Polyphosphazenes: Effect of molecular motions on thrombogenesis

W. M. Reichert
Macromolecular Research Center, University of Michigan, Ann Arbor, Michigan 48109
F. E. Filisko
Macromolecular Research Center, University of Michigan, Ann Arbor, Michigan 48109
S. A. Barenberg*
Department of Chemical Engineering, University of Michigan, Ann Arbor, Michigan 48109

The effect and interrelationship between primary (segmental backbone) and secondary (side chain) molecular motions on thrombogenesis, independent of morphological order/disorder, crystallinity, and/or associated water is elucidated using an amorphous hydrophobic polymer of poly[(trifluoroethoxy) (fluoroalkoxy)phosphazene], PNF. The results indicate that thrombogenesis for an amorphous hydrophobic polymer is sensitive and dependent on the degrees and types of primary and secondary molecular motions at the polymer interface.

INTRODUCTION

In defining and correlating the surface properties of polymeric materials to thrombogenesis, the critical surface tension, surface free energy, electrical conductivity, surface charge, surface topography, and surface chemistry have been proposed as important factors.[1] The aspects of primary (segmental backbone) and secondary (side chain) molecular motions on thrombogenesis, however, have received limited attention.[2,3] Merrill[2] originally postulated that molecular motions at the blood–material interface may influence the initial deposition of the blood plasma proteins. He likened these molecular motions "to a field of flowers blown by the wind." Barenberg[3] subsequently presented evidence that there is an effect of restricted and unrestricted side chain motions on thrombogenesis. In the above studies and in other studies,[4] however, the influence of the effect of molecular motions on thrombogenesis has been complexed by the presence of morphological order and/or disorder, degree of crystallinity, and amount of associated water. To date, no studies have been presented that isolate the effects of primary and secondary molecular motions on thrombogenesis from the above complexing factors.

* Present address: E.I. Du Pont, Experimental Station 323, Wilmington, DE 19898.

Journal of Biomedical Materials Research, Vol. 16, 301–312 (1982)
 CCC 0021-9304/82/030301-12$02.20

This article provides data on the effects and interrelationships between primary and secondary molecular motions on thrombogenesis, independent of morphological order and/or disorder and/or surface crystallinity. The polymer selected for this study was an amorphous hydrophobic polymer of poly[(trifluoroethoxy)(fluoroalkoxy)phosphazene], PNF,[5,6] (Fig. 1). The salient aspects of this polymer are as follows:

(1) Onset of the secondary molecular motions occurs between −160 °C and −120 °C.

(2) The side chain motion can be restricted by irradiation (ultraviolet, electron beam, or gamma).

(3) There is no apparent ultrastructure morphology.

(4) The PNF can be derivatized.[5]

(5) The polymer can be readily coated onto our extracorporeal test shafts[7] and irradiated thereon to alter the primary and secondary molecular motions.

(6) The contact angle measurements[8] indicate that the fluoroalkoxy pendant groups comprise the surface to be interfaced in the extracorporeal blood adsorption studies.

MATERIALS AND METHODS

The polymer used in this study was poly[(2,2,2 trifluoroethoxy)(telomer fluoroalkoxy)phosphazene], PNF, [CF_3CH_2O—,$CF_2H(CF_2)_xCH_2OPN$], supplied to us through the courtesy of Firestone Central Research.[9]

The polymer purification was accomplished by dissolving the PNF in Freon TA* followed by mixing in an equal volume of distilled deionized water, DDW, with the Freon polymer solution. The system was allowed to phase separate. The Freon layer was then eluted into hexane causing the polymer to precipitate out. The polymer was then vacuum dried. Solutions of the purified polymer (10% w/v) were prepared in acetone. Isotropic films were solvent cast in a closed environment in order to retard the rate of solvent evaporation and reduce surface contamination.

Ultraviolet irradiation of the PNF was done with a Hanovia 616A high-pressure mercury vapor lamp with an output dosage of 70 $\mu W/cm^2$ at a distance of 50 cm. The duration of exposure used in this study ranged from 0 to 30 h.

The dielectric measurements were performed on a automated difference dielectric system.[10,11] Films of the polymers were coated directly onto the cell

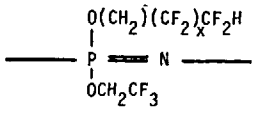


Figure 1. Poly[(trifluoroethoxy)(fluoroalkoxy)phosphazene]; x = 3, 5, 7.

* Miller Stephenson Chemical.

from acetone in a closed environment. The as-cast films were run, irradiated, and rerun. This then allowed us to observe the effects of irradiation on the same film. The temperature range investigated was from −200 °C to 100 °C over a frequency range from 0.234 to 20.0 kHz.

Different dielectric measurements were obtained by placing the unirradiated PNF spectra in the memory of the dielectric system and subtracting the unirradiated spectra from the irradiated.

Tensile stress measurements were performed on solvent-cast (10% w/v) films using an Instron Tensile Testor.

The amount of bound water associated with the PNF was measured using a Perkin-Elmer DSC II equipped with a subambient stage. The samples were scanned from 227 K to 303 K at 10 K/min under dry nitrogen. The heats of fusion of the sorbed water were calculated relative to the heats of fusion of indium.[12]

Mass spectroscopy of the gaseous by products produced from irradiating the PNF was performed using a Finnigan 4000 Mass Spectrometer.

Critical surface tension and attenuated total reflectance infrared spectroscopy, ATR, of irradiated and unirradiated solution cast PNF films were done in conjunction with Dr. Robert Baier.[8]

Angular resolved ESCA of the irradiated and unirradiated PNF surfaces was done in conjunction with Dr. Buddy Ratner.[13]

Morphological studies of the PNF were done on thin films of the polymer as cast from acetone (10% w/v) onto carbon-coated glass slides. The polymer-carbon thin films were floated off the glass slides in DDW and placed on either nylon (for x-ray dispersion studies) or copper grids, depending on the analysis to be performed.

Thin films of the polymer were cast from acetone onto carbon-coated glass slides. The films were placed in contact with isotonic saline for 4 h and then washed in DDW. A carbon film was subjected to the above saline wash treatment to serve as the experimental control.

Conventional and scanning transmission electron microscopy (CTEM and STEM) was done on a J.E.O.L. 100 C electron microscope. The scanning (secondary emission) electron microscopy was done on a J.E.O.L. U3 electron microscope.

The x-ray dispersion analysis was done on a J.E.O.L. 100 C equipped with a Princeton Gamma Tech detector and Nuclear Data software. The count times were on the order of 20 min at magnifications of 20,000, 30,000, and 50,000×.

The extracorporeal shafts,[7] 303 stainless steel and polypropylene, were prepared by dip coating the shafts into 10.0% (w/v) solutions of the polymer and allowing the excess to drip off. The shafts were inverted and placed in a closed system to retard the rate of solvent evaporation. This technique resulted in an isotropic coating of the polymer.

The animals used in these studies were conditioned male dogs of mixed breed. Access to the animals' circulatory system was accomplished via an acute shunt surgically implanted into the neck of the dog. The shunt was con-

structed of $^3/_{16}$ inch Silastic tubing anastomosed (via a silastic-coated stainless steel stint) to the carotid artery and jugular vein. Blood flow through the shunt was on the order of 1 L/min.

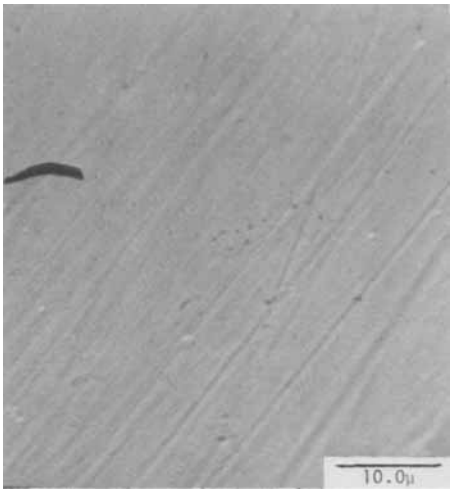
Twenty four hours prior to an experiment, 150 mL of blood was collected from the animal into a sterile Fenwal (450 mL) blood bag containing citrate-phosphate-dextrose. The platelet-rich plasma supernatant was then centrifuged at 1000 *g* for 15 min. The platelet-poor plasma was transferred, leaving a residual of 2 mL with each platelet pellet. The platelets were resuspended, to which 300–400 μC ^{111}In (Diagnostic Isotropes) was added. The system was incubated at ambient temperature for 60 min after which 10 mL of platelet poor plasma was added and centrifuged at 1000 *g* for 15 min. The platelets were resuspended in platelet-poor plasma and reinfused into the animal. Additionally, human ^{125}I labeled fibrinogen was injected into the animal 24 h prior to the experiment. The peak fibrinogen radioactivity after infusion was approximately 1×10^4 cpm/mL. No anticoagulants were used prior or during the experiments.

The PNF-coated shafts were assembled into the test chambers.[7] The test chambers were flushed with isotonic saline in order to displace the air interface. The blood flow through each of the chambers was adjusted to 200 mL/min using a Ward's doppler flow ultrasound cuff. The rotation of the shafts were maintained at 200 rpm. Under these conditions a laminar flow regime was maintained with a shear rate of 150 s^{-1}. Experiments were carried out for time periods of 60 min. After completion of each experiment, the chamber was flushed with isotonic saline.

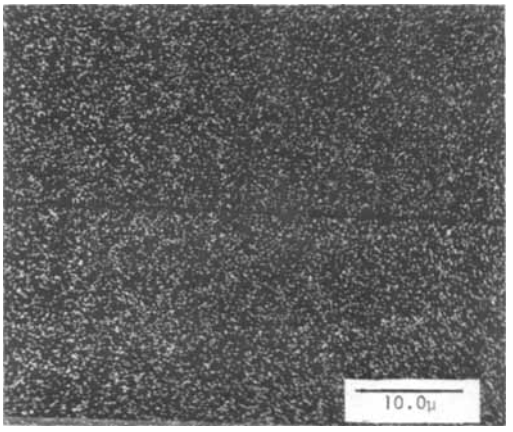
At completion of the test, the test shafts were removed and the relative radioactivity of each of the shafts was determined in a Nuclear Chicago Well counter. The shafts were fixed by placing them into buffered glutaraldehyde, sucrose, and sodium cocadylate in distilled deionized water, followed by staining in a 1.0% solution of buffered osmium tetroxide. Afterwards they were rinsed in distilled water, solvent exchanged, and critically point dried. The shafts were coated with a 20-nm layer of gold using a pulsatile sputterer; this prevented any local heating of the sample and surface charging of the sample during electron microscopy.

RESULTS

The PNF polymer morphology, Figure 2(a), as cast from acetone exhibited a homogeneous morphology with no apparent indication of any form of ultrastructure, i.e., no nodules or microdomains. The x-ray dispersion map, Figure 2(b), of the unirradiated PNF exhibited a homogeneous distribution of phosphorus with no apparent signs of aggregation or other impurities. The morphology of the 1-h and 24-h irradiated PNFs, as those above, also exhibited homogeneous morphologies.[12] The x-ray dispersion maps of the irradiated PNFs indicated no change in the phosphorus distribution. These results indicate that the blood-interfacing surface of the PNF was homogeneous with respect to ultrastructure morphology and surface chemistry. Additionally,



(a)



(b)

Figure 2. (a) Electron photomicrograph of PNF as cast from acetone. (b) Phosphorus x-ray dispersion map of Fig. 2(a).

the PNFs were morphologically clean at the scale investigated, i.e., there were no residual catalysts, salts, etc.

When the polymers were exposed to isotonic saline and washed it was found that the PNF sequestered ions.[14] The degree and amount is currently under investigation.[12]

The molecular motions of the PNF, Figure 3, (i.e., primary and secondary molecular relaxations) were documented by dielectric and dynamic (Rheovibron) mechanical measurements.[10,12] The −160 °C relaxation has been ascribed to the combined onset of the trifluoroethoxy, β', and fluoroalkoxy, β'', side chain motions.[10,15,16] The −50 °C relaxation has been ascribed[15] to the glass transition (segmental backbone) of the PNF.

In order to study selectively the effect of primary and secondary molecular motions on thrombogenesis, the PNF was subjected to low dose ultraviolet

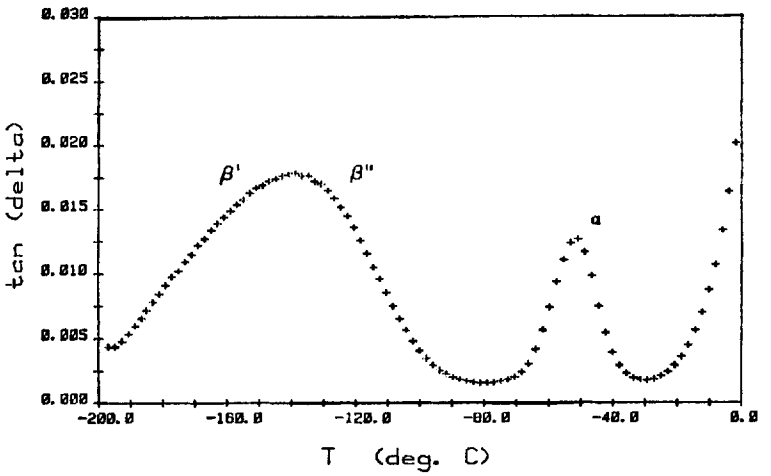


Figure 3. Dielectric spectra of PNF as cast from acetone; glass transition denoted by α, trifluoroethoxy and fluoroalkoxy side chain relaxations denoted by β' and β'', respectively.

irradiation, the same dose rate used in the extracorporeal studies. It was anticipated that this low dose treatment would selectively crosslink intramolecularly the pendant side chains followed at higher dose rates by intra- and intermolecular crosslinking.

As can be observed in Figure 4, the magnitudes of the α and β relaxations increased at 1 h ultraviolet irradiation followed by a decrease in magnitude at 30 h irradiation. The difference dielectric spectra, Figure 5, indicates that the two side chains were not equally affected by the ultraviolet light. At low exposures to UV irradiation (less than 6 h), the fluoroalkoxy side chains were selectively altered photochemically, while the trifluoroethoxy side chains

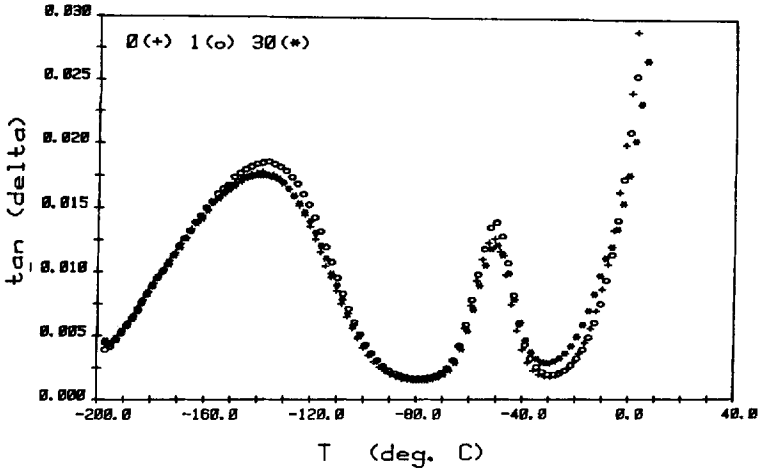


Figure 4. Dielectric spectra as a function of irradiation time. Note initial increase of β relaxation at 1-h irradiation followed by decrease at higher exposure levels.

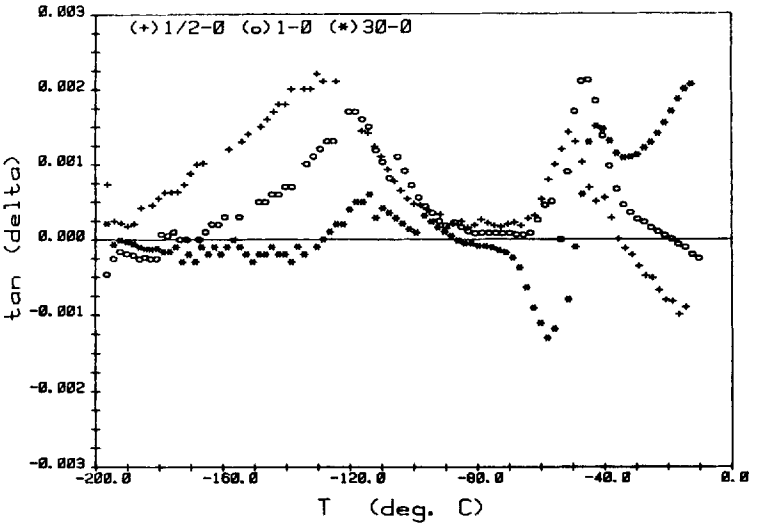


Figure 5. Difference dielectric spectra of the PNF as a function of irradiation time minus the unirradiated control.

remained relatively unaffected. Upon further irradiation (greater than 6 h) Figure 5, the pendant side chains underwent an intra- and intermolecular crosslinking as evidenced by the β decrease and α shift in the difference dielectric spectra. This is also supported by swelling studies[12] which show the 30-h irradiated sample swelling in acetone while the 0- and 1-h irradiated samples do not. It is therefore apparent that the pendant side chain motions are affected by ultraviolet irradiation and become restricted as a function of irradiation.

The results of the contact angle measurements (Table I), ATR and mass spectroscopy[12] of the irradiated and unirradiated PNFs indicate that there is no change in the surface chemistry, per se, nor is there irradiation-induced chain scission.

Preliminary analysis of the ESCA results[13] from irradiated and unirradiated PNF shows that the nitrogen-carbon and the phosphorus-carbon ratios remain relatively constant in the 0-, 1-, and 24-h irradiated samples. However, the fluorine-carbon ratio increases continuously ($0 < 1 < 24$) and the oxygen-carbon ratio of the 0- and 1-h irradiated samples are essentially the same, but slightly smaller than the 24-h irradiated sample. A more detailed analysis

TABLE I
Critical Surface Tension of the PNF as a Function of UV Irradiation

UV Irradiation Time (h)	Critical Surface Tension (dyn/cm^2)
0	16.5
1	14.4
24	15.6

TABLE II
Amount of Water as a Function of UV Irradiation

UV Irradiation Time (h)	mg bound water/mg polymer
0	0.01
1	0.01
24	0.05

of this data, as well as FTIR analysis of the PNFs will be presented in a collateral article.[11]

The tensile stress strain measurements,[12] not presented, indicate that the PNFs are becoming crosslinked upon irradiation as evidenced by the increase in the ultimate tensile properties.

Differential scanning calorimetry, DSC, was undertaken in order to ascertain the degree, if any, of bound water associated with the PNF polymer. As can be observed in Table II, the virgin PNF contained 0.01 mg bound water/mg polymer which increased to a maximum of 0.05 mg bound water/mg polymer. The reasons for the increase in bound water content as a function of irradiation is currently under investigation.[12] This change may be tied to the oxidation level of the polymer.

When the PNF polymer was exposed to canine blood for 60 min (Figs. 6–8, Table III), as a function of irradiation time dose, the relative thrombogenic responses were quite distinct. The virgin unirradiated PNF developed a large thrombus (Fig. 6) with no apparent signs of becoming limited. In contrast, the 1-h irradiated PNF developed a limited thrombus, such that in given island areas some platelet and leukocyte adhesion could be noted. The 24-h irradi-

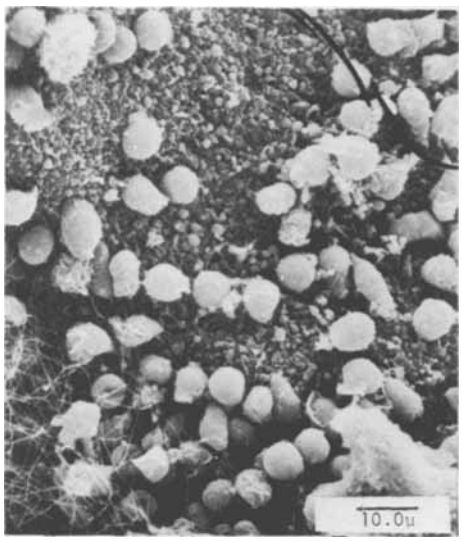


Figure 6. Scanning electron photomicrograph of unirradiated PNF exposed to canine blood for 60 min.

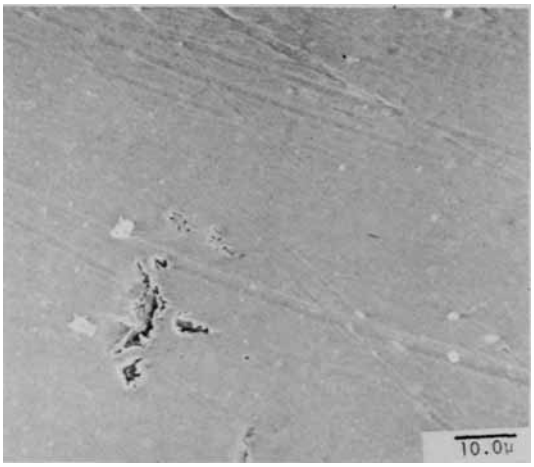


Figure 7. Scanning electron photomicrograph of 1-h irradiated PNF exposed to canine blood for 60 min.

ated PNF also exhibited morphologically a limited thrombogenic response like that of the 1-h irradiated sample. The labeled studies (Table III), however, indicated that the thrombogenic response of the 24-h irradiated PNF polymers was greater than that of the 1-h irradiated samples. The amount of fibrin and platelets, (Table III), of the 24-h irradiated polymer increased relative to the 1-h irradiated samples. Additionally, as can be observed in Table III, when the polymers were irradiated at all, the amount of labeled fibrin and platelets on the surface of the PNF polymers decreased substantially.

This decrease and subsequent increase in fibrin and platelet deposition appears concurrently with the observed changes in the interfacial secondary

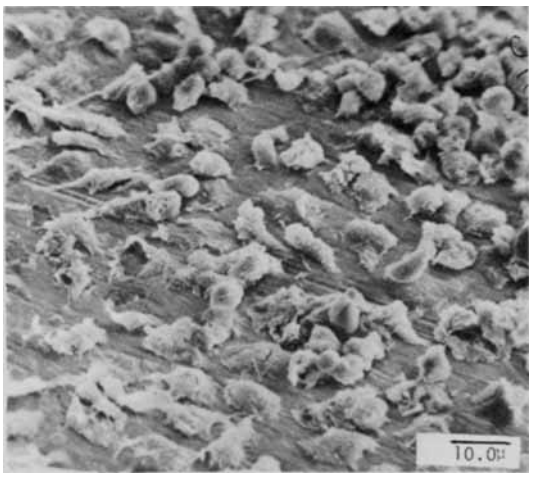


Figure 8. Scanning electron photomicrograph of 24-h irradiated PNF exposed to canine blood for 60 min.

molecular motions associated with the PNF polymers. This apparent interrelationship will be addressed below.

DISCUSSION

The results of the difference dielectric measurements, in conjunction with the morphological and x-ray dispersion observations, contact angle measurements, ATR (not presented), and ESCA measurements, indicate that in the extracorporeal studies the blood is exposed to essentially the same surface. The only apparent change is the difference in degrees and types of surface molecular motions. The surface molecular motions of the unirradiated PNF consisted of both the trifluoroethoxy and fluoroalkoxy side chains in the unrestricted state, whereas the surface molecular motions in the 1-h irradiated PNF consisted of the trifluoroethoxy side chains in the dynamic state with the fluoroalkoxy side chains partially restricted. In the case of the 24- and 30-h irradiated samples, the surface molecular motions of both the trifluoroethoxy and fluoroalkoxy side chains were partially restricted.

The extracorporeal results indicate that the initial adsorption of the plasma protein(s) and/or cellular elements onto the surface of the PNF polymers was mediated, in part, given the supra, by the relative degrees of surface molecular mobility associated with the fluoro pendant side chains. Specifically, when the PNF consisted of both the trifluoroethoxy and fluoroalkoxy side chains in an unrestricted state, coupled with the backbone motion, a large thrombus was observed (Fig. 6). When, however, the PNF was irradiated for 1 h, resulting in the partial restriction of the trifluoroethoxy molecular motion, a limited thrombogenic response was observed (Fig. 7). Upon further irradiation of 24 hours, where both the molecular motions of the trifluoroethoxy and the fluoroalkoxy side chains became restricted, a limited thrombogenic response was observed (Fig. 8). The radiolabeled studies (Table III), however, indicated an increased thrombogenic response for the 24-h irradiated sample relative to the 1-h irradiated PNF, but still substantially decreased relative to the unirradiated PNF. The reason for the variance in the thrombogenic response between the 1- and 24-h irradiated samples may be attributed to the increased amount of associated water in the 24-h irradiated sample. It has been shown[17-20] in studies on hydrophilic and hydrophilic/hydrophobic polymers that the degree and type of associated water influence hemocompatibility and platelet consumption. In this study it is possible that we are observing the initial thrombogenic effects attributed to the presence of structured/bound

TABLE III
Amount of Labeled Fibrin and Platelets on PNF-Coated Shafts after 60-min Exposure to Canine Blood as Function of UV Irradiation

UV Irradiation Time (h)	Fibrin/Clot (mg/clot)	Platelet/Clot $\times 10^{-8}$
0	2.3	63
1	0.1	7
24	0.2	20

water in conjunction with the variation in surface molecular mobility. It should also be noted that the presence and increase in the bound water as a function of irradiation time would tend to infer that the PNF is undergoing chain scission as a function of irradiation, exposing the carbonyl groups and thusly changing the surface chemistry of the surfaces being exposed in the extracorporeal studies. This does not, however, appear to be the case because the infrared results[8] do not show a carbonyl band nor do the mass spectroscopy results[12] indicate any low-molecular-weight chain scission products being formed upon irradiation. Additionally, the ESCA studies[13] suggest that the surface may be oxidized by ultraviolet irradiation for only the 24-h irradiated samples, and not for the 0- and 1-h samples. This oxidation may then explain the change in the bound water content. The steady change in the fluorine–carbon ratio as a function of exposure length indicates that the side chains on the surface are being effected by the ultraviolet irradiation. The above, in conjunction with the swelling studies,[12] indicates that the PNFs are undergoing intra- and intermolecular crosslinking as a function of irradiation in opposition to chain scission. Additionally, it should be noted that in both the virgin and 1-h irradiated samples the amount of bound water (Table II) was approximately the same, but the thrombogenic response (Figs. 6–8, Table III) was different. This then would imply that the observed decrease in thrombogenicity between the virgin and 1-h irradiated samples was independent of water content but dependent on the variation in molecular mobility of the surfaces. In the 24-h irradiated PNF, however, the bound water content increased to 0.05 mg bound water/mg polymer, suggesting we could be observing the initial effects of bound water on thrombogenesis, complexed with the variation in molecular mobility between the 1- and 24-h irradiated samples.

Therefore, in conclusion, the above results indicate that there is an initial effect of molecular motions on thrombogenesis independent of morphological order/disorder, crystallinity, and/or associated water (at the 0.01 mg bound water/mg polymer level). Whereas at higher levels of bound water (0.05 mg bound water/mg polymer), the effect of molecular motions on thrombogenesis is complexed by the presence of the bound water, as evidenced by the increased thrombogenic response.

This work was supported by a grant from N.I.H./N.H.L.B.I. 1 R01 HL 23288. Additionally, the authors wish to thank Richard Sicka and Firestone Research for their cooperation.

References

1. L. Vroman and E. F. Leonard, Eds., *The Behavior of Blood and Its Components at Interfaces*, Vol. 283, N.Y. Academy of Sciences, New York, 1977.
2. E. W. Merrill, "The Behavior of Blood at their Surfaces," *Ann. N.Y. Acad. Sci.*, **283**, 6–16 (1977).
3. S. A. Barenberg, J. S. Schultz, J. M. Anderson, and P. H. Geil, "Hemocompatibility: Macromolecular Motions and Order of the Polymer Interface," *Trans. Am. Artif. Intern. Organs*, **25**, 159–162 (1979).
4. E. W. Salzman, "Blood-material Interactions Lecture," Devices and Technology Branch, Contractors Meeting, Washington, D.C., 1979.

5. H. R. Allcock, *Phosphorus Nitrogen Compounds*, Academic, New York, 1972.
6. G. E. Singer, N. S. Schneider, and G. L. Hagnauer, "Polyphosphazenes: Synthesis, Properties and Applications," *Polym. Eng. Sci.*, **15**, 321–338 (1975).
7. J. S. Schultz, J. D. Goddard, A. A. Ciarkowski, J. A. Penner, and S. M. Lindenauer, "An *Ex Vivo* Method for the evaluation of Biomaterials in Contact with Blood," *Ann. N.Y. Acad. Sci.*, **283**, 494–523 (1977).
8. R. Baier, private communication.
9. Courtesy Firestone Central Research, Akron, Ohio.
10. W. M. Reichert, F. E. Filisko, and S. A. Barenberg, "Poly[(fluoroalkoxy)-phosphazenes]: Effect of Ultraviolet Irradiation," submitted.
11. W. M. Reichert, "Polyorganophosphazenes: Characterization and Hemocompatibility," Ph.D. dissertation, University of Michigan, Ann Arbor, MI, in preparation.
12. C. Garcia, J. M. Anderson, and S. A. Barenberg, "Hemocompatibility: Effect of Structured Water," *Trans. Am. Artif. Intern. Organs*, **26**, 294–298 (1980).
13. B. Ratner, private communication.
14. W. M. Reichert, F. E. Filisko, and S. A. Barenberg, "Hemocompatibility: Effect of Molecular Motions of the Polymer Interface," *Adv. Chem. Ser.*, in press.
15. T. M. Connelly, and J. H. Gillham, "Thermomechanical Transitions in Polyphosphazenes," *J. Appl. Polym. Sci.*, **20**, 473–488 (1976).
16. I. C. Choy and J. H. Magill, "Thermomechanical Transitions in Polyphosphazenes," *J. Appl. Polym. Sci.*, in press.
17. M. S. Jhon, J. D. Andrade, "Water and Hydrogels," *J. Biomed. Mater. Res.*, **7**, 509–522 (1973).
18. J. D. Andrade, H. B. Lee, M. S. Jhon, S. W. Kim, and J. B. Hibbs, "Water as a Biomaterial," *Trans. Am. Soc. Artif. Intern. Organs*, **19**, 1–7 (1973).
19. B. D. Ratner, A. S. Hoffman, S. R. Hanson, L. A. Harker, and J. D. Whiffen, "Blood Compatibility-Water Content Relationships for Radiation Grafted Hydrogels," *J. Polym. Sci.*, **66**, 363–375 (1979).
20. T. Okano, S. Nishiyama, I. Shinohara, T. Akaike, Y. Sakurai, K. Kataoka, and T. Tsuruta, "Effect of Hydrophilic and Hydrophobic Microdomains on Mode of Interaction between Block Polymer and Blood Platelets," *J. Biomed. Mater. Res.*, **15**(3), 393–402 (1981).

Received August 12, 1980
Accepted November 28, 1981