Thrombogenesis: An epitaxial phenomena. I.

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A semiempirical epitaxial model has been derived which correlates and interrelates the surface free energy, ultrastructural morphology, surface charge, surface chemistry, and surface molecular motions of a model triblock copolymer to thrombogenesis. This paper addresses the aspect of: (1) ultrastructure order versus disorder, (2) primary and secondary molecular motions, (3) surface and side chain chemistry, (4) thrombogenesis, and (5) the derived epitaxial model based on the above observations. This model can be extrapolated to include and predict the relative thrombogenic responses of various crystalline and semicrystalline polymeric substrates, i.e., aliphatic polyolefins, polyurethanes, and polypeptides.

INTRODUCTION

A semiempircal epitaxial model has been derived which correlates and interrelates the surface free energy, ultrastructure morphology, surface charge, surface chemistry, and surface molecular motions of a block copolymer to thrombogenesis. This paper addresses the aspect of: (1) ultrastructure order versus disorder, (2) primary and secondary molecular motions, (3) surface and side chain chemistry, (4) thrombogenesis, and (5) the resultant derived epitaxial model based on the above observations.

Epitaxial crystallization is defined as that of an oriented overgrowth of a crystalline phase upon a crystalline substrate. The term "epitaxy" literally meaning "on-arrangement" was introduced by Royer[1] who developed an early theory of epitaxial growth based on structural matching. The matching of crystallographic directions is sometimes accompanied by a match of crystallographic spacings.

The overall role of the substrate is to enhance the nucleation of a second crystalline phase by lowering the free energy by means of the force field near

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the surface. This force field is a function not only of the chemical constituents of the substrate, but also their geometric arrangement. The substrate acts not only as a heterogeneous nucleation catalyst upon which "normal" crystallization can occur, but may occasionally induce crystalline arrangements that are not observed under ordinary conditions. For example, polymers can crystallize epitaxially on alkali halide substrates above the temperature at which the normal single crystals would be disrupted in solution. Also, thermodynamically less stable crystal forms may be induced in the near-interfacial region if there exists a high degree of polymer molecular specificity with the surface template, i.e., a lattice match. A comprehensive review of polymer epitactic processes has been given by Mauritz.[2]

In a previous paper[3] we presented empirical evidence indicating a correlation between the effect of morphological order and macromolecular motions on the initial adsorption of the blood elements and ultimately on thrombogenicity. It is the intent of this paper to review, in part, our previous work in order to coherently present the epitaxial model which has been subsequently derived to the publication of our original work. The precept with which we will be presenting is that morphologically ordered polymeric systems, of given side chain chemistry, can sequester ions, which can subsequently serve as an ionic array/template for protein epitaxial crystallization.

The polymeric system used in this study was a semi-crystalline hydrophobic triblock copolymer of poly[(γ-benzyl-L-glutamate)$_{160}$ (acrylonitrile/butadiene)$_{60}$ (γ-benzyl-L-glutamate)$_{160}$], $A_{160}B_{60}A_{160}$, synthesized and characterized in our laboratories[4,5]. The salient aspects of this triblock copolymer are: (1) the ultrastructure morphology of the copolymer (long range order vs. short range order, i.e., phase separated versus phase mixed) can be controlled through solution casting from selected solvents, (2) the onset of secondary molecular motion (PBLG side chain) occurs between 12–25°C, (3) the PBLG side chain motion can be restricted by casting the copolymer from a nonpreferential solvent, (4) the pendant side chain groups of the PBLG block copolymer are projecting normal to the helical backbone, and (5) the copolymer can be extracorporeally evaluated with strict control of morphology and molecular motion.

MATERIALS AND METHODS

The polymer used in this study was a triblock copolymer, $A_xB_yA_x$, of poly[(γ-benzyl-L-glutamate)$_{160}$(acrylonitrile/butadiene)$_{60}$(γ-benzyl-L-glutamate)$_{160}$] synthesized by reacting a primary amine capped random copolymer of butadiene/acrylonitrile, ATBN,* with γ-benzyl-L-glutamate-*N*--carboxy anhydride, BLG-NCA, as described by Katchalsky.[6]

Dilute solutions (0.1% w/v) of the copolymer were prepared from dioxane and chloroform. The dioxane and chloroform systems represented reasonable examples of phase separation and phase mixing, respectively. Concentrated solutions (1.0% w/v) of the copolymer were prepared from dioxane and

* Courtesy of B.F. Goodrich Chemical Company, Brecksville, OH.

chloroform for use in both the physical property and extracorporeal studies.

The dynamic mechanical spectroscopy studies were done using a torsion (braid) pendulum.[7,8] The temperature range investigated was from 90–325 K. The samples were prepared by dipping fiberglass braids into dilute solutions of the copolymers and allowing them to air dry. The dried braids were placed in the pendulum and equilibrated under vacuum for a period of 24 hr. The dampening measurements were conducted in a helium atmosphere and were equilibrated at each temperature before each measurement.

Morphological and electron diffraction studies were done on thin films of the copolymer as cast onto carbon-coated glass slides in a solvent atmosphere. The thin films were vapor stained with osmium tetroxide.

Conventional and scanning transmission electron microscopy was done on a J.E.O.L. 100C electron microscope. The scanning electron microscopy was done on a J.E.O.L. U3.

The extracorporeal shafts, #303 stainless steel and polypropylene,[9,10] were prepared by dip coating the shafts into 1.0% w/v solutions (dioxane and chloroform) of the copolymer and allowing the excess solution to drip off. The shafts were inverted and placed in a closed system, retarding the rate of solvent evaporation. This technique resulted in an isotropic coating of the copolymer.

The animals used in these studies were conditioned male dogs of mixed breed. Access to the animals' circulatory system was accomplished initially via a chronic arteriovenous (currently via an acute) shunt surgically implanted in the neck of the dog. The shunt was constructed of 3/16 in. Silastic tubing anastomosed to the carotid artery and jugular vein. Blood flow through the shunt was on the order of 1 liter/min.

Twenty-four hours prior to an experiment, 500 ml of blood was withdrawn from the animal; the platelets were separated, washed, and labeled with ^{51}Cr. The labeled platelets with the erythrocytes were reinfused. The peak platelet radioactivity after infusion ranged from 10^2 to 10^3 cpm/ml of whole blood. Additionally, human ^{125}I labeled fibrinogen was injected into the animal 24 hr 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 experiment.

The copolymer coated shafts were assembled into test chambers. The test chambers were flushed and filled 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 ultrasound flow cuff. The rotation of the shafts were maintained at 200 rpm. Under these conditions a laminar flow regime (profile) was maintained. Experiments were carried out for periods of 5, 10, and 60 min. The 5 and 10 min experiments, not presented in this paper, were used to observe the initial deposition. After completion of each experiment the chamber was flushed with isotonic saline.

At the completion of the test, the 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. After 24 hr, the shafts were postfixed with buffered glutaraldehyde, sucrose, and sodium cocadylate in distilled deionized water, followed by staining in a 1.0% solution of buffered osmium tetroxide. Afterward they were rinsed in distilled deionized water and exchanged into an ethanol medium and finally with an amyl acetate medium. The exchanged shafts were critically point dried and coated with a 20 nm layer of gold using a pulsatile sputterer; this prevented any local heating of the sample. The critically point dried coatings were removed, embedded in Spurr (via propylene oxide) and ultramicrotomed on a Sorvall MT-2B with a diamond knife. The unstained (osmocated) thin sections were carbon coated and examined by conventional and scanning transmission electron microscopy.

RESULTS

Morphology

In Figure 1 the copolymer morphology, as cast from chloroform (nonpreferential solvent), exhibited a homogeneous morphology indicative of phase mixing between the ATBN and the BLG segments of the copolymer. The ATBN midblock domains were on the order of 15 nm in diameter. When the copolymer was cast from dioxane (Fig. 2) the morphology consisted of lamellar domains; this being indicative of phase separation between the ATBN and BLG segments of the copolymer. In the dioxane cast films the ATBN midblock layers were on the order of 15 nm thick while the alternating BLG layers were on the order of 50 nm thick.

Electron diffraction

The *d* spacings of the electron diffraction patterns of the above films indicated that the BLG segment of the copolymer was helical.

Dynamic mechanical spectroscopy

The dynamic mechanical properties were determined for the copolymer as cast from dioxane and chloroform. The predominate molecular relaxation occurred at 298 K. This relaxation has been ascribed to the onset of motion (side chain glass transition) of the entire side chain of BLG. The onset of molecular motion of the PBLG backbone[11] and/or disruption of the stacks of the benzene rings in the side chains[12] occurs at 408 K; in either case, the backbone conformation is assumed essentially rigid below 408 K. The point of this discussion is to establish that the only molecular motion occurring in the copolymers over the physiological temperature range affecting hemocompatibility are those that can be ascribed to the onset of side chain motion of PBLG.

Of particular interest in this aspect of the study was the significant differ-

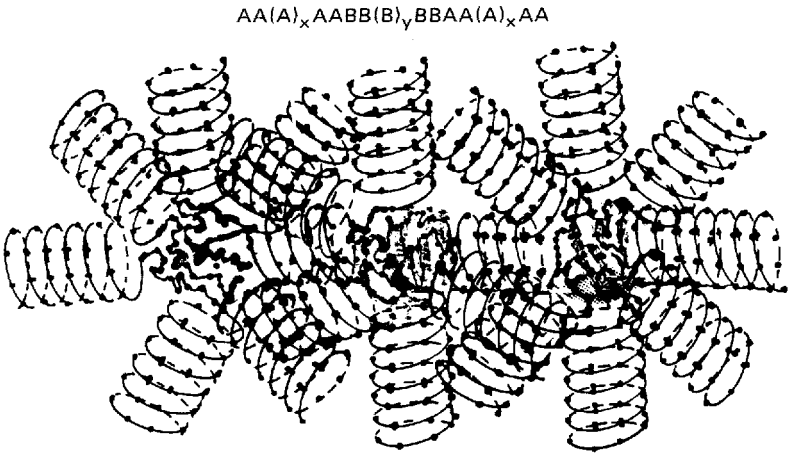


Figure 1. Electron photomicrograph of phase mixed chloroform cast copolymer (black areas ATBN, white areas PBLG). Above: schematic molecular model of triblock copolymer "A" PBLG segment, "B" ATBN midblock segment. Figures 1–4 reprinted with permission of *Trans. Amer. Soc. Artif. Int. Organs.*

ences observed in the spectra of the chloroform cast copolymer as compared to the dioxane cast copolymer. When the copolymer was cast from dioxane (Fig. 3) a large single relaxation occurred at 298 K, corresponding to the PBLG side chain glass transition. However, when the copolymer was cast from chloroform, the PBLG side chain relaxation underwent a splitting (this splitting

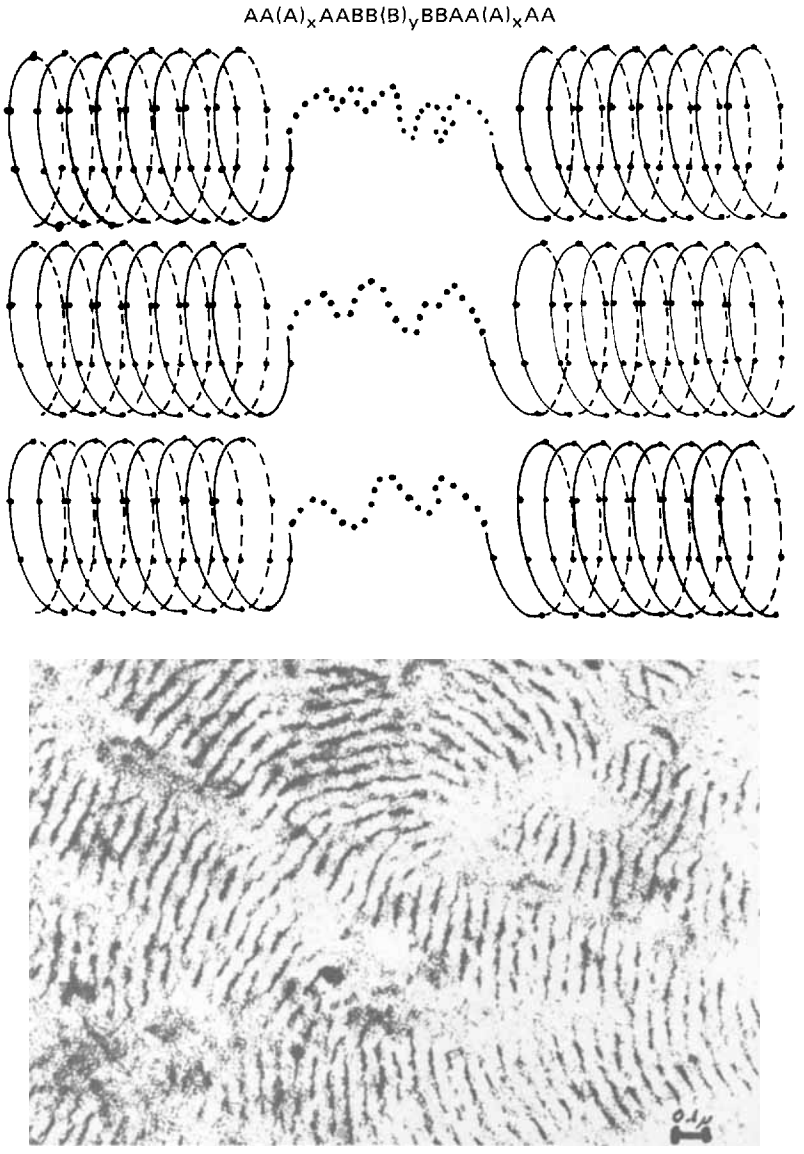


Figure 2. Electron photomicrograph of phase separated dioxane cast copolymer (black areas ATBN, white areas PBLG). Above: schematic molecular model of triblock copolymer "A" PBLG segment, "B" ATBN midblock segment.

has also been documented by dielectric and fourier transform infrared spectroscopy[4,5]), with a new dispersion maxima being observed at 315 K. This change then infers that at physiological temperatures the motion of the side chains of the chloroform cast copolymer are restricted (static), whereas the side chain motion of the dioxane cast copolymer is not, i.e., unrestricted (dynamic) at physiological temperatures.

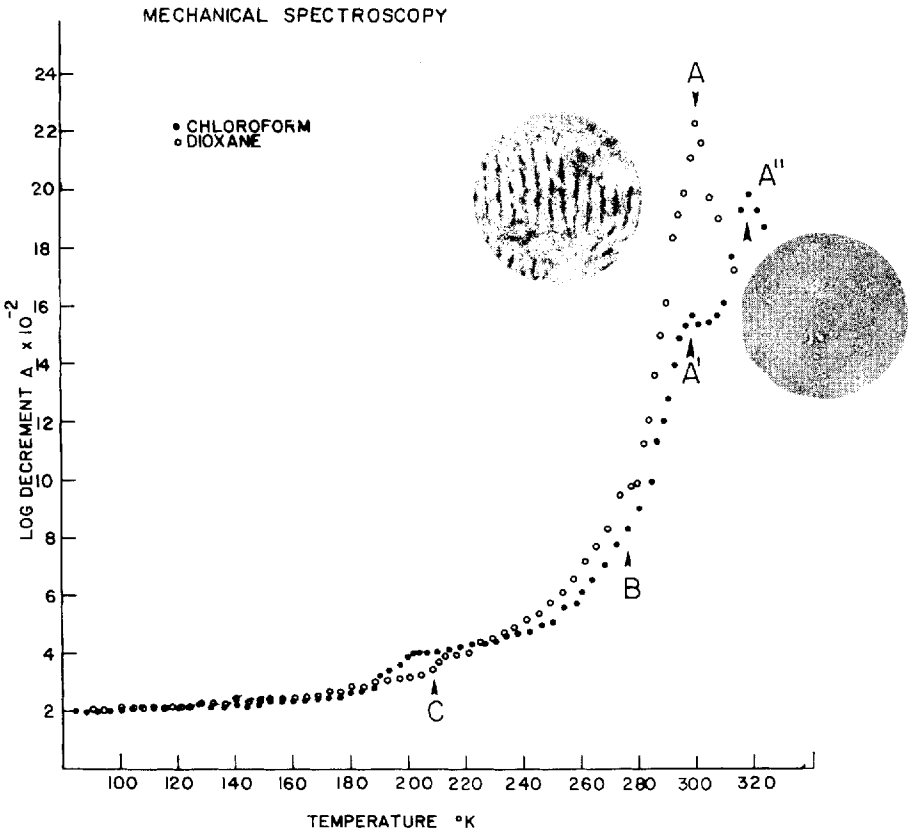


Figure 3. Dynamic Mechanical Spectra of the copolymer as cast from dioxane, o, and chloroform, o. A (dioxane), A′ and A″ (chloroform) onset of side chain motion; B, interfacial relaxation; C, glass transition temperature of ATBN.

This type of side chain motion (unrestricted and restricted), in conjunction with morphological order and/or disorder has been previously postulated[13] and demonstrated[3] to influence the initial sorption of the plasma protein(s) and subsequent interaction with blood. These interaction effects in conjunction with the results of the extracorporeal experiments will be discussed below.

Extracorporeal evaluation

When the copolymers were exposed to canine blood for 60 min (for 5 and 10 min results, see ref. 3) the dioxane cast copolymer developed a large thrombus, Figure 4(a), with no apparent signs of becoming limited. In contrast, Figure 4(b), the chloroform cast copolymer developed a limited pseudoneointima primarily composed of fibrin with no other apparent cellular interactions occurring. These series of experiments were replicated using different dogs and different substrate shafts, i.e., stainless steel and polypro-

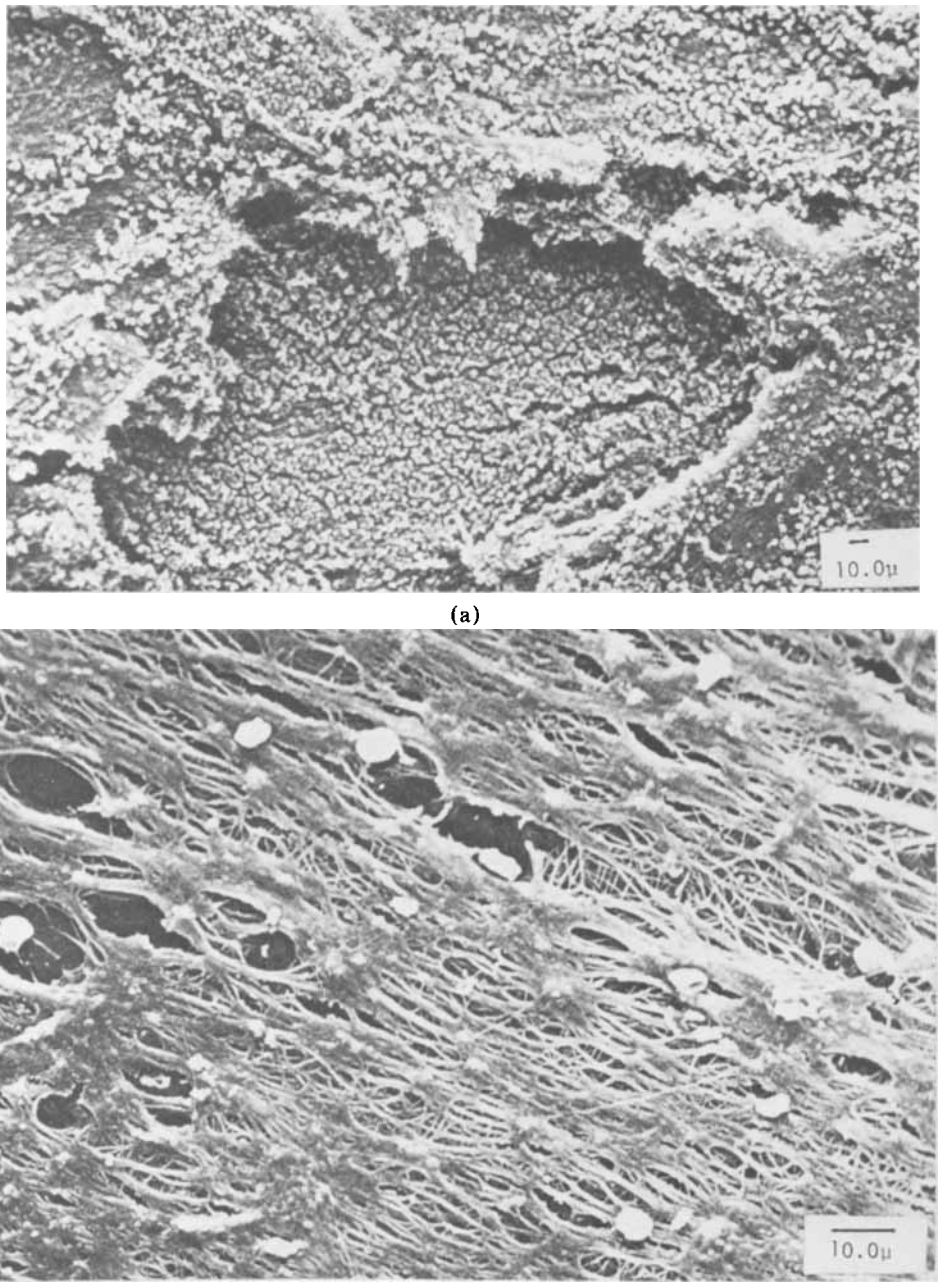


Figure 4. (a) Scanning electron photomicrograph of dioxane cast copolymer exposed to canine blood for 60 min. (b) Scanning electron photomicrograph of chloroform cast copolymer exposed to canine blood for 60 min.

pylene, with consistent reproducibility.

The above blood exposed copolymers were ultramicrotomed in cross section. As can be observed in Figure 5(a), the dioxane cast copolymer exhibited a

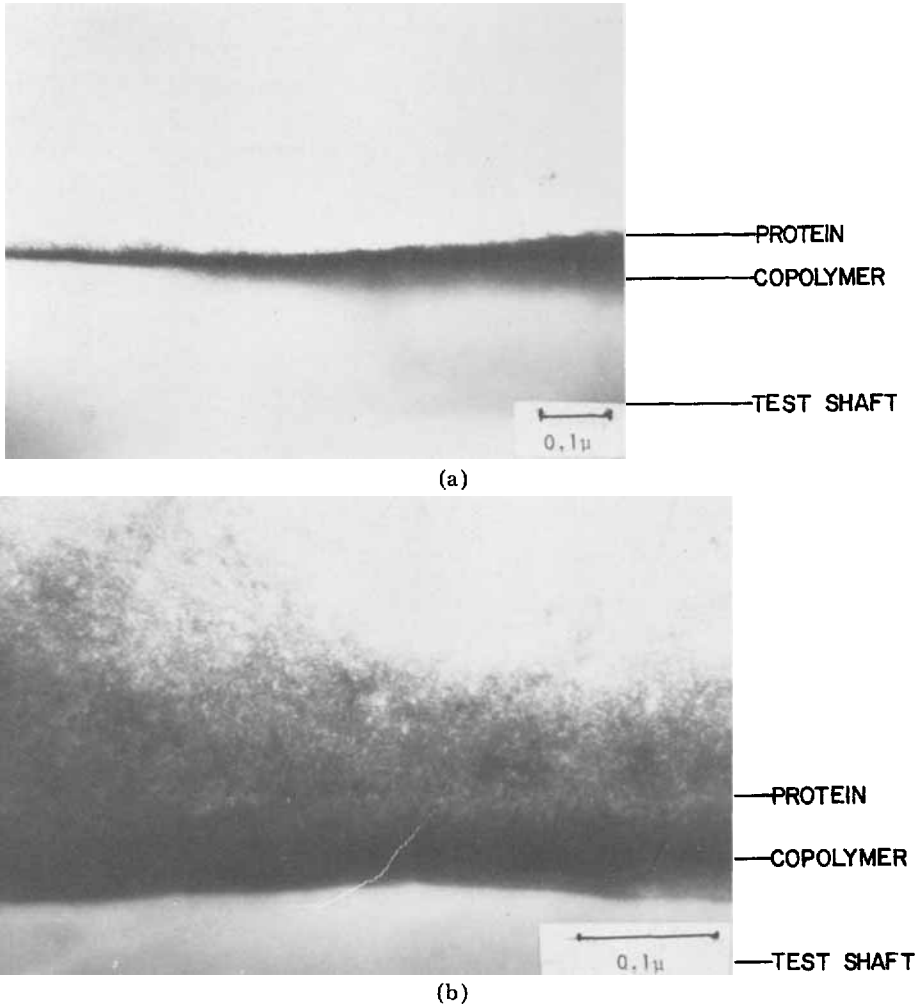


Figure 5. (a) Transmission electron photomicrograph of ultramicrotomed cross section of dioxane cast copolymer exposed to canine blood: note 10 nm osmophilic layer. (b) Transmission electron photomicrograph of ultramicrotomes cross section of chloroform cast copolymer exposed to canine blood: note 50 nm osmophilic layer.

tightly bound 10 nm osmophilic layer, whereas the chloroform cast copolymer, Figure 5(b), exhibited a loosely bound 50 nm osmophilic layer.

DISCUSSION

The above results indicate that an epitaxial model can be derived to correlate the above data. The model, at this juncture, takes into account the differences between the two types of secondary side chain (restricted/static and unrestricted/dynamic) molecular motions observed, the ultrastructural differences between the phase separated (ordered) and phase mixed (disordered) morphologies of the block copolymers, the electronegativity of the side chain

carbonyl oxygens, the possible ionic clustering effects due to the above electronegativity and the surface chemistry of the copolymers. The model does not take into account any effects due to the streaming potential and/or local surface pH differences. These points, as well as the general applicability of the model will be addressed in a subsequent paper.

The model is divided into four parts: (1) definition of the surface to be interfaced with blood, (2) the mode of the plasma protein(s) and/or electrolyte adsorption, (3) relaxational motion of the blood interfacing pendant side chains, and (4) protein denaturation and/or liquid crystalline order.

Therefore, the first aspect of the model is the definition of that surface* which is to be interfaced with blood. The dioxane cast phase separated copolymeric surface, Figures 6 and 7(a), represents a dynamic quasiordered hydrophobic array whereas the chloroform cast phase mixed copolymeric surface, not pictured, represents a static disordered hydrophobic array. The second aspect of the model incorporates the two concurrent types of adsorption occurring at the surface (1) that of the plasma proteins(s) and (2) that of either ions and/or polyelectrolytes. The sorption of the protein(s) occurs, in part, by a two step process. The proteins, Figure 7(b), would initially hydrophobically bond to the exposed phenyl groups in a metastable state [see arrow, Fig. 7(b)] and (2) the proteins in the metastable state would then surface diffuse to a lower free energy state located in the potential trough between the benzyl ester side chains [see arrow Fig. 7(c)]. These proteins would then align in a liquid crystalline fashion with the hydrophobic portion of the proteins being hydrophobically bonded to the phenyl groups and the hydrophilic portion of the sorbed protein(s) being exposed to the blood interface; this being the case for the dioxane cast phase separated copolymer. In the case where there is not an ordered array, as in the chloroform cast copolymer, the sorbed protein(s) would not sorb in an ordered array nor form a liquid crystal type of

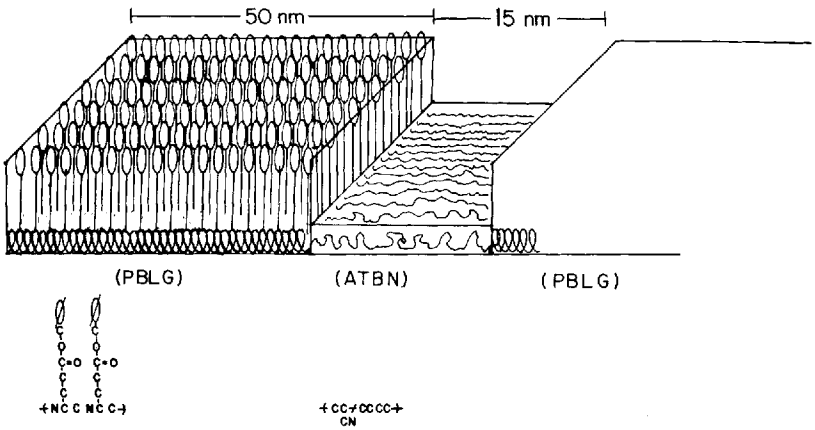


Figure 6. Schematic representation of dioxane cast copolymer.

* It should be noted that the ESCA work of O'Malley et al.[14] on triblock copolymers indicate a lateral phase separation, whereas our morphological (thin film) observations and electron diffraction studies indicate a horizontal phase separation for the triblock copolymers used in this study.

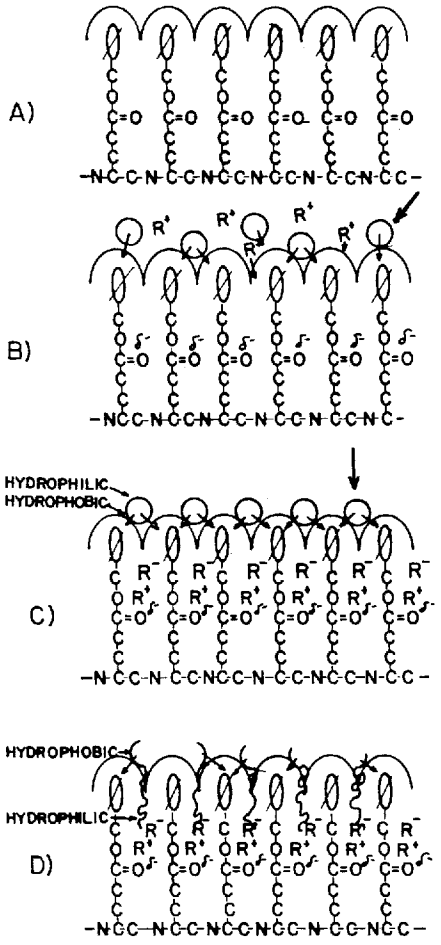
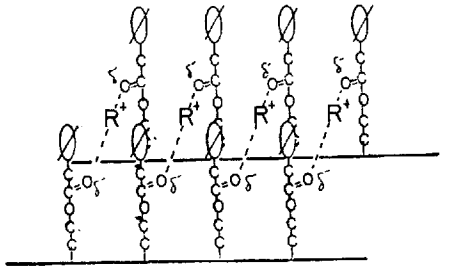


Figure 7. (a) Schematic representation of PBLG segment of the copolymer in cross section. (b) Schematic representation of PBLG segment of the copolymer in cross section: Spheres represent the initial sorption of the protein(s), R's represent sorption of the ions, arrow depicts the hydrophobically bonded protein in a metastable state. (c) Schematic representation of PBLG segment of the copolymer in cross section: Spheres represent hydrophobically bonded proteins, R's represent the ions and counterions. (d) Schematic representation of PBLG segment of the copolymer in cross section: Open ended spheres represent denaturation, R's ionic array. (e) Schematic representation of PBLG segment of the copolymer: R's representing immobilized ions which serve as the subsurface ionic array.

surface. The third aspect of the model takes into account the relaxational motion of the side chains and the electronegativity of the carbonyl oxygen. In that the side chain mobility of the solution interface may facilitate ionic diffusion to the proximity of the electronegative oxygens, Figures 7(c) and 7(d).

In the vicinity of these oxygens, the electrostatic potential energy of cations, e.g., Na^+,K^+,Ca^+, will be most favorable. As a result of this one might envision



E)

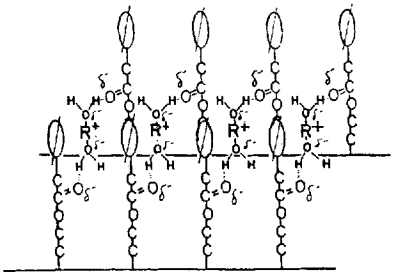


Fig. 7. (*Continued from previous page.*)

coordinations of a number of carbonyl groups about these cations, Figure 7(e), either directly or through hydrogen bonded water molecules. Additionally, since the macromolecular side chains are in an ordered packing mode, one might tentatively hypothesize that (assuming that the sodium ions are reasonably immobilized) a relatively ordered subsurface cationic array will exist.

It is then at this step that the differences between the effects of surface chemistry and secondary molecular motions as dictated by morphological order can be observed on thrombogenesis. In the case of the sterically ordered substrate, in conjunction with the subsurface cationic array, the sorbed proteins can assume a liquid crystalline (and/or paracrystalline state) and subsequently be subjected to further perturbations. Whereas in the case of the disordered substrate the sorbed proteins can not assume a paracrystalline state and subsequently will not, per se, be subjected to any further conformational changes. These differences and evidence will be discussed and presented below.

In the final state of the thrombogenic model two events may occur: the first is that the adsorbed protein(s) will undergo denaturation as a result of the subsurface hydrophilic cationic array, causing the hydrophilic portion of the protein to partially bury itself in between the side chains, thusly exposing the hydrophobic portion of the protein chain to the blood interface. The other consideration is that the adsorbed proteins are aligned on the substrate in a liquid crystalline fashion, which would serve as an ionic epitaxial template to the other plasma proteins. This then would result in a developing ordered

ionomeric clot. In the final case of the nonthrombogenic model there is not steric nor subsurface cationic ordered array, therefore the adsorbed proteins will remain in their adsorbed native conformation, presumably with the hydrophilic portions extending into the biologic interface.

The initial evidence for this model can be observed in Figures 5(a) and 5(b). When the ordered copolymer was exposed to canine blood a 10 nm bound osmophilic proteinaceous layer was observed. Whereas when the disordered copolymer was exposed under the same conditions, Figure 5(b), a 50 nm loose osmophilic layer was observed. It is presumed, a priori, at this juncture that the 10 and 50 nm bound layers are a result of the epitaxial (steric and cationic) and nonepitaxial aspects of the above model, respectively. The thickness of the loosely bound layer being a result of the sorption/desorption of other plasma proteins. It is important to note that these initially adsorbed layers mediate[15] the resultant thrombogenic response of these copolymers as can be evidenced by Figures 4(a) and 4(b). In order to further substantiate the above observations, detailed studies utilizing high resolution electron microscopy in conjunction with x-ray dispersion mapping and microcalorimetry of the above copolymers is currently underway in our laboratories. Additionally, angular resolved ESCA studies are currently underway in conjunction with Ratner's laboratory[16] to specifically ascertain the surface structure of the homo and block copolymers.

Figure 8 represents the synopsis of the model and the necessary methodology needed to extrapolate the model from the presented block copolymers to other crystalline and semi-crystalline hydrophobic polymers with a glass transition temperature greater than 37°C. In essence the flow chart, Figure 8, works by defining: (1) the polymer backbone and side chain chemistry and conformation in the solid state, (2) the surface crystallinity of the polymer, (3)

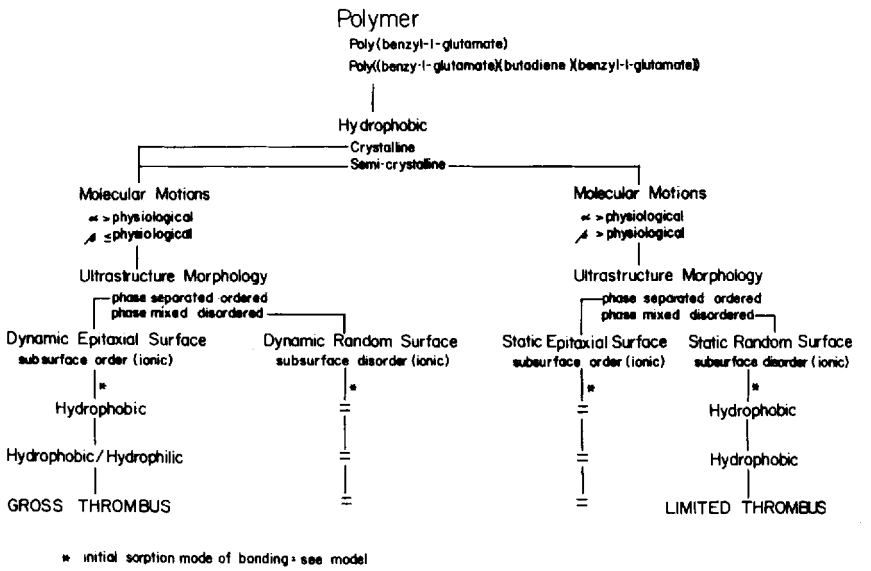


Figure 8. Flow chart synopsis of the development of the model.

the primary (segmental backbone) and secondary (side chain) molecular motions of the polymer relative to physiological temperatures, (4) the surface ultrastructural morphology, and (5) the steric/ionic order and/or disorder of the surface and its ability to sequester ions in either an ordered and/or disordered array. It is then at this juncture that the interfacing surface is defined in terms of serving as a template or not for epitaxial crystallization. If the surface appears as an ordered ionic/steric array the model predicts that a thrombogenic response will result. However, if the surface appears as a disordered array the model predicts that only a limited thrombogenic response will occur. The applicability of this model to other polymeric systems will be presented in a subsequent paper.

Therefore in summary, it appears that the interactions between the immediate substrate surface and hydrophobic groupings will contribute to the overall adsorption process. However, the dynamic state of the side chains would present a dynamic "liquid" steric template rather than the conventional "solid" steric template, with an ill defined periodic potential energy profile, in the near interfacial region. Whether or not this dynamic template would serve to induce an orientational influence on incoming sections of proteins has yet to be proven. However, the electrostatic force field of the substrate ions will be strong, long range and not suffer a drastic attenuation through the intervening layer of phenyl rings, due to the low dielectric constant of the phenyl rings. Therefore, given the assumptions of surface order and ionic distribution, an epitaxial adsorption orientation mechanism may appear credible as the initial nucleation event in thrombogenesis on biomaterials having microstructural crystallinity.

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