might be useful (0% and 50% fibrin). The rat abdominal aorta was selected as a well described microvascular model [5].

4a.Prostheses Implantation

Grafts with an ID of 1.5 mm were sterilized immediately before implantation as follows: One end of a 60 mm long segment of graft material was tied off with 6-O silk ligature and the other mounted on a sterile tubing adapter attached to a sterile 10 ml syringes. A syringe pump model 940 (Harvard Apparatus, South Natick, MA, U.S.A) were then used to ultrafiltrate liquid through the wall of the vertically oriented graft using 10 ml each of the following 4 solutions: (1) sterile isotonic saline, (2) 50% ethanol, (3) 0.1N hydrochloric acid, and (4) a final rinse of sterile isotonic saline. Grafts 1.5 cm long were implanted in the infrarenal abdominal aorta of four groups of six male Sprague-Dawley rats weighing 300-350 gm, using pentobarbital sodium anesthesia, standard microsurgical techniques, no anticoagulant, and according to the following implant intervals: (1) PU grafts, 4 weeks; (2) PU grafts, 8 weeks; (3) PU-FBN(50%) grafts, 4 weeks; (4) PU-FBN(50%) grafts, 8 weeks.

4b. Prostheses Retrieval and Histologic Evaluation

After the implant intervals the rats were deeply anesthetized with pentobarbitol sodium and transcardially perfused with heparinized PBS buffer and modified Karnovsky's fixative. The grafts were then excised, opened longitudinally under magnification, and pinned in the open position for gross examination, assessment of patency, and macrophotography. The grafts were then maintained in Karnovsky's fixative until processed for histology.

Specimens for light microscopy examination were fixed in glutaraldehyde, dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin and Gomori's trichrome.

RESULTS AND DISCUSSION

FBN-Based Bioartifical Polymer.

In our opinion, it is probably necessary to achieve complete cross-linking of the FBN used in the fabrication of PU-FBN blends to assure an adequately stable material to use for biomedical application. Cross-linked FBN is distinguished from FBNG mainly by its insolubility under physiological conditions and by its higher thermal stability. FBN prepared in the absence of FSF can be easily dissolved in 5 M urea. Due to cross-linking, the insoluble, FBN formed in the presence of FSF cannot be dissolved in 5 M urea and other similar solvents and is thermally stable below 170 °C [6]. The increase in the thermal stability of FBNG relative to cross-linked FBN, and the effect of the enzymatic activity of Th, FSF, and calcium chloride on the FBNG contained in the bulk of the prosthetic material was evidenced by DSC analysis. The results of the thermal characterization of FBNG, FBN, PU, a 2:1 PU-FBNG blend, and a 2:1 PU-FBN based bioartificial polymer are shown in Figure 4.

The FBNG, trace a, is characterized by a broad endotherm centered at 90°C and extending over a range of about 100 degrees, plus a smaller endotherm at 150□C. The endotherm at 90°C corresponds to that found by Donovan and Mihalyi [7] for FBNG solutions and is associated with denaturation of the central portion of the FBNG molecule. The specific heat change observed above 180°C is probably associated with thermal degradation. The FBN, trace b displays only a specific heat change at approximately 60°C and no endotherm at temperatures up to 180°C. This is expected because the structure of the FBNG molecule responsible for the endotherm at 90°C is no longer present in the cross-linked FBN molecule. It is known that the thermal degradation of FBN starts at about 170°C, therefore, the endotherm observed at approximately 220°C in the FBN trace can be tentatively associated with some kind of chemical transformation. The PU, trace c, shows no

significant endotherms over the entire temperature range investigated from -20°C to +280 °C. The 2:1 PU-FBNG sample, trace d, appears

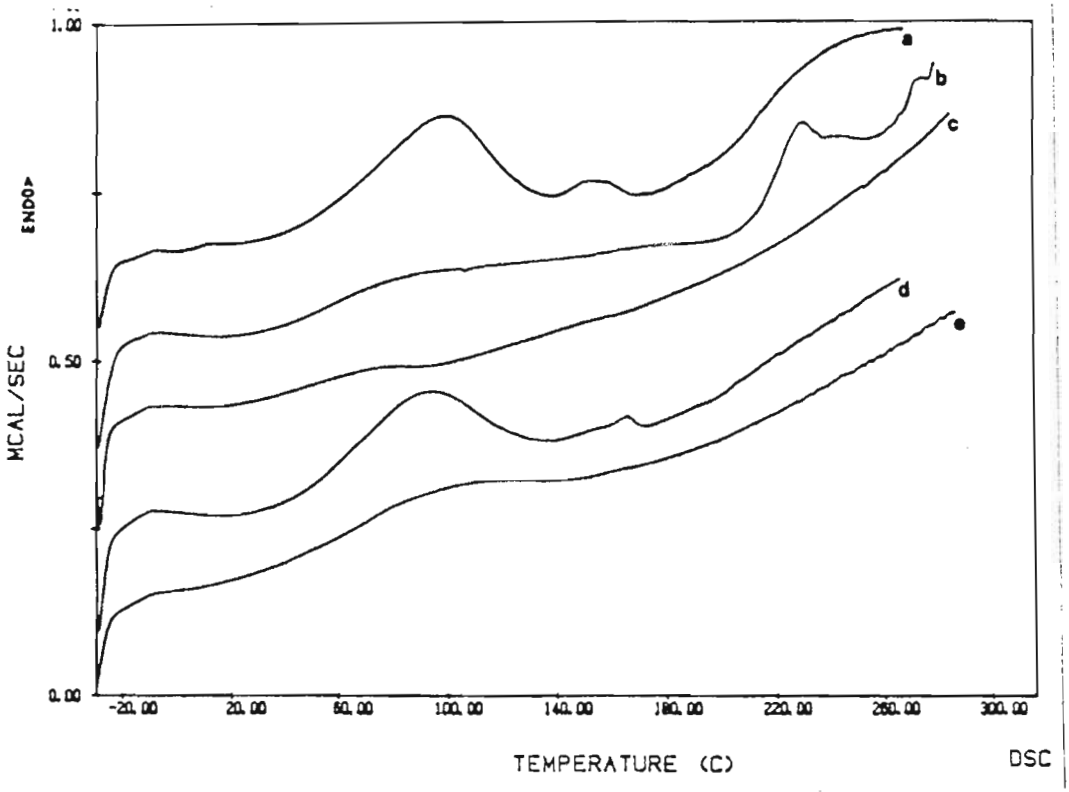


FIGURE 4. DSC thermograms. a) FBNG; b) cross-linked FBN; c) PU tube; d) 2:1 PU-FBNG tube (not cross-linked); e) 2:1 PU-FBN tube (cross-linked).

qualitatively similar to FBNG. It is noteworthy, however, that the enthalpy change associated with the 90°C endotherm is now much higher than expected on the basis of the amount of FBNG present in the blend. This indicates that some kind of interaction between FBNG and PU must be occurring on a molecular level. Trace e, referring to the 2:1 PU-FBN sample, is very similar to that for the cross-linked FBN from the enzymatically treated FBNG solution, confirming that treatment with the cross-linking solution led to an almost complete transformation of the FBNG within the bulk of the PU-FBNG tube. The thermal stability of the FBN appears much higher in the blended material than in the natural form as demonstrated by the absence of strong thermal effects above 180°C. This may be explained on the basis of molecular interactions between PU and FBN probably similar to those already mentioned in the case of FBNG.

In Figure 5 the tensile behaviors of a 2:1 and a 1:1 PU-FBN prostheses are compared with those of a pure PU prosthesis, a dog's femoral artery, and of cross-linked fibrin. One may note that increasing the amount of FBN in the material, the material's initial modulus decrease approaching the pattern of a natural vessel. From a mechanical point of viewing, materials # 2 and # 3 can be considered as composites in which the matrix is made of rubber and the filler of FBN.

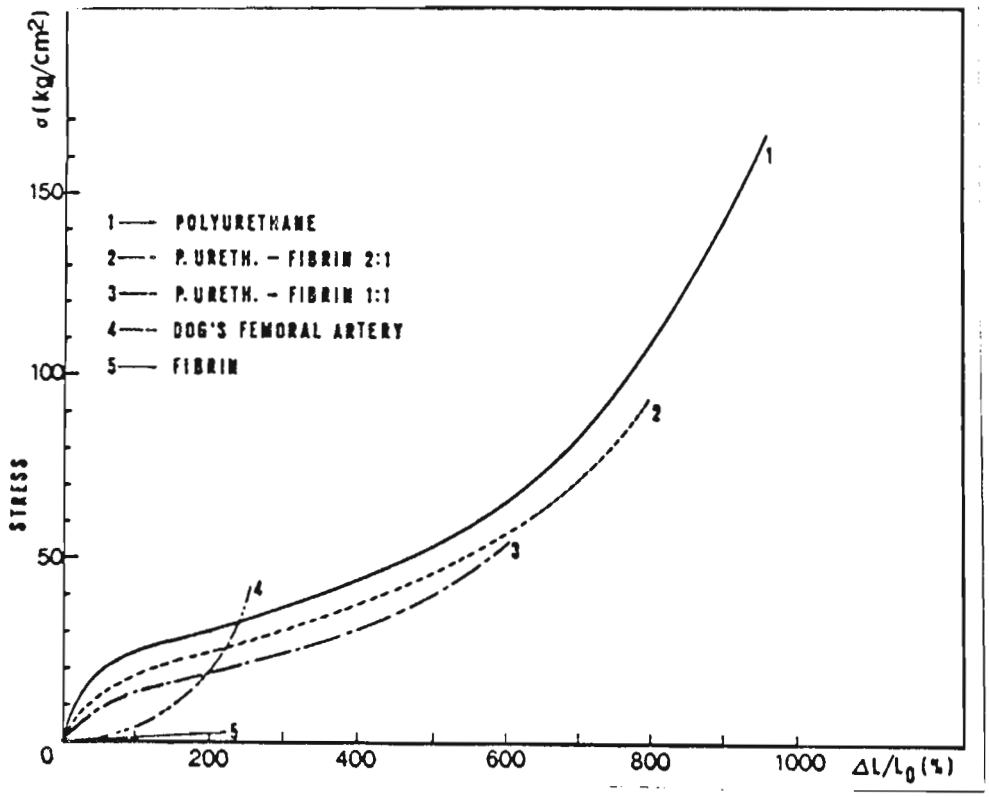


FIGURE 5. Stress-strain relationships.

The SEM of the cross-section of a prosthesis is shown in Figure 6. The tubular structure shows a thin (~10 μm) relatively smooth inner skin, a spongy porous wall approximately 350 μm thick, and an outer porous surface; the wall is made of circularly oriented layers of material which forms an open-cell porous structure.

The results of the gross examination of the explanted grafts are shown in Table 1.

The patent, non-aneurysmal grafts [both PU and PU-FBN(50%)] were completely covered with a transparent, glistening neointima without any evidence of exposed polymer when visualized at 25 power

magnification (Figure 7). All aneurysmal grafts contained at least some mural thrombus, often filling saccular aneurysms up to 4 mm in

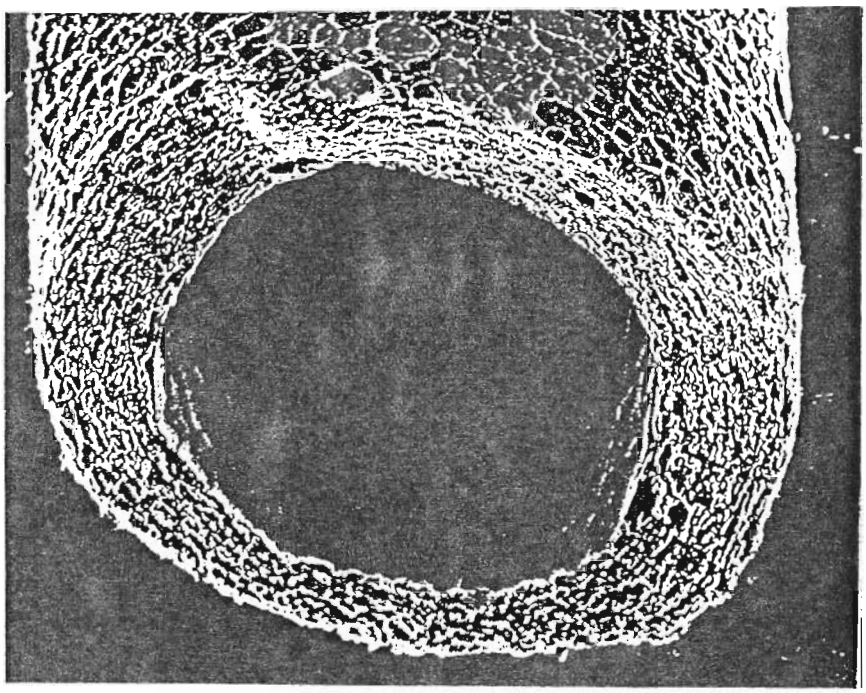


FIGURE 6. SEM of the cross-section of a 1.5 mm prosthesis.

TABLE I

GRAFT	4 WEEKS IMPLANTS	8 WEEKS IMPLANTS
PU	5/6 grafts patent, 0 aneurysmal.	3/6 grafts patent, 0 aneurysmal.
PU-FBN (50%)	3/6 grafts patent, 4/6 aneurysmal.	4/6 grafts patent, 5/6 aneurysmal.

diameter and involving 50% of the length of the graft (Figure 8). All patent, aneurysmal grafts were lined with at least some glistening neointima as described above, and in 4 cases this covered 80-100% of the luminal surface (Figure 8). No grafts in these experiments showed any perigraft hematomas or graft rupture. Light microscopic evaluation of the patent grafts demonstrated mild inflamatory responses to the materials with minimal collagenous encapsulation of

the grafts and few giant cells. The internal capsules of the PU grafts was approximately 30 cell layers thick and appeared composed

FIGURE 7. Gross macro-photograph of a patent, non-aneurysmal PU-FBN(50%) graft after 8 weeks implantation.

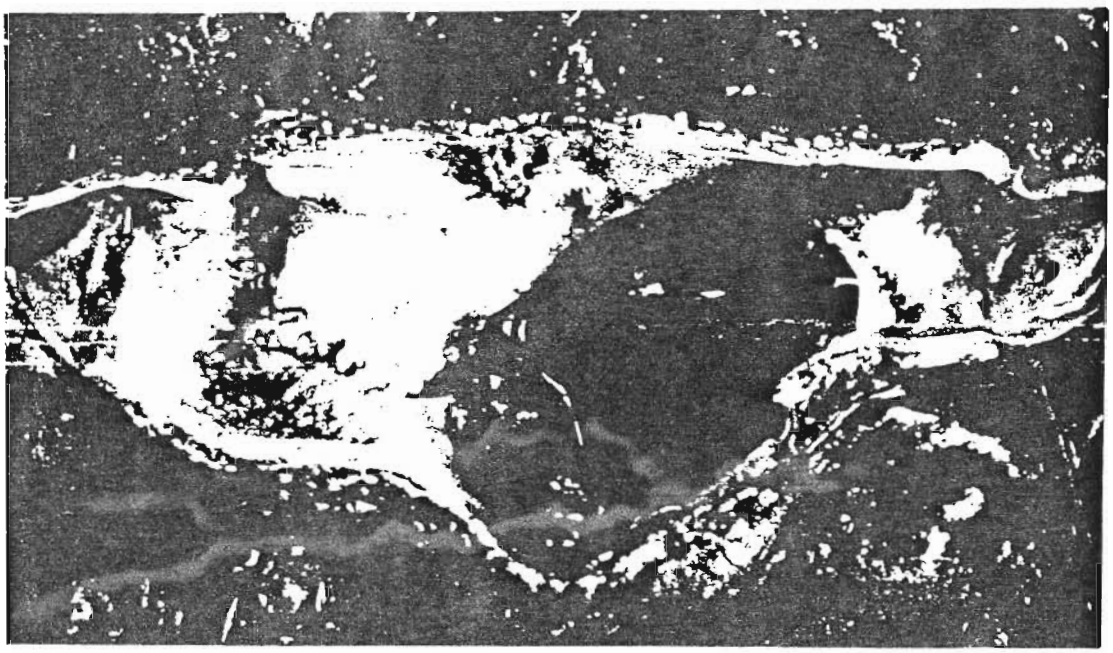


FIGURE 8. Gross macro-photograph of a patent, aneurysmal PU-FBN(50%) graft after 8 weeks implantation.

of a multilayered collagenous structure, almost completely lined with edothelial-like cells overlying multiple layers of myofibroblastic

cells. A localization of the inflammatory response to the external surface of the prostheses was also noted (Figure 9). The internal capsule of the PU-FBN(50%) grafts was 4-5 cells layers thick composed of little or no apparent myofibroblastic cells layer lined with a confluent layer of endothelial-like cells. The material itself was extensively invaded by organized collagenous tissue and was totally undetectable in many area of the grafts wall (Figure 10).

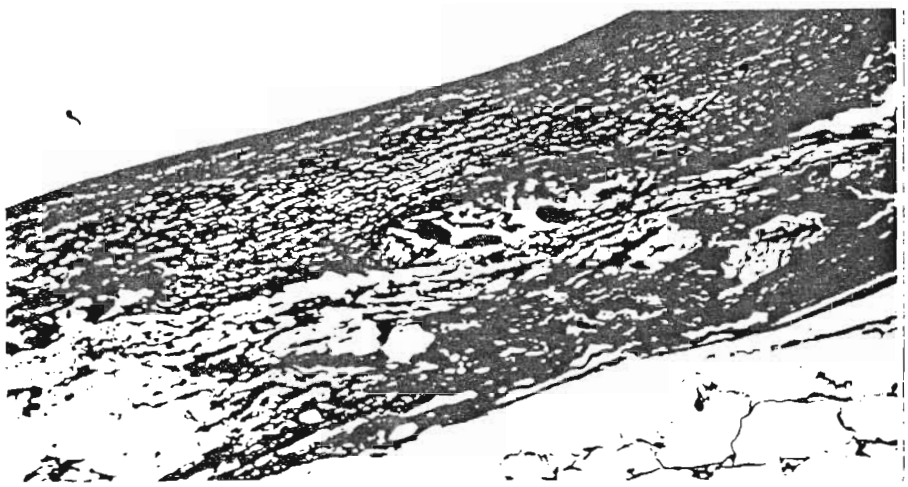


FIGURE 9. H&E stain, low power of a PU graft after 8 weeks implantation.

FIGURE 10. H&E stain, low power of a PU-FBN(50%) graft after 8 weeks implantation.

CLG-Based Bioartificial Polymers

The results of the thermal analyses of CLG, PAA, PAAM, PVAL and CLG-based blends are shown in Figures 11-15. In all these figures the

CLG thermogram is characterized by an endothermal peak centred at 381 K and extending over a range of 20 degrees. This peak corresponds to the denaturation transition of the protein (unfolding of the triple-helix structure). The enthalpy of denaturation is 34.4 J/g. This value, along with that of the denaturation temperature, correspond to those found by Finch and Ledward for dry bovine achilles tendon collagen [8].

In Figure 11 are plotted the thermograms obtained for CLG-PAA mixtures of different composition ratios. It can be noted that increasing the amount of PAA from 40 to 80% by weight causes a shift of the denaturation temperature of CLG from 381 K up to 396 K, while no second-order transition is observed for these systems. A glass transition temperature is observed for pure PAA at 387 K.

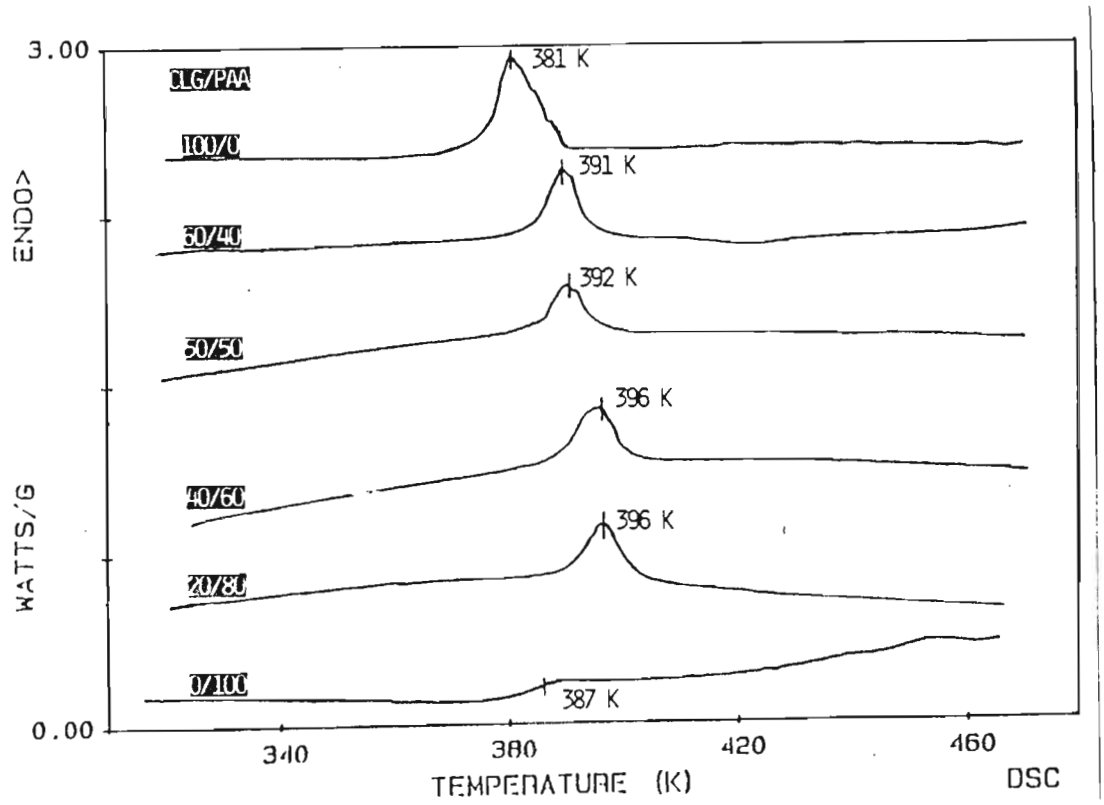


FIGURE 11. DSC thermograms of CLG/PAA blends (heating rate 5 K/min).

A similar behavior is shown by CLG-PAAM blends (Figure 12), where increasing the amount of PAAM from 25 to 75% by weight results in a shift of the denaturation temperature of CLG from 382 K up to 394 K. A

glass transition temperature can be noted for PAAM at 380 K.

The thermal characterization of CLG-PVAL mixtures is shown in Figure 13. The upper trace refers to the already described pure CLG,

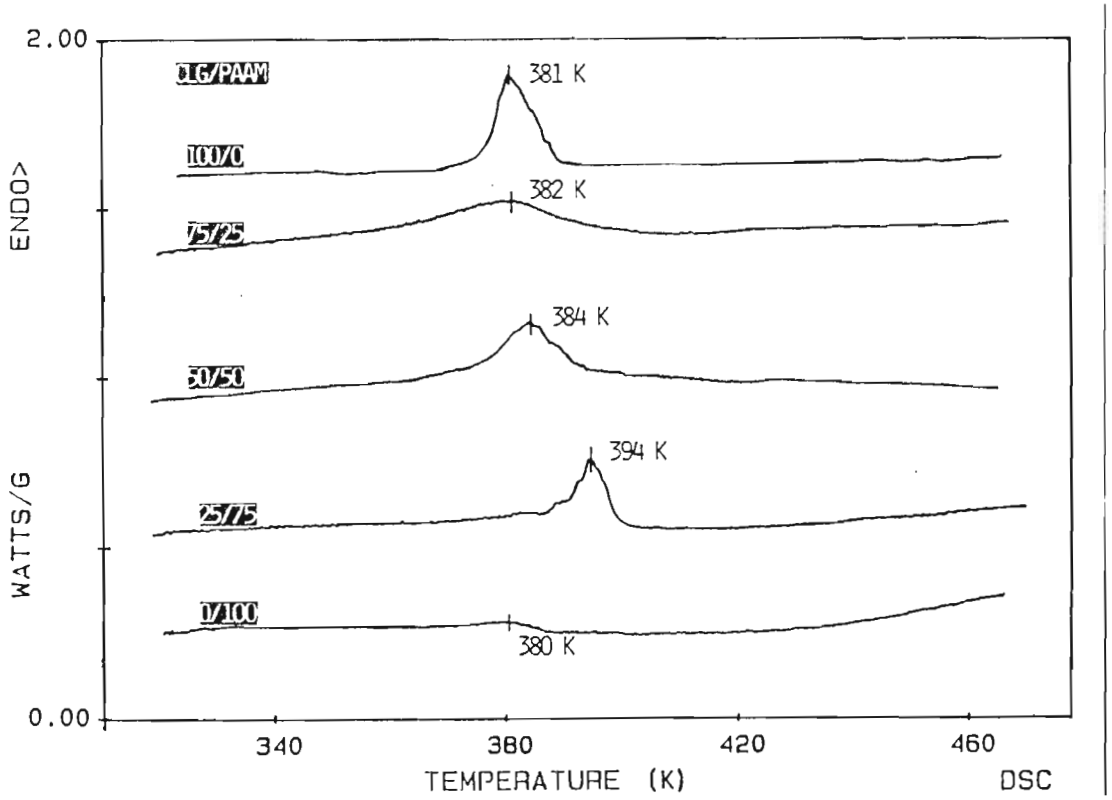


FIGURE 12. DSC thermograms of CLG/PAAM blends (heating rate 5 K/min).

while the other ones refer, from the bottom to the top, to pure PVAL and to three reference composition blends (25,50.75% by weight of PVAL). PVAL shows an endothermal peak centred at 483 K, corresponding to its melting transition. Increasing the amount of CLG in the mixtures the melting temperature of PVAL decreases up to 469 K (3:1 CLG-PVAL blend), while an increase in CLG denaturation temperature up to 404 K can be observed when the PVAL content goes from 0 to 75% by weight.

Figure 14 shows the variation of the denaturation enthalpy of CLG blends with PAA and PAAM, as a function of composition. The denaturation enthalpy is essentially unaffected by the presence of the synthetic polymer until its content reaches 40% and 50% for PAA and PAAM respectively. When the amount of the synthetic component is further increased, CLG denaturation enthalpy shows a marked rise,

especially for the CLG-PAA system.

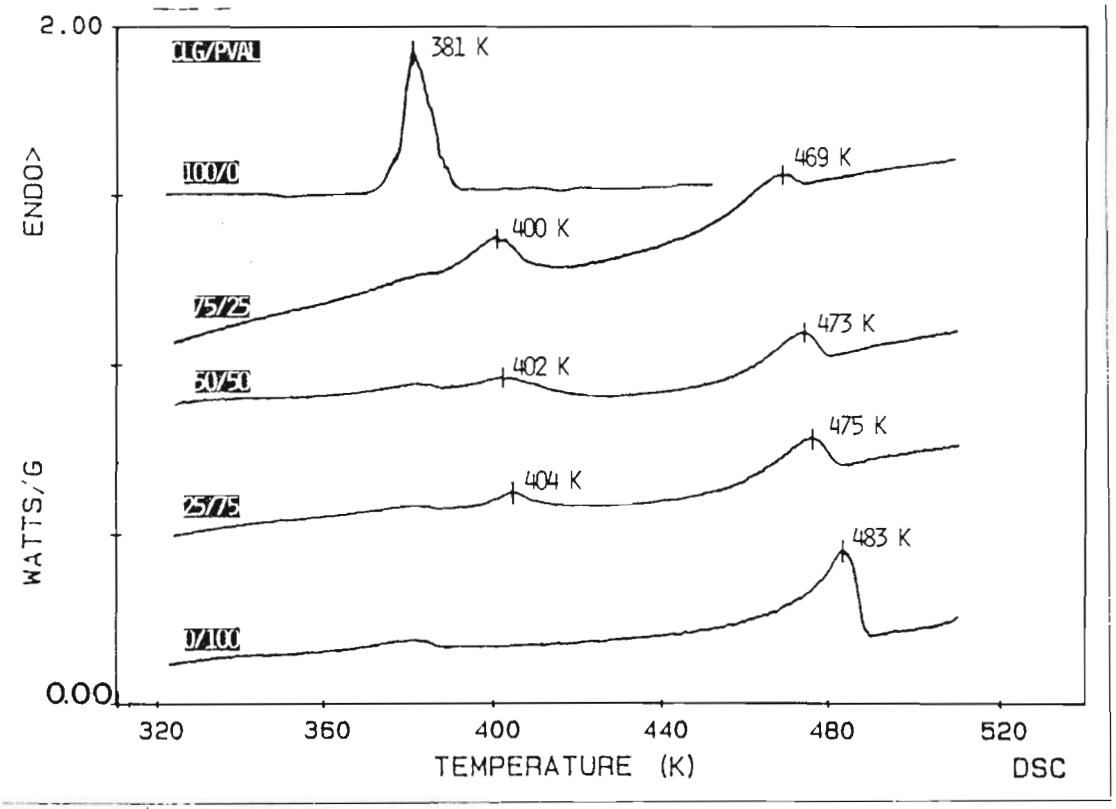


FIGURE 13. DSC thermograms of CLG/PVAL blends (heating rate 5 K/min).

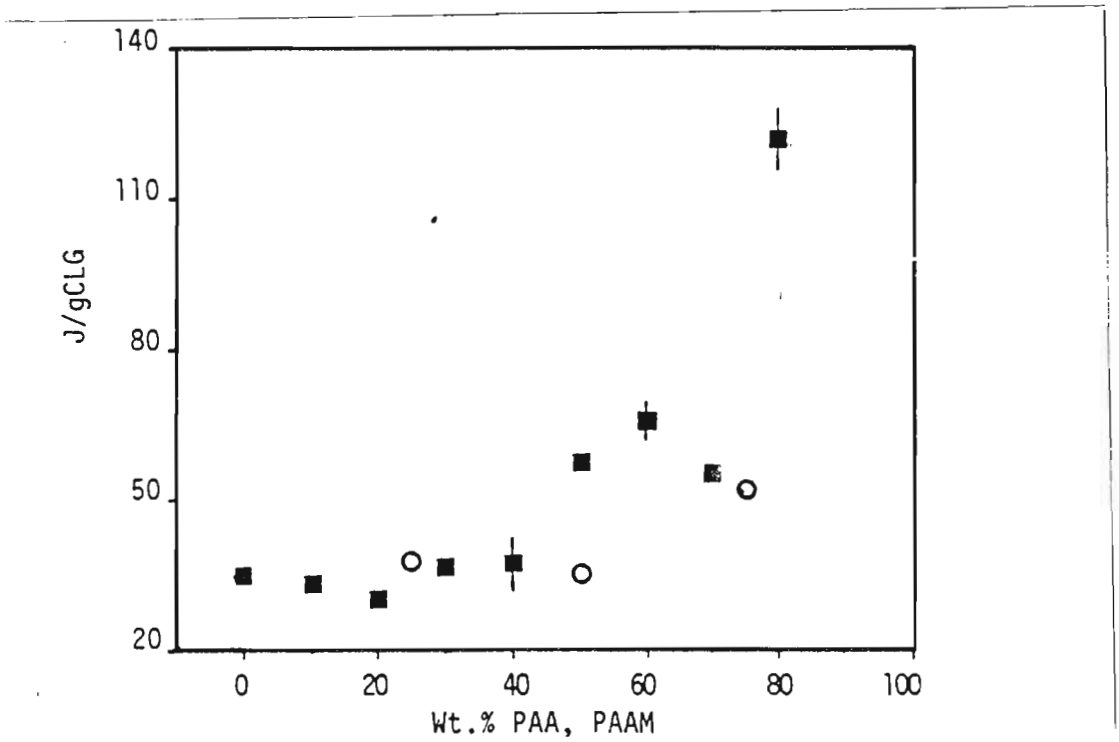


FIGURE 14. Variation of the denaturation enthalpy of CLG in mixtures with PAA (■) and PAAM (○), as a function of composition.

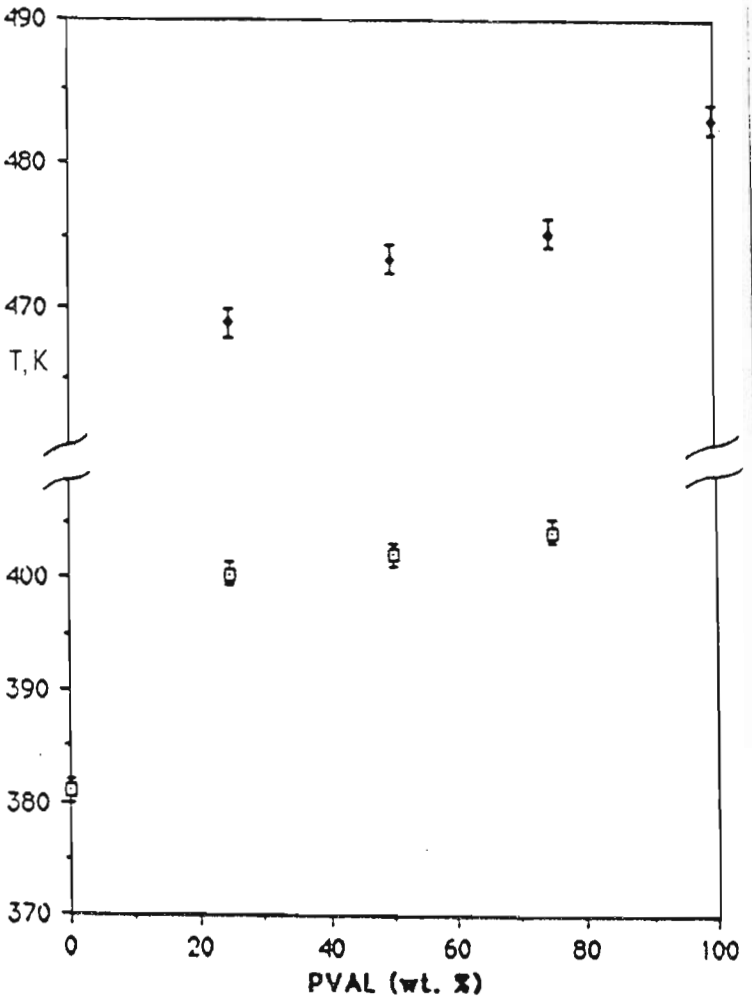


FIGURE 15. Variation of the melting temperature of PVAL (◆) and denaturation temperature of CLG (□) in CLG-PVAL blends, as a function of composition.

Figure 15 reports the temperatures of the peak maxima observed for CLG-PVAL blend thermograms versus blend composition. The plot indicates a marked increase of the denaturation temperature of CLG for blends containing a concentration of PVAL varying from 0 up to 25%, while denaturation temperature values remain quite constant with increasing PVAL content. The melting temperature of PVAL linearly decreases when the amount of CLG was increased.

SEM analysis of fracture surfaces of samples of the mixtures indicates that the overall morphology of these materials is affected by the chemical nature of the synthetic component. In particular blends with PAA and PAAM show a different morphology compared to pure polymers and a homogeneous distribution of the components (Figures 16-19).

These results could be explained by the occurrence of intermolecular interactions between polar groups of the macromolecular compounds [9].

CONCLUSIONS

The results of this study demonstrate that blends of biological and synthetic polymers can be used to prepare a new class of biomaterials,

called "bioartificial polymeric materials", which, through opportune techniques, can be processed to fabricate biomedical devices.

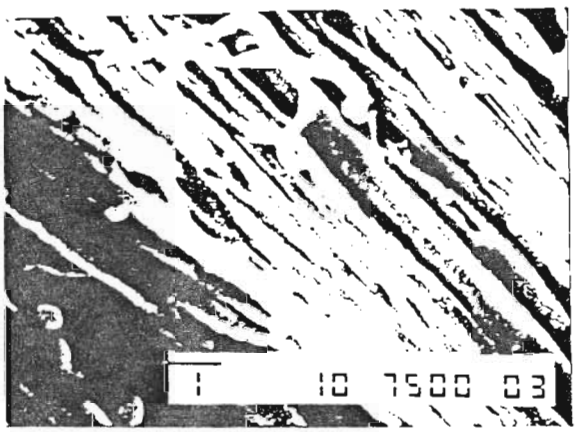


FIGURE 16. SEM micrograph of a fracture surface of bovine achilles tendon collagen.

FIGURE 17. SEM micrograph of a fracture surface of 1:1 CLG/PAA blend.

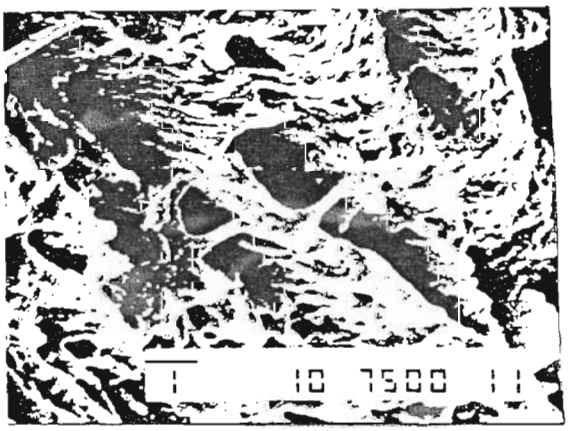


FIGURE 18. SEM micrograph of a fracture surface of 1:1 CLG/PAAM blend.

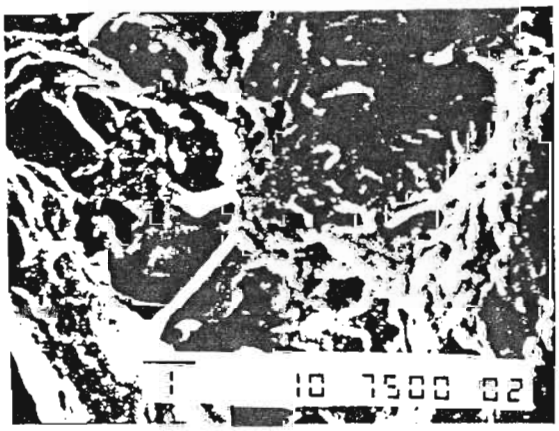


FIGURE 19. SEM micrograph of a fracture surface of 1:1 CLG/PVAL blend.

The major advantage of this approach is the ease these materials can be prepared in different composition ratio, which consequently results in materials showing physico-chemical, mechanical, and biocompatibility properties varying over a wide range. In the case of FBN based bioartificial polymers, FBNG has been suspended in a PU solution which, through a phase-inversion and spraying technique, has been processed to produce highly porous vascular prostheses. DSC analysis of the enzymatically cross-linked material shows a higher thermal stability of the PU-FBN blend in respects of pure FBN, indicating chemical interactions occurring at molecular level. Varying the content of FBN in the synthetic matrix, the PU tensile behaviors have been modified, and as a result we obtained materials which show stress-strain relationships ranging from those of a relatively elastic PU tube to those of a natural vessel. In vivo studies evidence a mature internal capsule and a better organization of the tissue in the regenerated arterial wall of PU-FBN grafts in comparison with PU grafts, suggesting a facilitating effect of FBN for tissue ingrowth. Regarding the CLG-based bioartificial polymers, CLG has been blended with various water-soluble polymers and used to prepare cast films. At this point of the research the materials are still in an early phase of characterization, however, DSC data consistently show a protective effect of the synthetic component in respect of the biological one, which is expressed by an increase of the denaturation temperature of CLG. This phenomenon is particularly pointed out for CLG-PAA and CLG -PVAL blends. A similar effect has been described by Shinoda et al. for mixtures of poly(acrylic acid) and poly(methacrylic acid) with polyaminoacids (10). It is conceivable that due to this characteristic the material might be easier to process at higher temperature than that used to prepare sheets, porous tubes, and hollow fibers with pure CLG.

The drawbacks and uncertainties of the bioartificial polymeric materials also need to be mentioned. Aneurysmal changes seen in the grafts containing 50% of FBN clearly show that this percentage is too great for arterial grafts. It is also of interest to note that while the PU polymer used to make these grafts is considered by other users to be biostable, it is rapidly degraded and resorbed in our experiments. The presence of fibrin in the matrix of the graft appears

to significantly hasten this degradation process. This might also be true for CLG-based devices which once implanted in the body might stimulate an intense tissue reaction leading to partial or total material degradation, which might or might not be desirable depending on the implant area and on the purpose the device has been designed.

At this point of the research we have little informations regarding the problems we raised, and fabrication of devices with an opportune range of FBN or CLG content to be characterized with in vivo experiments is planned.

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