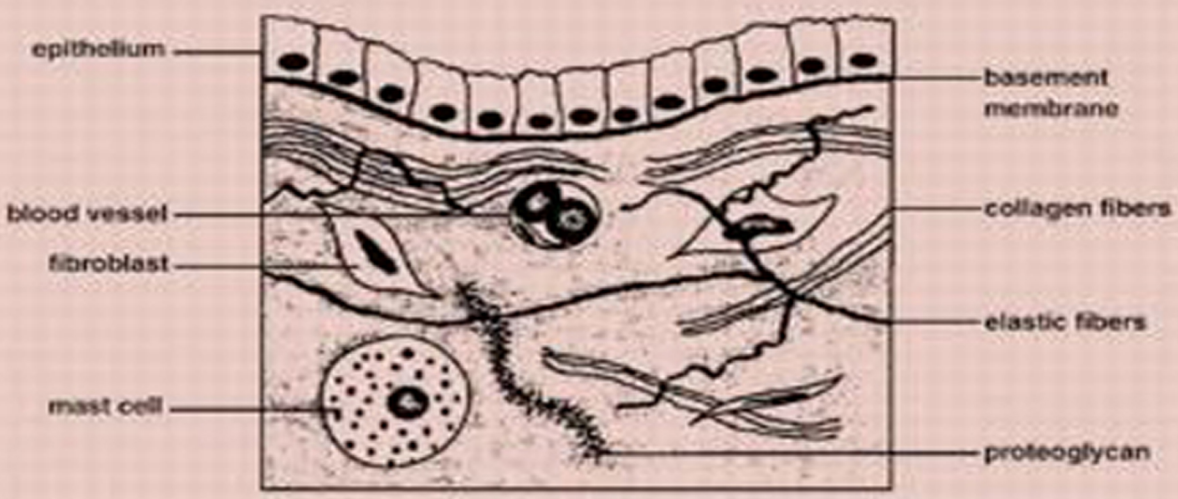
Tissue Engineering and Novel Delivery Systems



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Preface

Tissue Engineering and Novel Delivery Systems contains cutting-edge chapters by leading practitioners who deal with critical issues concerning medical applications of biomaterials. The topics range from synthetic biopolymers used in controlled-release delivery systems to natural equivalents used in tissue reconstruction. Each chapter derives its targeted discussion from basic science and engineering exposure, as well as clinical experience.

The text offers a wealth of valuable data and experience that will be of use to all bioengineers, materials scientists, and clinicians concerned with the properties, performance, and use of biomaterials as delivery or reconstructive vehicles—from research engineers faced with designing biomaterials to physicians and surgeons charged with shepherding the use of the biomaterial into the applied clinical settings. The chapters provide rich insights into our experiences today with a broad spectrum of modern biomaterials applications from forever challenging delivery systems to exciting tissue replacements. The book features discussion of the following:

Basic science, engineering, and medical applications of biomaterials
The properties, performance, and use of engineered biopolymers for delivery systems
Developments in tissue-engineered biomaterials
Emerging concepts in biomaterials

Biomaterials are the subject of intense and demanding development. New challenges brought forth from the frontiers of a society that embraces the concept of premium healthcare drive intellectual curiosity and experimentation. No longer do engineered tissues and targeted delivery vehicles seem untenable. This book focuses on materials in or on the human body—materials that define the world of "biomaterials" and cover a wide range of biomaterials both natural and synthetic. The diversity of the field necessitates multidisciplinary contributions from science, engineering, and practical medical approaches. As a result, scientists, engineers, and physicians are among the authors. They provide a full and detailed accounting of the state of the art in this rapidly growing area and reflect the diversity of the field.

The users of this book will represent a broad base of backgrounds ranging from the basic sciences (e.g., polymer chemistry and biochemistry) to the more applied disciplines (e.g., mechanical and chemical engineering, pharmaceutics, and medicine). To meet varied needs, each chapter provides clear and fully detailed discussions. This in-depth but practical coverage should also assist recent inductees to the biomaterials circle. This volume conveys the intensity of this fast-moving field in an enthusiastic presentation.

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Role of Extracellular Matrix Remodeling in Advanced Biocompatibility

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I. DEFINITIONS

For many years the extracellular matrix (ECM) was thought to serve only as a structural support for tissues. However, as early as 1966, Hauschka and Konigsberg [1] showed that interstitial collagen promoted the conversion of myoblasts to myotubes, and shortly thereafter it was shown that both collagen [2] and glycosaminoglycans [3] play a crucial role in salivary gland morphogenesis.

Based on these (and other) pieces of indirect evidence, Hay [4] put forth the idea that the ECM was an important component in embryonic inductions, a concept which implicated the presence of binding sites (receptors) for specific matrix molecules on the surface of cells. By this time, the stage was set to begin investigating in detail the mechanisms by which extracellular matrix molecules influence cell behavior. Bissell et al. [5] proposed the model of "dynamic reciprocity" between the ECM on the one hand and the cytoskeleton and nuclear matrix on the other. In this model, ECM molecules interact with receptors on the surface of cells which then transmit signals across the cell membrane to molecules in the cytoplasm; these signals initiate a cascade of events through the cytoskeleton into the nucleus, resulting in the expression of specific genes, whose products, in turn, affect the ECM in various ways [6,7].

Cell–ECM interactions participate directly in promoting cell adhesion, migration, growth, differentiation, and programmed cell death; in modulation of the activities of cytokines and growth factors, and in directly activating intracellular signaling. All these activities are connected in some way to biological compatibility.

The molecular complex called ECM has as basic components collagens and other glycoproteins implicated also in the resistance to tensile and compressive mechanical forces.

The macromolecular components of the polymeric assemblies of the ECM are in many cases secreted by cells as precursor molecules that are significantly modified (proteolysis processed, sulfated, oxidized, and cross-linked) before they assemble with other components onto functional polymers [8]. The formation of matrix assemblies in vivo is therefore in most instances a unidirectional, irreversible process, and the disassembly of the matrix is not a simple reversal of assembly, but involves multiple, highly regulated processes. One consequence of this is that polymers reconstituted in the laboratory with components extracted from extracellular matrices do not have all the properties they have when assembled by cells in vivo.

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The ECM in vivo is also modified by cells as they proliferate, differentiate, and migrate, and cells in turn continuously interact with the matrix and communicate with each other through it (Fig. 1) [9].

The ECM is therefore not an inert product of secretory activities, but influences cellular shape, fate, and metabolism in ways that are as important to tissue and organ structure and function as the effects of many cytoplasmic processes. In the past the ECM was primarily appreciated for its challenge to biochemists interested in protein and complex carbohydrate structure; a detailed characterization of ECM constituents is now considered essential for understanding cell behavior in the context of tissue and organ development and function. Some of these constituents are obviously important for their structural properties (as collagens, elastin, and fibrillin), while others (matrix-bound FGFs, TGF-β, BMPs) are signaling molecules. In a third category are multidomain molecules (as fibronectin, laminin, thrombospondin, tenascin, syndecans, and other proteoglycans) that are both structural constituents as well as regulators of cell behavior.

These molecules and their organization vary with tissue type (mesenchymal cells are immersed in an interstitial matrix, whereas epithelial and endothelial cells contact a basement membrane only through their basal surfaces), and their distribution is not static, but rather varies from tissue to tissue and during development from stage to stage [10–13].

This diversity of composition, organization, and distribution of ECM results not only from differential gene expression for the various molecules in specific tissues, but also from the existence of differential splicing and post-translational modifications of those molecules. For example, differential splicing may change the binding potential of proteins to each other [11,14]

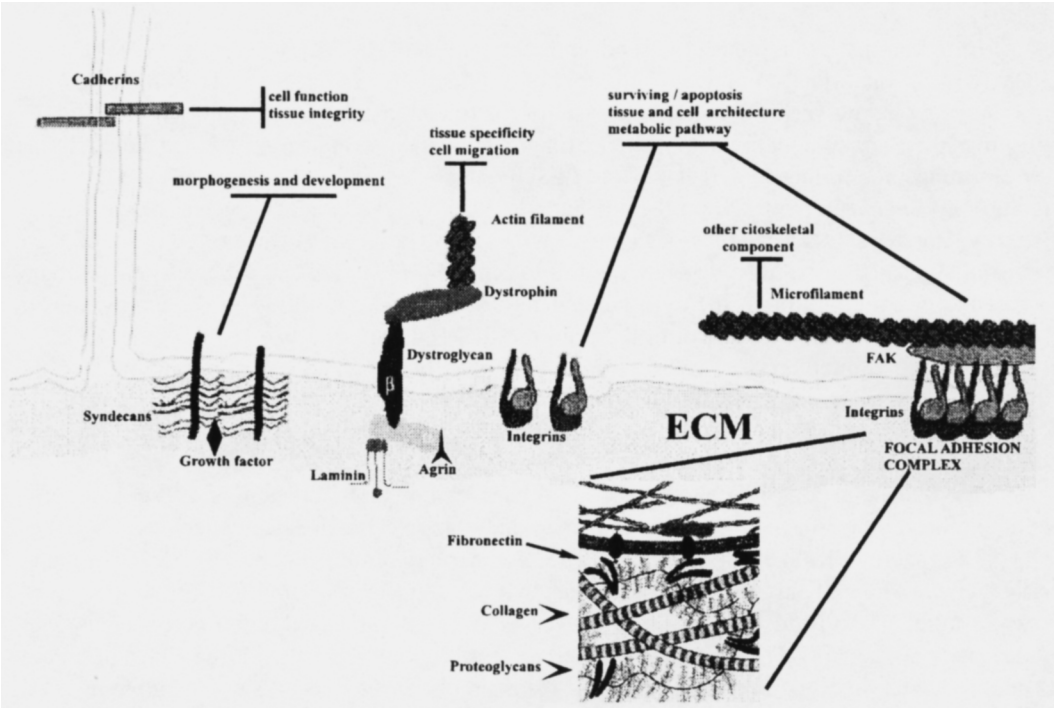

Figure 1 General organization and functions of some receptions involved in interactions between the cell and extracecullar matrix.

or to their receptors [15]; variations in the glycosylation can lead to changes in cell adhesion [16].

In addition, the presence of divalent cations such as Ca^{2+} [17,18] can affect matrix organization and influence molecular interactions that are important in the way ECM molecules interact with cells [19].

The ECM of bone is a complex structure composed primarily of type I collagen, but also containing other constituents, including proteoglycans (biglycan and decorin), glycoproteins (osteonectin, osteopontin, fibronectin, thrombospondin and bone sialoprotein), and factors that may be essential for the mineralization process, such as bone protein [20]. The remodeling of bone ECM–hardened connective tissue involves coupling of the degradation of the extracellular matrix with the synthesis of new matrix components. In certain bone diseases, such as osteoporosis, an imbalance occurs such that a disproportionate amount of matrix is degraded compared to the amount synthesized [21].

Growth factors and cytokines interact with the ECM in a variety of ways which allows them to mutually affect each other [22,23]. On the one hand, ECM can serve as a reserve by binding growth factors and cytokines and protecting them from being degraded [24], by presenting them more efficiently to their receptors [16,25] , or by affecting their synthesis [26]. In this way the ECM can affect the local concentration and biological activity of these factors. For example, when neutrophils adhere to fibronectin, they produce higher levels of tumor necrosis factor (TNF) [22]. On the other hand growth factors and cytokines can stimulate cells to alter the production of ECM molecules, their inhibitors, and/or their receptors [27]. TGF-β up-regulates the expression of matrix molecules [28] and of inhibitors of enzymes that degrade ECM molecules [29].

In a number of cases, only specific forms of these growth factors and cytokines bind to specific ECM molecules. Platelet-derived growth factor (PDGF) [30] and the 9E3 protein [31,32] (a chicken cytokine that is overexpressed during wound repair) fit in this category. The latter is secreted as a 9-kDa protein but can be processed to 6 kDa by plasmin; both forms are found in association with interstitial collagen, but only the smaller form binds to laminin, and neither form binds to fibronectin [33].

Importantly, binding of specific forms of these factors to specific ECM molecules can lead to their localization to particular areas and affect their biological activities.

Many cell types undergo cell death when deprived of adhesion to the appropriate extracellular matrix. For example, endothelial and epithelial cells die by apoptosis when detached from substrate or when the attachment is prevented by growing them in suspension. This phenomenon is named *anoikis* from the Greek word for homelessness. It is involved in a wide diversity of tissue-homeostatic, developmental, and oncogenic processes [34].

Normal cell and tissue homeostasis reflects a dynamic balance of cell proliferation, differentiation, and apoptosis; in this context anoikis maintains the correct cell number of high-turnover epithelial tissues. The clearest evidence for this is that the breakdown of anoikis contributes to neoplasia [35,36].

The substrate-dependent cell growth is mediated by surface receptors belonging to the integrin family. Meredith et al. [37] demonstrated that detachment-induced apoptosis in human endothelial cells was blocked by plating cells on an immobilized antibody to β1 integrin.

II. COMPONENTS OF THE EXTRACELLULAR MATRIX

The extracellular matrix is an intricate arrangement of glycoproteins, collagens, proteoglycans, and growth factors that act not only as a physical scaffold for the attachment and organization

of cellular structures, but also as a mediator of intracellular signaling through cell surface receptors that recognize these ECM molecules. Most ECM glycoproteins promote cell adhesion and cause cytoskeletal reorganization, leading to signals that direct differentiation and promote cell survival. ECM is composed of fibrillar protein primarily important for cell adhesion and structural characterization of the matrix, represented by collagen, laminin, and fibronectin. These proteins are accompanied by other proteins primarily involved in the dynamic relationship proprieties of ECM (growth factor activity modulation, cellular growth, differentiation, and migration), represented by proteoglycan, vitronectin, and several type of glycoprotein that mediate cell–matrix or cell–cell interaction. All these protein elements are immersed in an amorphous environment represented by glycosaminoglycan chains that form a matrix gel structure [38].

The ability of cells to adhere to the ECM is a critical determinant of cytoskeletal organization and thus of cellular morphology (Fig. 2). In addition to regulating cell shape, cell–ECM interactions also regulate the ability of a cell to proliferate, migrate, and differentiate. Furthermore, cell–matrix interactions that support cytoskeletal organization of focal adhesions are essential for survival of anchorage-dependent, nontransformed cells [39].

This wide range of activities suggests that the ECM is a key contributor to overall cellular physiology. Correspondingly, the ability of matricellular proteins to modulate cell adhesion and cytoskeletal organization suggests an important role for these proteins in essential processes.

A. Fibrillar Proteins

The collagens are a superfamily of extracellular fibrillar proteins that plays a dominant role in maintaining the integrity of various tissues and also has a number of other important functions. The superfamily actually includes more than 20 collagen types with altogether at least 38 distinct polypeptide chains and more than 15 additional proteins that have collagen-like domains.

The collagens are characterized by the presence of one or several domains termed "triple helix" that are made of three polypeptide chains folded around each other, all differing by their molecular structure and by the way helical and globular domains are arranged. In any case, however, at least one triple helical domain exists. It is formed by the association of three polypeptide chains, each of them containing a glycine every three residues and many proline

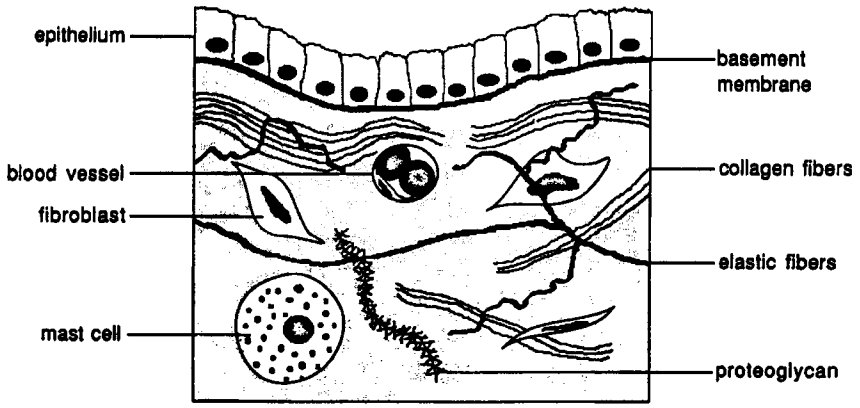

Figure 2 Schematic drawing of connective tissue. Variation in the organization, amount, and composition of the ECM depends, on local physiological requirements. The ECM can be mineralized in bone and dental tissue, elasticized in skin, and rendered transparent in the lens of the eye.

or hydroxyproline residues, this specific molecular characteristic attests for the belonging of the protein to the collagen group. Probably these domains are useful for the association of peptide chains in register prior to their folding; however, they participate in the transport of the elementary molecules from the synthesizing cells to their final place in the connective tissue, and they contribute to insertion of the molecules into their specific place inside the growing fibrils [40],[41]]. Most collagens form polymeric assemblies, such as fibrils, networks, and filaments, and the superfamily can be divided into several families based on these assemblies and other features. Most notable are (1) fibrils that are found in most connective tissues and are made by alloys of fibrillar collagens (types I, II, III, V, and XI) and (2) sheets constituting basement membranes (type IV collagen) and Descemet's membrane (type VIII collagen). Collagen fibers as they are evidenced by histological methods, for instance in tendons, are of complex structure. Most of their constituting subunits are type I tropocollagen molecules, but they also contain in their center a filament of type V collagen that seems to serve as a guide during their edification. On the surface of the fibers are molecules of type III collagen that limit the growth in diameter and also type XII molecules that serve to bind the fibers to the surrounding substances. The collagen type multiplicity is explained by their various functions (mechanical role for tendons and ligaments, functions of wrapping around muscle cells, basement membrane role as a support for endothelial cells, function of glomerular filter, etc.) [42].

Collagens are not only essential for the mechanical resistance and resilience of multicellular organisms, but are also signaling molecules defining cellular shape and behavior. The communication between collagens and cells is achieved by cell surface receptors. Three types of cell surface receptors for collagen are known: integrins, discoidin domain receptors, and glycoprotein VI. All three types independently trigger a variety of signaling pathways upon collagen binding. Besides regulating numerous cellular responses, both integrin and discoidin domain receptors monitor the integrity of the collagenous extracellular matrix by triggering matrix degradation and renewal. Some recently discovered mechanisms of locally controlled expression of collagen, collagen-binding receptors, and collagen-degrading proteases in the cellular microenvironment have been described [43].

The laminins are a family of glycoproteins that provides an integral part of the structural scaffolding of basement membranes in almost every animal tissue. Each laminin is a heterotrimer assembled from alpha, beta, and gamma chain subunits, secreted and incorporated into cell-associated extracellular matrices. A different genetic regulation leads to the expression of different laminin forms and determines the formation of extracellular matrices with variable laminin composition and thereby different biological properties [44]. The laminins can self-assemble, bind to other matrix macromolecules, and have unique and shared cell interactions mediated by integrins, dystroglycan, and other receptors. The many interactions of laminins are mediated by binding sites, often contributed by single domains, which may differ between different forms of laminin. By virtue of their receptor interactions, they initiate intracellular signaling events that regulate cellular organization and differentiation. Through these interactions, laminins critically contribute to cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and promotion of tissue survival [45].

Fibronectin is a high molecular glycoprotein present in the blood, in connective tissue, and at cell surface. It is a dimer of subunits of around 250 kDa, each composed of a series of independently folding modular domains. It is composed of multiple homologous repeats and contains many functional domains. Because of its ability to interact with many ligands—including cells, heparin, fibrin, collagen, DNA, and immunoglobulin—fibronectin can play its role in a variety of biological processes. As circulating glycoprotein may function as a nonspecific opsonin design to facilitate the uptake of tissue debris by phagocytic cells (Fig. 1) [46].

Fibronectin is synthesized by many types of differentiated cells and is believed to be involved in the attachment of cells to the surrounding extracellular matrix. It appears in different isoforms due to alternative mRNA splicing and subsequent post-translational modification. This glycoprotein interacts with cell surfaces as shown by the fact that fibronectin–collagen complexes, or fibronectin alone when insolubilized on a surface such as plastic, enhances the attachment of various types of cells to such surfaces. It seems that fibronectin, through its binding to collagen and to the cell surface, forms a bridge between the cell and its surrounding matrix. The abundance of fibronectin in basement membrane structures and the developmental changes observed in its expression lead one to believe that the attachment (or lack of it) of cells to fibronectin plays a significant role in morphogenetic events or in normal development [46,47].

Proteoglycan consists of a core protein and an associated glycosaminoglycan (GAG) chain of heparan sulfate, chondroitin sulfate, dermatan sulfate or keratan sulfate, which are attached to a serine residue. Proteoglycans may be found adhering to cell surface or Matrigel. The core proteins of cell surface proteoglycans may be transmembrane (e.g., syndecan) or GPI anchored (e.g., glypican). Many different cell surface and matrix proteoglycan core proteins are expressed in several tissues, some of them have tissue specificity expression as neurocan. The level of expression of these core proteins, the structure of their GAG chains, and their degradation are regulated by many of the effectors that control the development and function of tissue (Fig. 1) [48].

Regulatory proteins bind GAG including many growth factors and morphogens (fibroblast growth factors, hepatocyte growth factor/scatter factor, members of the midkine family), matrix proteins (collagen, fibronectin, and laminin), enzymes (lipoprotein lipase), and microbial surface proteins [49]. Structural diversity within GAG chains ensures that each protein–GAG interaction is as specific as necessary. The GAG–protein interactions serve to regulate the signal output of growth factor receptor tyrosine kinase and hence cell fate as well as the storage and diffusion of extracellular protein effectors. In addition, GAGs clearly coordinate stromal and epithelial development, and they are active participants in mediating cell–cell and cell–matrix interactions. Since a single proteoglycan, even if it carries a single GAG chain, can bind multiple proteins, proteoglycans are also likely to act as multireceptors which promote the integration of cellular signals [50].

B. Classic (Non fibrillar) Proteins

The syndecans are a family of transmembrane heparan sulfate/chondroitin sulfate proteoglycans involved in the control of cell growth and differentiation. The biological activities of syndecan involve interactions with a variety of extracellular ligands, such as growth factors and matrix components, that are mainly mediated by the heparan sulfate moieties. The core proteins of the syndecan family proteoglycans are involved in signaling [51]. In particular the cytoplasmic tail of the ubiquitously expressed syndecan-4 is distinct from the other syndecans in its capacity to bind phosphatidylinositol 4,5-bisphosphate (PIP2) and to activate protein kinase C (PKC) alpha. These properties may confer on syndecan-4 specific and unique signaling functions (Fig. 1) [52].

The glypicans are a family of heparin sulfate proteoglycans (HPSGs) that are linked to the cell surface by a glycosylphospatidylinositol (GPI) anchor. They play a critical role in developmental morphogenesis and seems to regulate growth factor distributions in extracellular space. In general glypicans are expressed predominantly during development. Expression levels change in a stage- and tissue-specific manner, suggesting that glypicans are involved in the regulation of morphogenesis [53].

Neurocan is a chondroitin sulfate proteoglycan of the lectican family and a component of the extracellular matrix of the central nervous system. It is mainly expressed during modeling and remodeling stages of this tissue. Neurocan can bind to various structural extracellular matrix components, such as hyaluronan, heparin, tenascin, and growth and mobility factors, and can interact with several cell surface molecules [54].

Vitronectin is a multifunctional adhesive glycoprotein—a plasma serum component—that can also function as a matrix component. The extracellular matrix protein vitronectin is recognized as an adhesive substrate by cells expressing specific vitronectin receptors of the integrin family (alpha v beta 1, alpha v beta 3, alpha v beta 5 or alpha IIb beta 3). Osteoclasts to osteopontin exposed on the bone surface via the classic vitronectin receptor alpha v beta 3, may be crucial to their bone resorption activity [55]. Cell interaction with vitronectin may induce spreading and migration and represents an important component in the tissue provisional matrix. In the matrix vitronectin also has an important regulatory role with respect to growth factors (e.g., vitronectin-bound IGF binding protein-5 (IGFBP-5) by modifying the responsiveness to insulin-like growth factor I [56]).

As plasma serum is well known, the primary role of vitronectin is the regulation of vascular cell function [57]. In the ECM, vitronectin has an important role in cell growth and differentiation in specific processes that involve repair, as in promoting keratinocytes migration after lesion or in regulating the tissue integrity of bone matrix and osteoblast proliferation [58].

The galectins are a family of carbohydrate-binding proteins that are distributed widely in metazoan organisms. Each galectin exhibits a specific pattern of expression in various cells and tissues, and expression is often closely regulated during development. Although these proteins are found mainly in the cell cytoplasm, some are secreted from cells and interact with appropriately glycosylated proteins at the cell surface or within the extracellular matrix. These receptors include cell-adhesion molecules such as integrins and matrix glycoproteins such as laminin and fibronectin isoforms. Recent studies have increased understanding of the roles of the galectins in regulating cell–cell and cell–matrix adhesion. These interactions are critically involved in modulation of normal cellular motility and polarity as well as during tissue formation, and loss of adhesive function is implicated in several disease states including tumour progression, inflammation, and cystic development in branching epithelia such as kidney tubules [59–61].

Agrin is an extracellular matrix protein identified and named based on its involvement in the aggregation of acetylcholine receptors (AChRs) during synaptogenesis at the neuromuscular junction. Recent studies have demonstrated that agrin is a large extracellular heparan sulfate proteoglycan, with a molecular mass in excess of 500 kDa and a protein core of 220 kDa. Emerging evidence indicates that agrin's function is not limited to its role in AChR aggregation during synaptogenesis, as the majority of agrin expression occurs in the developing central nervous system, especially in developing axonal tracts [62,63].

Osteopontin (OPN) is a phosphorylated acidic glycoprotein that has been implicated in a number of physiological and pathological events, including maintenance or reconfiguration of tissue integrity during inflammatory processes. As such, it is required for stress-induced bone remodeling and certain types of cell-mediated immunity. It also acts in dystrophic calcification, coronary restenosis, and tumor cell metastasis. An RGD-containing protein, OPN exists both as an immobilized ECM molecule in mineralized tissues and as a cytokine in body fluids; it is not a significant part of typical nonmineralized ECM. Several studies have demonstrated that OPN delivers a prosurvival, antiapoptotic signal to the cell. OPN influences cellular functions in a unique manner by mimicking key aspects of an ECM signal outside the confines of the ECM [64].

C. Matricellular Proteins

These proteins are structurally unrelated glycoproteins that function as adaptors and modulators of cell–matrix interactions and are associated with remodeling, morphogenesis, and vascular growth. These protein are expressed primarily during development, during growth, and in response to injury, and they are not abundant in the normal adult animal, except in tissues with continued turnover, such as bone [65,66].

One key feature of matricellular proteins is that they function as both soluble and insoluble proteins. As substrates, these proteins are only capable of supporting the initial and intermediate stages of cell adhesion, attachment, and spreading. Focal adhesion and stress fiber formation, characteristics of strong cell adhesion, are rarely observed when cells are plated on these substrates. When presented in mixed substrata, the matricellular proteins can also antagonize the proadhesive activities of other matrix proteins. Interestingly, these matricellular proteins actually have deadhesive effects when presented as soluble proteins to cells in a strong adhesive state. These structurally diverse proteins include thrombospondins (TSPs), the tenascins, and SPARC (secreted protein, acidic and rich, in cysteine), all of which exhibit highly regulated expression during development and following cellular injury. TSP1, tenascin-C, and SPARC stimulate reorganization of actin stress fibers and disassembly of focal adhesion complexes but have only minimal or negligible effects on cell shape [65].

Probably the regulatory role of the matricellular components is manifested primarily in the capacity of responses to injury, as opposed to normal development, when the organization of copious amounts of secreted, structural proteins becomes necessary over a relatively short period of time.

Trombospondins are a small family of secreted, modular glycoproteins consisting of TSP1 and TSP2. It can interact with specific cell surface receptors, cytokines, growth factors, and proteases, and the availability of each of these diverse molecules may help to define their function in a given environment. TSP1 was first identified as a thrombin-sensitive protein that was released in response to activation of platelets by thrombin. It is incorporated into fibrin clots and binds to a number of plasma proteins including fibrinogen, plasminogen, and histidine-rich glycoprotein. Instead TSP2 seems to be required for the generation of normal platelets from megakaryocytes. TSPs are induced in response to injury; they are able to modulate cell function interacting in the matrix environment as proadhesive or deadhesive factors [67,68].

Tenascins (TN) are a family of large extracellular matrix glycoproteins that comprise five known members. They display highly restricted and dynamic patterns of expression in the embryo, particularly during neural development, skeletogenesis, and vasculogenesis. These molecules are reexpressed in the adult during normal processes such as wound healing, nerve regeneration, and tissue involution, as well as in pathological states including vascular disease, tumorigenesis, and metastasis. In concert with a multitude of associated ECM proteins and cell surface receptors, TN proteins impart contrary cellular functions, depending on their mode of presentation (i.e., soluble or substrate bound) and the cell types and differentiation states of the target tissues. Expression of tenascins is regulated by a variety of growth factors, cytokines, vasoactive peptides, ECM proteins, and biomechanical factors [69,70].

SPARC (secreted protein, acidic and rich in cysteine) is a multifunctional glycoprotein that modulates cellular interaction with the extracellular matrix by its binding to structural matrix proteins, such as collagen and vitronectin, and by its abrogation of focal adhesions, features contributing to a counteradhesive effect on cells [71].

SPARC inhibits cellular proliferation and regulates the activity of growth factors, such as platelet-derived growth factor, fibroblast growth factor (FGF) 2, and vascular endothelial growth factor (VEGF). The expression of SPARC in adult animals is limited largely to remodeling

tissue, such as bone, gut mucosa, and healing wounds, and it is prominent in tumors and in disorders associated with fibrosis. SPARC is a prototypical matricellular protein that functions to regulate cell–matrix interactions associated with development, remodeling, cell turnover, and tissue repair; thereby it is able to influence many important physiological and pathological processes [71,72].

III. CELL–EXTRACELLULAR MATRIX INTERACTIONS

Interactions of cells with extracellular matrix molecules play a crucial role during development and wound healing. It is the continuous crosstalk between cells and the surrounding matrix environment that leads to the formation of patterns, the development of form (morphogenesis), and the acquisition and maintenance of differentiated phenotypes during embryogenesis. Similarly, during wound healing these interactions contribute to the processes of clot formation, inflammation, granulation tissue development, and remodeling. Many different lines of experimental evidence have shown that the basic cellular mechanisms that result in these events involve cell adhesion/deadhesion, migration, proliferation, differentiation, and programmed cell death.

A. Receptors

Cells ultimately dictate the location and composition of regional matrices. These matrices in turn communicate with cells and regulate their attachment, movement, growth, and gene expression.

Integrins encompass a family of cell surface transmembrane glycoproteins molecules which play a crucial role in cell–cell and cell–extracellular matrix interaction in order to create and maintain tissue architecture. Of these heterodimeric transmembrane glycoproteins (consisting of an alpha and beta chain) as yet at least 20 different types have been described, all with a different pattern of reactivity with extracellular matrix components. The same integrin heterodimer can recognize several ECM proteins, and a particular ECM ligand may be recognize by more than one integrin [73,74],[35]. It has become clear that through integrin–ligand interaction cell function is also modulated. Furthermore, in pathological conditions integrins play a role of some significance. Otherwise integrins mediate leucocyte traffic in developing inflammatory processes and function in neoplastic growth when it comes to invasion and metastasis. As a result of the ability of integrins to specifically bind various ligands, they mediate specific binding of cells to each other and to the extracellular matrix. The subunit of integrin transduces intracellular signals as a consequence of binding of the cytoplasmic tail to components of the cytoskeleton, including actinin and actin, as well as with components of several intracellular signaling pathways, including focal adhesion kinase (FAK), src-family kinases, ras, and phosphoinositol-3-kinase (PI-3K). Overall these data indicate that cells need to adhere to ECM via integrins and undergo some minimal degree of cytoskeleton organization to survive (Fig. 1) [75]. These receptors mediated cell adhesion to several structural proteins in ECM, and the several types of integrin receptors are often associated with cell or tissue specificity (e.g., beta 1 integrins appear to be the predominant adhesion receptor subfamily utilized by human osteoblast-like cells to adhere to collagen and laminin and in part to fibronectin [76]).

This broad spectrum of activity is achieved by combining the ability to create mechanically functional junctions (cell–matrix and cell–cell) and signal-transducing capabilities. Osteoblasts and osteoclasts express specific integrin receptors, and the pattern of expression varies depending on the stage of cell differentiation. Interactions of integrins with bone–matrix adhesive proteins are thought to be important for regulating the tissue integrity and may provide a local, responsive regulatory system of osteoblastic differentiation as well [77].

Integrin-mediated binding to the matrix is required for growth cells and survivor, because prevention or disruption of integrin-mediated attachment or functional blocking with antagonists leads to a form of apoptosis termed anoikis [78,79].

Dystroglycan connects the extracellular matrix and cytoskeleton. It was originally identified as the extracellular and transmembrane constituents of a large oligomeric complex of sarcolemmal proteins associated with dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene. During the last few years, dystroglycan has been demonstrated to be a novel receptor of not only laminin, but also agrin, two major proteins of the extracellular matrix having distinct biological effects. Dystroglycan plays a critical role in organizing extracellular matrix molecules on the cell surface and in basement membranes, and at least two human pathogens utilize dystroglycan to gain access to host cells [80,81]. As a receptor of laminin/agrin, it has been implicated in such diverse and specific developmental processes as epithelial morphogenesis, synaptogenesis, and myelinogenesis. These findings point to the fundamental role of dystroglycan in the cellular differentiation process shared by many different cell types (Fig. 1) [82].

Discoidin domain receptors (DDRs) are a large family of ECM receptors that transmit signals through the use of an intrinsic tyrosine kinase function. The subgroup DDRs is distinguished from other members of the receptor tyrosine kinase family by a discoidin homology repeat in their extracellular domains such as typically found in a variety of other transmembrane and secreted proteins. Recently, various types of collagen have been identified as the ligands for the two mammalian discoidin domain receptor tyrosine kinases, DDR1 and DDR2. Both receptors display several potential tyrosine phosphorylation sites that are able to relay the signal by interacting with cytoplasmic effector proteins [83].

Glycoprotein VI (GPVI) is a receptor glycoprotein that has received particular attention. It is expressed on platelets in association with a signaling adapter, the Fc receptor gamma chain (Fc R-γ). The platelet response to collagen is a primary event in hemostasis and thrombosis, but the precise roles of the numerous identified platelet collagen receptors remain incompletely defined. The GPVI expression confers both adhesive and signaling responses to collagen in a graded fashion that is proportional to the GPVI receptor density. These results resolve some of the conflicting data regarding GPVI-collagen interactions and demonstrate that (1) GPVI-Fc R-γ expression is sufficient to confer both adhesion and signaling responses to collagen and (2) GPVI-mediated collagen responses are receptor density dependent at the receptor levels expressed on human platelets [84].

Cadherins are the receptors involved in cell–cell adhesion, as mediated by the cadherin–catenin system; this is a prerequisite for normal cell function and the preservation of tissue integrity. With recent progress in our understanding, beta-catenin as a component of a complex signal transduction pathway may serve as a common switch in central processes that regulate cellular differentiation and growth. The function of the cadherin–catenin system in cell adhesion as well as in intracellular signaling appears to be subjected to multifactorial control by a variety of different mechanisms, and data on a hormonal control of these signaling pathways suggest an important regulatory influence in many cellular systems (Fig. 1) [85].

B. Growth Factors

Growth factors play a key role in regulation of their activity. However, growth factor signaling can also be regulated outside of cells by extracellular matrix proteins and proteolytic enzymes. The ability of extracellular proteins to process complex information in the absence of new protein synthesis is illustrated in blood clotting and complement pathways. An increasing number of growth factors, including insulin-like growth factors, fibroblast growth factor, transforming

growth factor beta (TGF-β), and hepatocyte growth factor, have been found to associate with the extracellular matrix proteins or with glycosaminoglican heparan sulfate. Rapid and localized changes in the activity of these factors can be induced by release from matrix storage and/or by activation of latent forms. These growth factors, in turn, control cell proliferation, differentiation, and synthesis and remodeling of the extracellular matrix. It is therefore likely that much of the information processing necessary for construction of complex multicellular organisms occurs in the extracellular environment. This suggests that the extracellular matrix plays a major role in the control of growth factor signaling. A direct interaction of growth factors with the matrix environment is based on the presence of growth factor binding proteins that may regulate the kinetic release and storage of growth factors in accordance with the immediate needs of cells [86].

An interesting example of interaction among growth factor and matrix may be observed in bone ECM, where a major mechanism for storage of TGF-β is via its association with latent TGF-β binding protein1 (LTBP1). LTBP1 proteolysis by plasmin, elastase, and metalloproteinase (MMP) may be a physiological mechanism for release of TGF-β from ECM-bound stores, potentially the first step in the pathway by which matrix-bound TGF-β is rendered active [87].

Growth factor interaction with ECM can involve the single molecular component such as glycoproteins, matricellular proteins, and proteoglycans that together with specificity of the matrix inside the different tissutal district contribute to regulate the action of several growth factors on cell physiology. The multifunctional adhesive glycoprotein vitronectin (VN), which is found in the circulation and widely distributed throughout different tissues, has been implicated in the regulation of vascular cell functions, and these activities could be related to interactions with various growth factors. In vitro, soluble VN interfered with TGF-β binding to isolated extracellular matrix and was found to associate with TGF-β1 and TGF-β2 as well as with other growth factors such as vascular endothelial growth factor, epidermal growth factor, or basic fibroblast growth factor in a saturable manner. In particular, binding of TGF-β was maximal for the heparin-binding multimeric isoform of VN, whereas VN in a ternary complex with thrombin and antithrombin or plasma VN exhibited weaker binding. Plasminogen activator inhibitor 1 (PAI-1) or heparin interfered with binding of VN to TGF-β, and soluble PAI-1 was able to dissociate VN-bound TGF-β [88].

The receptors important in adesive interaction with the cell–matrix are also involved in growth factor activity, both through interactions with the growth factor receptors, as well as direct interaction with the growth factor itself. A crosstalk between integrins and growth factor receptors was evidenced as an important signaling mechanism to provide specificity during normal development and pathological processes. Evidence from several model systems demonstrates the physiological importance of the coordination of signals from growth factors and the extracellular matrix to support cell proliferation, migration, and invasion in vivo. Several examples of crosstalk between these two important classes of receptors indicate that integrin ligation is required for growth factor–induced biological processes. Furthermore, integrins can directly associate with growth factor receptors, thereby regulating the capacity of integrin/growth factor receptor complexes to propagate downstream signaling [89].

Syndecans interact with growth factors, such as fibroblast growth factor, insulin-like growth factor, and epidermal growth factor. These interactions are required for biological activity, because these factors must first interact with the heparan sulfate chains of the syndecans before they can interact with their high-affinity signaling receptors (Fig. 1) [90].

C. Cytoskeleton and Signal Transduction

Cell matrix adhesion occurs at many specialized sites termed *focal contacts* or *focal adhesions*. They consist of multimolecular protein complexes of transmembrane adhesion receptors anchor-

ing intracellular cytoskeletal structural proteins such as talin, vinculin, and α-actinin and signal transduction molecules including c-Src, FAK, p130cas, and paxillin. Moreover, many of these components and their variants are expressed in a cell type–restricted fashion, introducing a high level of complexity (Fig. 1) [91,92].

At the cytoplasmic faces of the adhesion site more than 50 proteins have been reported to be associated with focal contacts and related to ECM adhesions. The major transmembrane ECM receptors in these sites belong to the integrin family of proteins. Most of these contain multiple domains through which they can interact with different molecular partners, potentially forming a dense and heterogeneous protein network. The molecular and structural diversity of this "submembrane plaque" is regulated by a wide variety of mechanisms, including competition between different partner proteins for the same binding sites, interactions triggered or suppressed by tyrosine phosphorylation, and conformational changes in component proteins, which can affect their reactivity. Indeed changes can also be driven by mechanical force generated by the actin- and myosin-containing contractile machinery of the cells, or by external forces applied to the cells, and regulated by matrix rigidity [93]. Recent advances reveal that components of cell adhesion complexes display multiple interactions and functions, which cooperate to mediate both cell adhesion and signaling. Cell–matrix and cell–cell adhesions can serve as both recipients and generators of signaling information, using hierarchical and synergistic molecular interactions regulated by aggregation, conformational changes, phosphorylation, and tension, to act as signaling centers from which numerous intracellular pathways emanate to regulate cell growth, survival, and gene expression in normal and pathological conditions [35,94].

The readily apparent differences between the cytoskeletal structures of attached versus suspended cells suggest that survival signaling in anoikis is likely to be extensively regulated by the cytoskeleton. Such regulation may be affected by the multiple cytoskeletal changes apparent in transformed cells. Indeed, substantial evidence now exists showing that both signaling molecules and apoptosis regulators are associated with the cytoskeleton, and as such may together regulate anoikis by serving as sensors of cytoskeletal integrity [95].

IV. EXTRACELLUAR MATRIX REMODELING

Biomaterials implanted into the human body participate in the process of wound healing. Healing is a complex and long-lasting process of tissue repair and remodeling in response to injury or implant. The wound response is aimed at reconstituting a tissue closely similar to the original one and can be divided into several distinct but overlapping phases such as blood coagulation, inflammation, cellular proliferation, and ECM deposition and remodeling (Fig. 3). The optimal wound healing process brings the complete tissue integration of the implant.

Normal wound healing starts blood coagulation and coagulation factors like factor XII [96,97] and thrombin [98] modulate wound healing by acting as mitogens and chemoattractants. Thrombin also stimulates procollagen production by fibroblasts [99]. The presence of an artificial foreign surface may alter the wound healing process by selective protein adsorption to the material [100–102] , thus amplifying or down-regulating subsequent cell reactions to the adhering proteins [103–105]. In this context the chain of cellular reactions (adhesion receptors, intracellular signaling pathways, release of intercellular signal mediators, and the effect of such mediators on surrounding tissue) is of prime importance for the understanding of the effect of implanting foreign materials into the body. Knowledge of the signaling pathways from material to tissue is also important for the possibility of engineering wound healing in desired directions, e.g., healing with differentiated tissue rather than scar formation [106]. During the initial contact between implant materials and whole blood (Fig. 4), proteins adsorb within fractions of a second

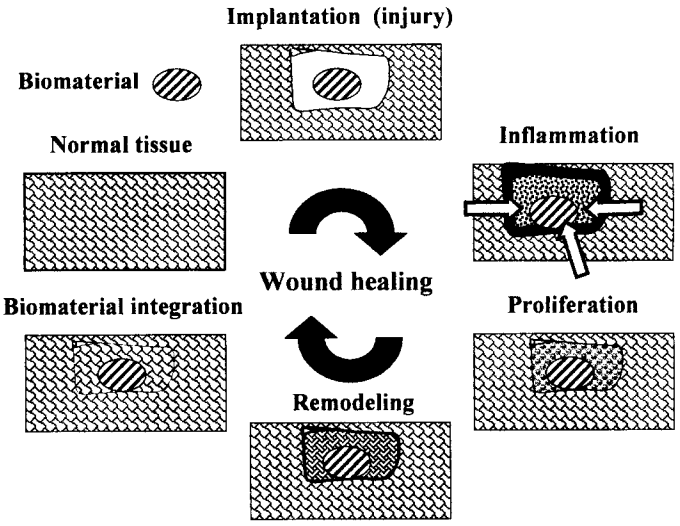

Figure 3 Wound healing phases after biomaterial implantation.

and platelets adhere to the surface-adsorbed proteins within the first few seconds of blood material contact [104]. Polymorphonuclear leukocytes (PMNLs) are found at the material surface after 10 min of blood exposure, and the cells are activated within 30 min of exposure [104], starting periprostetic inflammation. The PMNLs may become activated either directly, through some adhesion receptors, or via platelet-derived mediators (serotonin, platelet factor 4, lysophosphatidic acid, P-selectin, von Willebrand factor), and they produce reactive oxygen species as a part of the activation. Recently, phagocyte-derived oxygen species have received increasing recognition for their role in host defense and tissue injury [107]. The oxygen metabolites, notably O_2 and reaction products of this molecule, play an important role in the intracellular and extracellular killing of microorganisms [108] and may also serve as mediators of the immune system and modulators of cellular activities such as cell adhesion [109], phagocytosis [110], and signal transduction [111].

Many aspects of acute and chronic inflammatory processes seem to be mediated by oxidants released by phagocytes through their ability to cause cellular production of cytokines [112,113]. In macrophages, the transcription factor NF-kB can be activated by H_2O_2 generated by the respiratory burst [114]. An immediate response to oxygen radicals has also been demonstrated for macrophages adhering to plastic surfaces where exposure to H_2O_2 lead to spreading via MAP kinase–dependent intracellular signaling pathways [115]. The superoxide anion may also activate phospholipase A2 (PLA2), resulting in membrane damage and generation of lipid inflammatory mediators [116]. Platelet functions, of importance in the early contact between material and blood, can be significantly altered by exposure to reactive oxygen species (ROS), including eicosanoid biosynthesis. Eicosanoids such as PGE_2 have diverse effects on the regulation and activity of T lymphocytes present in the wound site. Eicosanoids and cytokines (TNF-α, IL-1β) can regulate the proliferation and apoptosis of T cells that are cleared at the end of the immune response and therefore regulate the duration and intensity of inflammation [117]. Fibroblasts are the main effector cells in wound healing after wound formation; fibroblasts migrate to the wound site where they produce extracellular matrix and proinflammatory cytokines [118]. The regulation of extracellular matrix deposition is a key event in many physiological

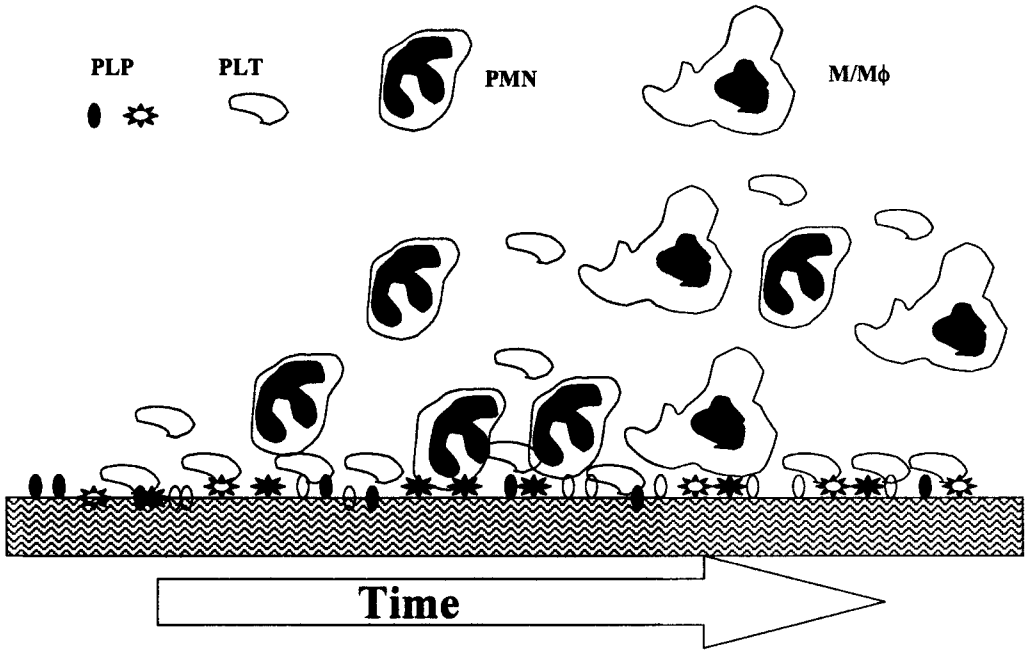

Figure 4 Initial interaction between whole blood and biomaterial surface. PLP, plasmatic proteins; PLT, platelets; PMN, polymorphonuclear leukocytes; M/Mϕ, monocytes/macrophage

and pathological conditions. During wound healing ECM molecules need to be rapidly synthestized during the formation of early granulation tissue and also during the final replacement by mature connective tissue and tissue remodeling. At the end of wound healing, fibroblast are cleared by apoptosis [119]. Cell migration, angiogenesis, degradation of provisional matrix, and remodeling of newly formed granulation tissue all require controlled degradation of the extracellular matrix. A tight balance between connective tissue synthesis and breakdown is required for the normal functioning of all tissues. In fact an excessive deposition of connective tissue is a hallmark of fibrosis [120], while an excessive matrix degradation is the main cause of tissue reasorption and prosthesis aseptic loosening [121]. One way to control this balance is by the release of mediators from inflammatory cells or connective cells which can influence collagen and matrix metalloproteinase production very effectively in both paracrine and autocrine fashions, as demonstrated for cytokines and growth factors such as the trasforming growth factor β family, the interleukins, tumor necrosis factor, platelet-derived growth factor, and others [122].

A. The ECM as Peri-Implantar Site

Periprosthetic bone loss can occur as a result of a reduction in the load transmitted to bone, so-called stress shielding. Periprosthetic bone loss also occurs as a result of an inflammatory reaction to small particles, such as those produced by the various wear modes. To varying degrees, both processes occur simultaneously in complex mechanical/biological systems such as joint replacements, and the adverse effects can be additive. Bone with decreased density secondary to stress shielding may be more susceptible to osteolysis. Relative motion between an implant and bone can cause bone loss through both mechanical and biological mechanisms [123].

Active coupling of bone formation and resorption and increased osteocytes with abundant bone canalicular projections were found combined with the presence of immature bone matrices (osteoid and low-mineralized bone areas) in periprosthetic bones from loose hip joints. These results indicated that active osteoclastic bone resorption and/or defective bone formation are coupled with monocyte/macrophage-mediated foreign body type granuloma in the synovial-like interface membrane of loose hip joints. Thus, this unique high-turnover periprosthetic bone remodeling with bad bone quality probably is caused by the result of cellular host response combined with inappropriate cyclic mechanical loading. The fragile periprosthetic bone may contribute to hip prosthesis loosening [124].

The tissue adjacent to total hip and knee prostheses consists of synovial tissue, "fibrous membrane" (variably organized and variably vascularized fibrous tissue), lymphocytes (occasionally), and foreign body inflammatory cells (macrophages and giant cells) that are present roughly in proportion to the number of small particles [124]. Prosthetic particles elicit a cascade of responses at the cellular and tissue levels. The cell whose function is central to the biological reaction to prosthetic wear particles appears to be the macrophage. The mononuclear stem cell, which originates in the bone marrow, is the progenitor for both mononuclear macrophages and osteoclasts. Macrophages phagocytose small wear particles and may fuse to form foreign body multinucleated giant cells, usually in association with larger particles (Fig. 5) [126]. Osteoblasts and fibroblasts also may be important in the response to wear particles, resulting in altered formation of bone and connective tissue [127]. Although lymphocytes are occasionally present, their role in the inflammatory reaction is unclear.

The histological features of the fibrous membrane coincide with the context of its formation and evolution. The quiescent membrane is composed of a thin layer of fibrous tissue and its occurrence is compatible with the biofunctionality of the implant.

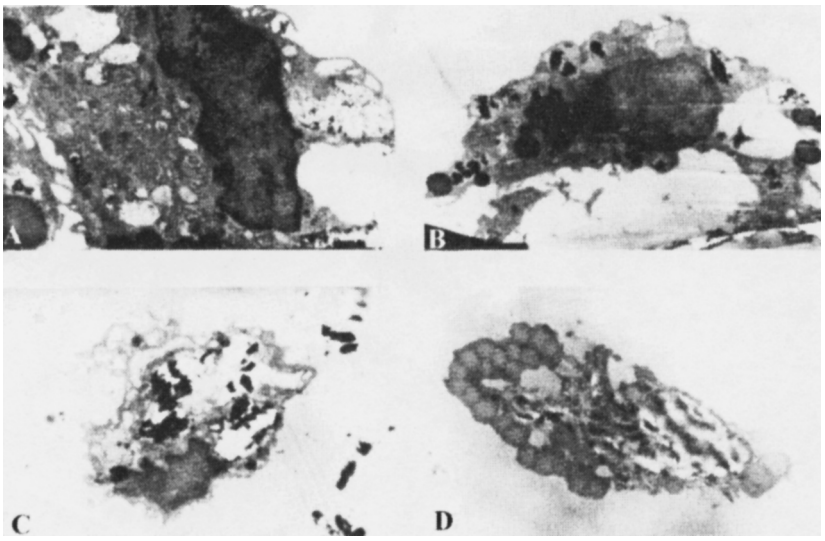

Figure 5 TEM micrograph of macrophages incubated in the presence of biomaterial. (A) Control macrophages in absence of biomaterials; (B, C, and D) marcophages exposed to different biomaterials. The biomaterial is fragmented in small particles inside the vacuoles (B) or appears as large solid clumps inside irregularly shaped vacuoles (C) or the scarce cytoplasm has plenty of vacuoles containing lipid particles. Image at 3500×. TEM observations showed that only smaller particles were actively ingested by cells, while larger particles elicited no visible reaction.

The aggressive or lytic membrane develops when tissue-irritating, small, irregularly shaped and edgy breakdown products are deposited at the interface. The thick lytic membrane consists of an inflamed fibrous tissue, scattered within which are myriad granulomas, and its surface facing the implant displays a synovial-like aspect (Fig. 6) [128]. The mono- and polykaryonic macrophages, constituting the granulomatous response, ingest and abut on the wear particles. Among the intermediary substances of inflammation elaborated by the lymphocytes and macrophages of the lytic membrane, factors which stimulate the osteoclasts play the pivotal role in as much as progressive bone resorption is associated with progressive growth of the quiescent membrane and, hence, with incremental interfacial motion, interfacial deposition of wear particles, and inflammatory granulomatous response. The ensuing vicious circle culminates in aseptic loosening of the arthroplasty. The morphological features of the lytic membrane, though characterized by a stereotypical reaction pattern, are in their details closely linked with the nature of the diverse components of the composite joint replacement. The histological appearances of the bone–implant interface of stable and loose arthroplasties; the tissular reactions to polymethylmethacrylate, polyethylene, polyacetal, metals, and hydroxyapatite; as well as the characteristics of cemented and cementless porous-coated, press-fit, and hydroxyapatite-coated prostheses are described in many works [129,130].

Most periprosthetic bone resorption is effected by osteoclasts, but there is evidence that macrophages and foreign body giant cells are capable of direct, low-grade bone resorption. In vitro studies have indicated that activated macrophages release cytokines, including interleukins and prostaglandins, which play a role in the recruitment and differentiation of cells and stimulate bone resorption, but the specificity of the cytokine response and the regulatory mechanisms has not been defined. Under certain conditions, macrophages appear to directly release interleukin 1 beta and tumor necrosis factor. Several cytokines, including interleukin 1 beta, stimulate

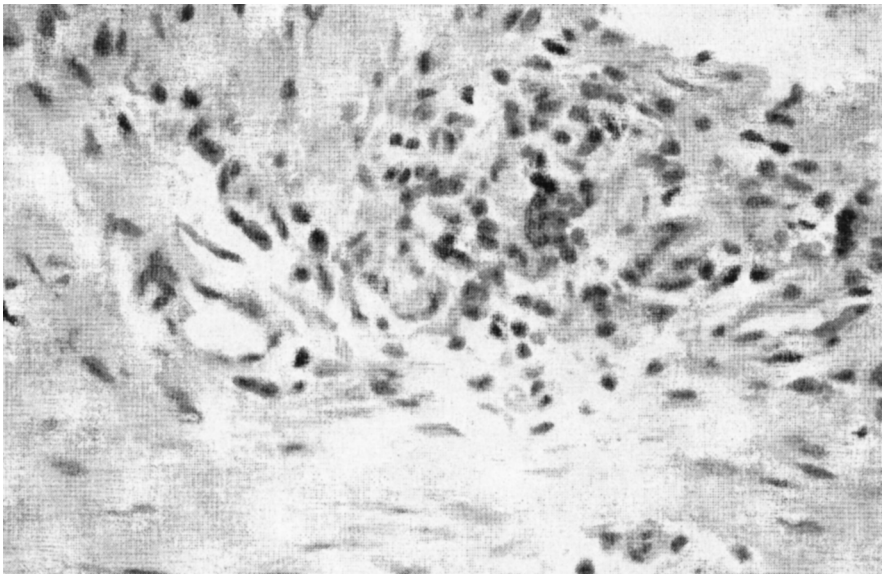

Figure 6 Immunoistochemistry of the new tissue formed at the implant material interface. Lymphocytes were identified using a monoclonal mouse antibody anti-human leukocyte common antigen (LCA), Figure shows strongly labeled lymphoid cells (generally small lymphocytes) that were numerous in the granulation tissue of the perimplantar membrane. Light microscopy at 250×.

osteoclast maturation. Although cytokines released by macrophages may directly stimulate bone resorption by osteoclasts, other effects may be mediated by intermediary cells such as fibroblasts or osteoblasts [131]. Matrix metalloproteinases (collagenase, gelatinase, and stromelysin), which are capable of effecting bone resorption, are also produced by interfacial membrane tissue around failed total hip and knee replacements. On the basis of the knowledge of such cellular and biochemical mechanisms of bone resorption, there has been increasing interest in and investigation of pharmacological agents that may modify these cellular responses. Other work indicates that, in addition to bone resorption, there is also a decrease in bone formation in association with periprosthetic osteolysis.

It appears that all of the materials used in total joint replacement are capable of inducing an inflammatory foreign body reaction if the particles are within a certain size range and there are enough of them. The bone resorbing ability of macrophages in vitro is a function of the size, shape, and composition of the particles, and it is dose dependent. It has been previously recognized that there is an upper size limit for particle reactivity, but there may also be a lower size limit.

Analyses of matrix metalloproteinase and tissue inhibitors of metalloproteinase interaction show imbalance between the enzymes and the endogenous inhibitors in favor of matrix metalloproteinase in peri-implantar tissue [132]. This induces pathologic connective tissue remodeling in the interface. The data suggest that matrix metalloproteinase and tissue inhibitors of the metalloproteinase system participate in the extracellular matrix degradation and tissue remodeling in artificial hip joints, and may contribute to the periprosthetic weakening, implant loosening, and osteolysis around implants. More evidence for their active involvement is sought by intervention studies with type-specific matrix metalloproteinase inhibitors [133].

Matrix metalloproteinases have been shown to play a role in aseptic loosening of total hip replacement (THR). Extracellular matrix metalloproteinase inducer (EMMPRIN) can up-regulate expression of several MMPs but has little effect on their tissue inhibitor (TIMP). EMMPRIN expression is up-regulated in interface tissue, and that locally accumulated EMMPRIN may modulate MMP-1 expression. An imbalance in the activity of MMPs and TIMP may lead to tissue destruction and periprosthetic osteolysis. These biological responses, combined with mechanical stress caused by micromotion and oscillating fluid pressure, may eventually cause aseptic loosening of THR [134].

B. ECM and Matrix Metalloproteinase

The timely breakdown of ECM is a key process for embryonic development, morphogenesis, and tissue resorption and remodeling. The matrix metalloproteinases play a central role in these processes. The expression of most matrices is transcriptionally regulated by growth factors, hormones, cytokines, and cellular transformation [135,136]. The proteolytic activities of MMPs are controlled during activation from their precursors and inhibition by endogenous inhibitors, α-macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs). Almost 28 different MMPs have been individuated in vertebrates (21 in humans, Table 1) [137], and in most cases MMPs are synthesized as proenzymes and secreted as inactive pro-MMPs. The MMPs' primary structure comprises several domain motifs (Fig. 7). The propeptide domain (about 80 amino acids) presents a highly conserved PRCG(V/n)PD sequence. The cys in the sequence binds the catalytic zinc to maintain the latency of pro-MMP [138]. The catalityc domain (about 170 amino acids) contains a zinc binding motif, HEXXHXXGXXH, and a conserved methionine, which forms a "Met-turn" structure [139]. The catalytic domains of MMPs have an additional structural zinc ion and 2–3 calcium ions, which are required for the stability and the expression of enzymatic activity. MMP-2 and MMP-9 have three repeats of fibronectin type II domain inserted

in the catalytic domain. These repeats interact with collagens and gelatins [140,141]. The C-terminal hemopexin-like domain (about 210 amino acids) has an ellipsoidal disk shape with a four-bladed β-propeller structure; each blade consists of four antiparallel β-strands and an α-helix [142]. The hemopexin domain is an absolute requirement for collagenases to cleave triple helical interstitial collagens [143], although the catalytic domains alone retain proteolytic activity toward other substrates [144].

The hemopexin domain of MMP-2 is also required for the cell surface activation of pro-MMP-2 by MTl-MMP [145]. A transmembrane domain is found in the MT-MMPs which anchors those enzymes to the cell surface.

Metalloproteinase genes are inducible by many stimuli such as growth factors, cytokines, chemical agents, and physical stress. MMP gene expression is down-regulated by trasforming growth factor beta, retinoic acids, and glucocorticoids [135,136]. Recent studies emphasize also that cell-matrix and cell-cell interactions can modulate the MMP gene expression in fibroblasts [146,147] , endothelial cells [148], lymphocytes [149], macrophages [150], and neoplastic cells [151].

Certain signaling pathways lead to expression of a particular MMP genes. For example, addition of soluble antibody to α5/β1 integrin causes disruption of the actin cytoskeleton and an increased expression of MMP-1 in rabbit synovial fibroblasts [152]. This is because of activation of the GTP-binding protein Racl, which generated reactive oxygen species and induced activation of NF-KB [153]. This leads to induction of IL-lβ, an autocrine inducer of MMP-1 expression.

Inflammatory cytokines, such as TNF-α and IL-1, also trigger a ceramide-dependent expression of MMP-1 in human skin fibroblast mediated by three distinct MAP kinase pathways, i.e., ERK1/2, stress-activated protein kinase SAPK/JNK, and p38 [154].

Apart from a few members activated by furin, most MMPs are secreted from the cell as inactive zymogens. Secreted pro-MMPs are activated in vitro by proteinases and by nonproteolytic agents such as SH-reactive agents, mercurial compounds, reactive oxygen, and denaturants. In all cases activation requires the disruption of the Cys-Zn^{2+} (cysteine switch) interaction and the removal of the propeptide [155]. In vivo, most pro-MMPs are likely to be activated by tissue or plasma proteinase or bacterial proteinases. It has been suggested that the urokinase type plasminogen activator (uPA)/plasmin system is a significant activator of pro-MMPs [156]. In-

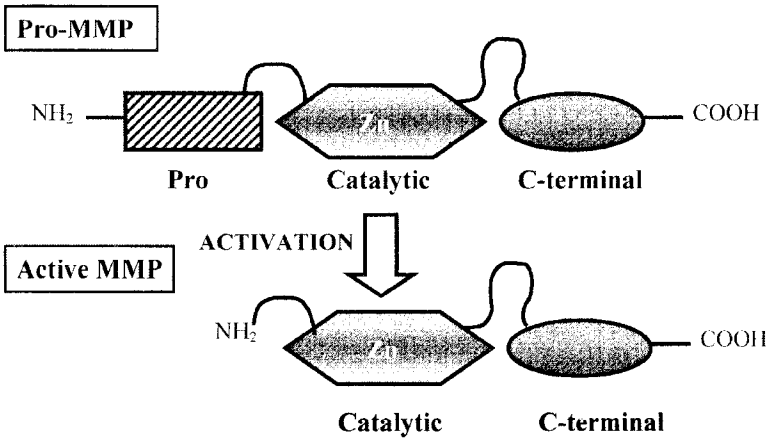

Figure 7 Basic domain structure of MMPs.

Table 1 Human MMPs, Substrate and Exogenous Activators

Enzyme	Substrates	Activated by
Collagenases		
Collagenase-1 (MMP-1)	Collagen I, II, III, VII, VIII, X, aggregan, serpins, 2M	MMP-3, -7, -10, plasmin kallikrein, chymase
Collagenase-2 (MMP-8)	Collagen I, II, III, aggregan, serpins, 2M	MMP-3, -10, plasmin
Collagenase-3 (MMP-13)	Collagen I, II, III, VII, VIII, X, XIV, gelatin, FN, laminin, large tenascin osteonectin, serpins	MMP-2, -3, -10, -14, -15, plasmin
Stromelysins		
Stromelysin-1 (MMP-3)	Collagen IV, V, IX, X, FN, elastin, gelatin, laminin, aggrecan, nidogenfibrillin, osteonectin, 1PI, myelin basic protein, OP, E-cadherin	Plasmin, kallikrein, chymase, tryptase
Stromelysin-2 (MMP-10)	As MMP-3	Elastase, cathepsin G
Stromelysin-like MMPs		
Stromelysin-3 (MMP-11)	Serine protease inhibitor, 1PI	Furin
Metalloelastase (MMP-12)	Collagen IV, gelatin, FN, laminin, vitronectin, elastin, fibrillin, 1PI, myelin basic protein, apolipoprotein A	ND
Matrilysins		
Matrilysin (MMP-7)	Elastin, FN, laminin, nidogen, collagen IV, tenascin, versican, 1-IP, OE-cadherin, TNF-	MMP-3, plasmin
Matrilysin-2 (MMP-26)	Gelatin, 1-IP, syntetic MMP- substrates, TACE-substrate	ND
Gelatinases		
Gelatinase A (MMP-2)	Gelatin, collagen I, IV, V, VII, X, FN, tenascin, fibrillin, osteonectin, monocyte chemoattractant protein 3	MMP-1, -13, -14, -15, -16, -tryptase?
Gelatinase B (MMP-9)	Gelatin, collagen IV, V, VII, IX, XIV, elastin, fibrillin, osteonectin 2	MMP-2, -3, -7, -13, plasmin, trypsin, chymotrypsin, cathepsin D
Membrane-type MMPs		
MT1-MMP (MMP-14)	Collagen I, II, III, gelatin, FN, laminin, vitronectin, aggrecan, tenascin, nidogen, perlecan, fibrillin, 1-IP, 2M, fibrin	Plasmin, furin
MT2-MMP (MMP-15)	FN, laminin, aggrecan, tenascin, nidogen, perlecan	ND
MT3-MMP (MMP-16)	Collagen III, FN, gelatin, casein, cartilage proteoglicans, laminin-1, 2M	ND
MT4-MMP (MMP-17)	Fibrin, fibrinogen, TNF precursor	ND
MT5-MMP (MMP-24)	Proteoglycan	ND
MT6-MMP (MMP-25)	Collagen IV, gelatin, FN, fibrin	ND
Other MMPs		
MMP-19	Gelatin, aggrecan, COMP, collagen IV, laminin, nidogen, large tenas	Trypsin
Enamelysin (MMP-20)	Amelogenin, aggrecan, COMP	ND
MMP-23	McaPLGLDpaARNh2 (synthetic MMP-substrate	ND
MMP-28	Casein	

FN, fibronectin; 2M, 2-macroglobulin; 1PI, 1-proteinase inhibitor; COMP, cartilage oligomeric matrix protein; ND, not determinated; TACE, TNF-converting enzyme; OP, osteopontin.

stead, the activation of pro-MMP-2 is thought to take place on the cell surface thanks to the action of membrane-anchored MMP (MT1-MMP) [157]. Tissue inhibitors of metalloproteinases are small (21–30 kDa) endogenous regulators of MMP activiy in the tissue, and four homologous TIMP (TIMP-1 to 4) have been identified [158].

Tissue inhibitors of MMPs inhibit cell invasion in vitro, tumorigenesis, metastasis in vivo, and angiogenesis [158]. TIMPs exhibit additional biological functions. T1MP-1 and T1MP-2 have mitogenic activities on a number of cell types, whereas overexpression of these inhibitors reduces tumor cell growth [158]. These biological activities of TIMPs are independent of MMP-inhibitory activities [159,160]. TIMPs seem to be therefore regulators not only in matrix turnover, but also in cellular activities.

Although the main function of MMPs is removal of ECM during tissue resorption and progression of many diseases, it is notable that MMPs also alter biological functions of ECM macromolecules by specific proteolysis. For example, MMP-2 released by growth cones promotes neurite outgrowth by inactivating neurite-inhibtory chondroitin sulfate proteoglycans, thereby unmasking the neurite-promoting activity of laminin [161]. Specific cleavage of the Ala586–Leu587 bond in the α_2 chain of laminin 5 by MMP-2 induces migration of normal breast epithelial cells by exposing a cryptic promigratory site [162]. The cleaved form of laminin 5 was found in tumors and in tissues undergoing remodeling but not in quiescent tissues [162]. Cleavage of type I collagen by MMP-1 and by MMP-13 initiates keratinocyte migration during reepithelialization [163] and osteoclast activation [164], respectively. Further insights into the biological and pathological function of MMPs have been provided by the use of transgenic animals and gene transfer techniques [165]. The expression of MMP-9 appears to be critical in later embryonic skeletal tissue development [166] and MMP-9–deficient (MMP-9-/-) mice exhibited phenotypic defects with a delayed, long bone growth associated with an abnormally thickened growth plate, which was accompanied by delayed apoptosis of hypertropic chondrocytes, vascularization, and ossification [167].

V. ECM AND ADVANCED BIOCOMPATIBILITY

The ECM cells, components, and enzymes form a dinamic microenvironment that interact with implanted biomaterials. Biomaterial can alter the normal ECM turnover or remodeling causing a reaction in the periprosthetic tissue that leads to the prosthesis loosening or it can promote a "positive" interaction that leads to prosthesis integration and optimal wound healing. Therefore biomaterial interaction with ECM is a key aspect in biocompatibility as demonstrated by a growing amount of evidence.

One of the most striking indications of the importance of ECM remodeling in the biocompatibility is the aseptic loosening of joint implants. Aseptic loosening is a current problem of major concern in patients with osteoarthritis and rheumatoid arthritis who had total hip replacements for the end-stage destructive arthritis. The problem has been increasing in recent years and pathologists observe the tissues obtained at revision arthroplasties from the femoral and/or cup components. It is well recognized that bone lysis occurs around the loose cemented prosthesis, where the fibrous membranes containing a histiocytic reaction to cement and polyethylene debris are formed at the bone–cement interface [168].

Various factors may contribute to the enhanced osteolysis around the cemented hip prostheses: they include localized mechanical stresses [169], micromotion between the cement and bone [170], abrasion particulate from the artificial joint surfaces [171], fragmentation of the cement [172], and hypersensitivity to metal [173].

A synovial-like membrane is reported to occur often at the bone–cement interface, and it releases prostaglandin E_2 (PGE_2) and collagenase [174,175] , both of which may be involved in bone resorption [175]. Prostheses that can be inserted without cement were developed to diminish the adverse effects of cement. However, aseptic loosening of the uncemented prostheses due to osteolysis has also been observed [176]. Examination of tissue from the cementless bone–implant interface in individuals with clinical evidence of loosening has demonstrated the formation of a membrane characterized by the presence of foreign body giant cells containing polyethylene debris and mononuclear histiocytic infiltrates within a fibrous tissue stroma [177].

Different studies [132,178–182] have shown that the cementless interface membranes as well as cemented ones produce significantly higher levels of collagenase, gelatinolytic activity, PGE_2, and interleukin-1 than the control tissue. These studies indicate that cells in the interface membranes produce matrix MMPs such as MMP-1, MMP-2, and MMP-9 as well as TIMPs. However, information is limited on the identification of the MMPs responsible for the enzymic activities or the source of the MMPs in the membranes [183,184]. Recently also an important role for MMP-1 and MMP-13 has been suggested in the loosening of artificial hip joints [185]. However the production of PGE_2 and IL-1 suggests the indirect role of the membrane in bone resorption through stimulation of osteoclasts by the factors at the local site. On the other hand, recent studies using an in vitro bone resorption assay have shown that macrophages and macrophage polykaryons derived from the joint capsule of hip arthroplasties or granulomas induced by bone cement can directly resorb bone [186]. Another mechanism inducing periprosthetic tissue remodeling is the wear of implant materials, release of implant particles and debris into periprosthetic tissues with the formation of reactive granulation tissue against a foreign body, and the activation of cells to produce cytokines and enzymes [187,188]. Some studies demonstrated that granulomatous tissue cells such as infiltrated macrophages have the ability to produce inflammatory cytokines and substances (i.e., IL-1 and TNF [189,190]) and activate osteoclasts. Osteoclasts were believed to cause bone resorption around implants and a recent study reported a strong expression of MMP-9 mRNA in the osteoclasts [191] as observed in the destructive joint of rheumatoid arthritis [192].

It was pointed out that the balance between MMPs and TIMPs was important to avoid the catastrophic effects of MMPs in various tissues [165]. In fact it has been hypothesized that an imbalance between MMPs and TIMPs at the edge of the interface granulation tissue and local overexpression of MMPs may cause tissue destruction around implants [193]. It is noteworthy that the cemented interface tissue has the ability to produce TIMPs [183].

It has been also observed that polymer surface chemical carachteristics can modulate the expression of MMPs in human osteoblast-like cells [194], fibroblasts [195], and peripheral blood cells [196] (Fig. 8) by direct contact, possibly through an autocrine mechanism or a surface differential adsorption. Recently a new intriguing role for MMP and TIMP espression at the biomaterial–bone interface has been suggested in the absence of any kind of disease [193]. Osteointegration at the site of orthopedic implants is dependent on the recruitment, attachment, and differentiation of osteogenic cells following implantation. The presence of fibrocartilage tissue and calcified bone within the interface, together with the presence of MMP-1 and MMP-2 and their inhibitors (TIMP-1 and TIMP-2) within fibroblasts, chondrocytes, and osteoblasts, indicates a possible important role of these enzyme in the osteo-integrative phenomenon.

In conclusion, ECM forms a dynamic site of interactions with biomaterials; its role is not a structural "static" support for tissues, but rather a very complex entity rich in factors that mediate the relation of intra- and extracellular signals. The study of the ECM environment can help in the understanding and improvment of biocompatibility.

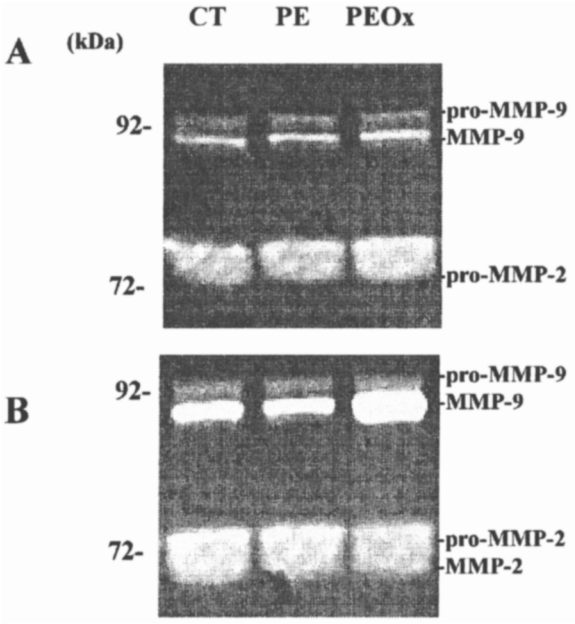

Figure 8 Representative gelatin zymograpy obtained at 24 (A) and 48 (B) h. PBMCs conditioned medium in the presence of polystyrene (CT), UHMWPE (PE), and UHMWPE-oxidized (PEOx) MMP-2 (gelatinase A) and both inactive and active MMP-9 (gelatinase B). Both inactive (pro-MMP-2 and-9) and active (MMP-2 and-9) forms are indicated along with molecular weight markers for inactive forms. (From Ref. 196.)

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2

Elastic Protein-Based Biomaterials: Elements of Basic Science, Controlled Release, and Biocompatibility

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I. INTRODUCTION

A. The Unique Opportunity Provided by Elastic Protein-Based Materials

The basic model protein of focus here, $(Gly\text{-}Val\text{-}Gly\text{-}Val\text{-}Pro)_n$, [or simply $(GVGVP)_n$], exhibits both dominantly entropic elasticity and the energy conversions of thermomechanical and chemomechanical transduction. It achieves these functional capacities with only mildly hydrophobic (Val and Pro) and neutral (Gly) side chains, that is, without any functional groups, save the peptide moiety itself. These fundamental functions achieved with such simplicity of composition become remarkably compounded due to the capacity of protein biosynthesis to position precisely any one of 20 different amino acid side chains at any location along the protein sequence. The result is a family of elastic, and even plastic, protein-based materials of near endless functional versatility. The application of a wide range of experimental methodologies, of thermodynamic and statistical mechanical analyses, and of molecular mechanics and dynamics calculations to protein-based polymers of such demonstrated functional diversity provides a unique opportunity

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to gain a level of understanding and utilization afforded by few, and possibly no other, chain molecules. All of this potential can be turned toward biomaterials applications. This review provides one glimpse into this potential.

B. Elastic Protein Mechanics Characterized by Two Interlinked but Distinct Physical Processes

The most effective use of elastic protein-based biomaterials requires an understanding of the molecular mechanics responsible for two interlinked but distinct physical processes—the development of entropic elastic force and contraction achievable by diverse energy sources[1]. The physical basis of desirable ideal (entropic) protein elasticity has been a concern for half a century and has been intensely contested for the last three decades. There are three contending mechanisms for protein elasticity: the classical (random chain network) theory of rubber elasticity [2,3], the solvent entropy theory [4,5], and the damping of internal chain dynamics on extension [6,7]. Data and analyses, briefly reviewed below, argue that entropic elastic force arises due to a damping of internal chain dynamics on extension [1,8,9]. Observation of entropic elasticity in atomic force microscopic (AFM) single-chain force-extension studies eliminates the random chain network mechanism, because its formulation requires a random network of chains with a Gaussian distribution of end-to-end chain lengths [10]. Isometric contraction by inverse temperature transition, that is, the development of entropic elastic force at fixed length whether due to thermally or chemically driven hydrophobic association, eliminates the solvent entropy mechanism as a source of entropic elastic force [1].

Versatile protein contractility arises due to hydrophobic association of protein sequences with the consequence of extension of interconnecting chain segments resulting in an increase in entropic elastic force due to damping of internal chain dynamics within the interconnecting chain segments. Versatility of energy inputs that drive contraction arises out of the many variables that control hydrophobic association/disassociation in aqueous systems [11,12].

C. Proposed Basis of Remarkable Biocompatibility of Elastic Protein-Based Materials

The basic elastic protein-based polymer sequence, $(GVGVP)_n$, originally observed in the mammalian elastic protein, elastin [13,14], exhibits a remarkable biocompatibility [15]. For example, it has not been possible to obtain monoclonal antibodies, even with very weak titers, to this elastic protein-based polymer [16]. When adequately purified, the phase-separated (hydrophobically associated) state of this elastic protein-based polymer appears to be simply ignored by the host. As will be argued herein, the dynamic nature of the phase-separated state at physiological temperatures constitutes a barrier to interaction that would otherwise be required for identification as a foreign material. Without eliciting an apparent inflammatory response, the hydrophobically disassociated (solution) state proteolytically degrades within the host. Thus, to control the state is to control the rate of degradation.

D. Hydration-Mediated Apolar–Polar Repulsion Provides for Special Controlled Release Devices

There exists a competition for hydration between apolar (hydrophobic) and polar (e.g., charged) groups constrained to coexist along the protein chain sequence [11]. In 1937 Butler reported the key realization that hydration of hydrophobic groups is a favorable exothermic reaction [17], but that solubility ultimately becomes lost due to the increase in order of water on going from

bulk water to hydrophobic hydration. In short, a limited amount of hydrophobic hydration occurs, but too much hydrophobic hydration drives insolubility on association of hydrophobic groups, obviously, with essentially complete loss of hydrophobic hydration.

Charged species, such as carboxylates ($—COO^-$) and amino ($—NH_3^+$) groups, when sufficiently proximal to hydrophobic groups, destructure hydrophobic hydration in the process of achieving adequate hydration of their own [11,18]. This has two consequences: increased solubility of hydrophobic groups as their potential for hydrophobic hydration decreases and pKa shifts for the ionizable species. When there is abundant bulk water available, charged species form with a characteristic pKa, but when ionizable species must destructure hydrophobic hydration to achieve adequate hydration, they are at a higher free energy, as reflected by significant pKa shifts and associated positive cooperativity apparent in the degree of ionization versus pH curve. In the process of attempting to minimize their free energies of hydration, hydrophobic groups and charged groups each seek out water unperturbed by the other. This constitutes an apolar–polar repulsive free energy of hydration, ΔG_{ap}.

Neutralization, for example by ion pairing, of a charged group with a hydrophobic-induced pKa shift lowers the free energy of the charged group, relaxes the need for hydration, and can allow for reconstitution of so much hydrophobic hydration that hydrophobic association results. For elastic protein-based polymers hydrophobic association is observed as a phase separation, called an inverse temperature transition because it is a transition to a more ordered state for the protein part of the system as the result of raising the temperature. When an oppositely charged drug neutralizes polymer charge, the drug provides the chemical energy for phase separation, that is, for formation of the drug delivery vehicle.

As will be shown below, the phase-separated state of drug plus elastic protein-based polymer becomes a drug delivery vehicle. The level of release depends on several factors—the surface area of the drug delivery vehicle available to the surrounding medium, the ionic composition of the medium, and, most fundamentally, the decrease in free energy on ionpairing of drug with polymer. Of course, lower release levels correlate with larger decreases in free energy on ionpairing of drug with polymer. The decrease in free energy on ionpairing is proportional to the hydrophobic-induced pKa shift exhibited by the ionizable groups of the polymer in the absence of drug. When the polymer is not cross-linked, the polymer, which is the drug delivery vehicle, disperses as the drug is released [16], unless ion exchange occurs to maintain the phase-separated state. The special cases of the loading and release of dexamethasone phosphate and betamethasone phosphate, reported below, demonstrate the effectiveness of this design of drug delivery vehicle using positively charged elastic protein-based polymers.

E. The Formation of Nanoparticles

Under selected conditions of concentration of poly(GVGVP) and temperature, quasi–elastic light scattering demonstrated the occurrence of stable nanoparticles [19]. As will also be reported here, quite uniform nanoparticles can be stably formed and fixed by cross-linking using the compositions—$(GVGVP)_{251}$, glutamic acid residue–containing polymer with increased hydrophobicity exhibiting large pKa shifts, and lysine-containing polymers with pKa-shifted amino functions. This means that controlled release of the type described above becomes possible with cross-linked nanoparticles.

II. MECHANICS OF ELASTIC PROTEIN-BASED BIOMATERIALS

Two elements of mechanics—the basis of ideal elasticity and the process of hydrophobic association—dominate consideration of elastic protein-based materials. Proper design and effective use

of elastic protein-based polymers as biomaterials requires clarification of these two elements of mechanics. Furthermore, elastic protein-based polymers become model chain molecules with which to determine the molecular basis of entropic elasticity and with which to develop the comprehensive hydrophobic effect for chain molecules in general.

A. Mechanism of Entropic Elasticity

As noted in the section I, there are basically three proposed mechanisms for entropic elasticity. The key arguments delineating these mechanisms are briefly considered in the immediately following text.

1. The Classical Rubber (Random Chain Network) Theory of Entropic Elasticity.

In this argument, stretching of a random network of chains causes the chains to become more aligned and shifted from a Gaussian distribution of end-to-end chain lengths between cross-links [10]. In this mechanism the chains are random; the chains do not exhibit regular structure. So the presence of a regular structure, as revealed by the presence of mechanical resonances in the dielectric relaxation spectra of the elastic protein-based polymers (see Figs. 1A and B), eliminates this mechanism. Furthermore, the ideal (entropic) elasticity exhibited by a single chain instead of requiring a network of random chains, as demonstrated previously [8,9], also eliminates this mechanism.

Nonetheless, calculations of the distribution of end-to-end chain lengths, as the result of rotations about one bond at a time, do provide approximate estimates of entropic elastic force. The fundamental flexibility of the chain (the chain entropy) is reasonably evaluated in this way. The natural constraint of the elasticity experiment, however, requires consideration of the coupling of rotations about two or more bonds at a time. In the elasticity experiment, the ends of an individual chain are fixed in space and then extended by a fixed amount with the result of an increase in force. So the difference in elastic force occurs without an experimental sampling of a distribution of end-to-end chain lengths. The computation reasonably becomes one of determining the difference in chain entropies calculated at two different extensions of the same chain length. The way that this happens of course is by rotation about one bond while allowing compensating rotation about another bond or bonds in such a way that the ends remain fixed in space.

This, in fact, becomes a representation of the mechanism referred to as the damping of internal chain dynamics on extension [6,7], as discussed below. Simply stated, as the distance between the ends of a chain of a given number of repeating units becomes greater, rotation about bonds becomes more limited. In terms of statistical mechanics the volume in phase space becomes less, and volume in phase space is the definition of entropy. So increase in elastic force occurs with a decrease in chain entropy of chain segments that sustain the force. Some argue that it is the decrease in entropy of chain and surrounding water, as considered below.

2. The solvent entropy mechanism (the decrease in entropy on stretching due to bulk water forming lower entropy hydrophobic hydration around newly exposed hydrophobic groups).

Weis-Fogh and Andersen [4] proposed that a decrease in solvent entropy occurred on extension of elastin as the result of exposure of hydrophobic groups to water. On stretching, an exothermic hydration of hydrophobic groups occurs with a decrease in the entropy of water. This mechanism maintains computational adherents up to the present time [5,20].

Experimental studies, wherein a chosen solvent mixture largely removes the solvent entropy change, demonstrate an increase in entropic elastic force rather than a decrease [21]. Such a result seriously questions the validity of this source of negative entropy for the development of entropic elastic force (see Fig. 2A). The isometric contraction experiment, the development

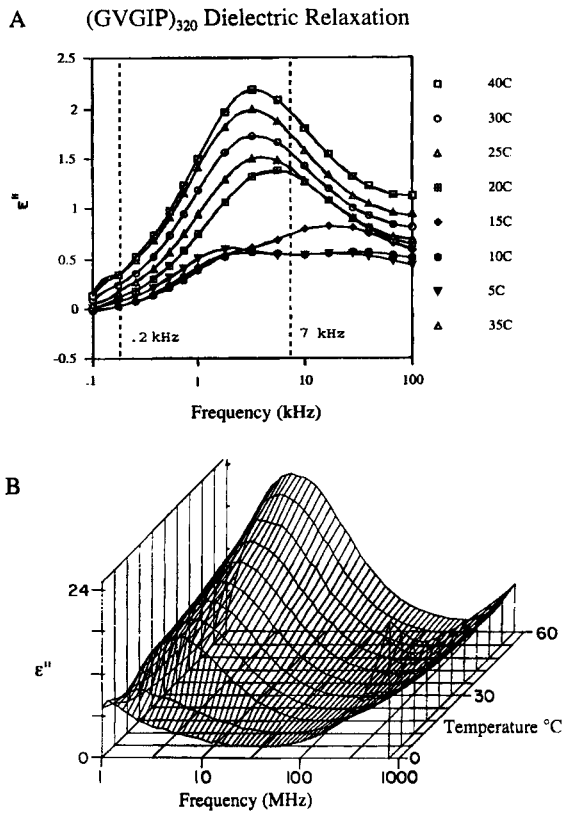


Figure 1 Dielectric relaxation spectra of the $(GVGIP)_n$ member of the family of elastic protein–based polymers showing the development of mechanical resonances on formation of the hydrophobically associated, phase-separated, and more ordered state achieved by raising the temperature. (A) The low frequency range of the spectrum (0.1 to 100 kHz) with the acoustic frequency range being in the lower two-thirds of the frequency range. As the temperature is raised from below to above the temperature range of the inverse temperature transition of hydrophobic association, there develops an intense relaxation centered near 3 kHz. (From Ref. 9.) (B) The 1-MHz to 1-GHz frequency range of the dielectric relaxation spectrum showing the development of an intense relaxation centered near 5 MHz that continues to grow in intensity on raising the temperature up to 60°C. (From Ref 33.)

of entropic elastic force at fixed length, however, eliminates the solvent entropy mechanism for any polymer in water that exhibits a hydrophobic association transition during the isometric contraction.

For amphiphilic polymers in water such as protein, isometric contraction results when hydrophobic association due to an inverse temperature transition occurs at fixed length. This can be shown both with thermally driven and chemically driven hydrophobic association when the protein or other amphiphilic polymer in water is kept at fixed length. In particular, the thermoelasticity experiment used to assess the fraction of entropic elastic force is carried out under conditions of fixed length. As the temperature is raised from below to above the onset temperature for hydrophobic association, the elastic force develops until the hydrophobic association transition is complete and a plateau results in a plot of ln(force/T°K) versus temperature

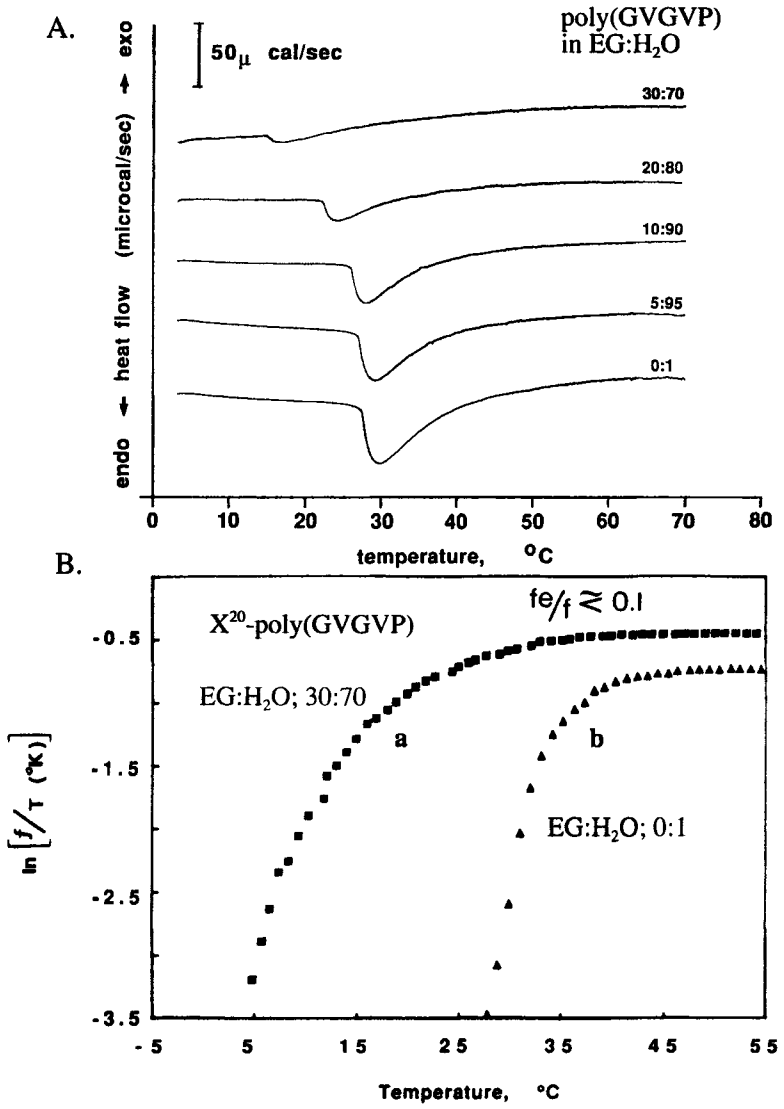


Figure 2 (A) Differential scanning calorimetry data on the endothermic heat of the transition of poly(GVGVP) in water and in increasing amounts of ethylene glycol (EG). The EG is increased until the heat of the transition approaches zero. Since the heat of the transition divided by the temperature at which the transition occurs is the entropy of the transition and since the heat of the transition is almost entirely due to the heat required to destructure hydrophobic hydration to form bulk water, the result is to remove the solvent entropy contribution to the transition from the hydrophobically associated to the disassociated state. (B) Thermoelasticity study of an elastic matrix of poly(GVGVP) formed on γ-irradiation cross linking. The sample is equilibrated at 40°C and stretched to a fixed length. The temperature is reduced, while keeping the sample at the extended length, equilibrated at a temperature below that of the phase transition; then, continuing to maintain a constant length, the temperature is slowly raised. The development of force plotted as the log[f/T], f divided by the temperature in degrees Kelvin °K, is given as a function of temperature. The near zero slope reached at higher temperatures indicates that the developed force is 90% or more entropic in origin. Very significantly the rapid increase in force as the hydrophobic folding transition proceeds occurs while the solvent entropy change must be positive. During the hydrophobic folding of the inverse temperature transition, more ordered, low-entropy hydrophobic hydration becomes less ordered, higher-entropy bulk water, as the 90% entropic elastic force develops due to a decrease in entropy. This eliminates solvent entropy change as the source of the entropic elastic force. (From Ref. 21.)

(see Fig. 2B). From the slope of the plateau in Fig. 2B, the developed force calculates to be 90% or more entropic for cross-linked poly(GVGVP) [21].

During a hydrophobic association transition, low entropy (more ordered) hydrophobic hydration becomes higher entropy (less ordered) bulk water. In the above experiment, an elastic force that is 90% entropic, due to a decrease in entropy, develops during the hydrophobic association transition as hydrophobic hydration becomes higher entropy bulk water. Chemically driven isometric contraction gives the same result [1,22]. Under conditions of isometric contraction due to hydrophobic association, the solvent entropy change is of the wrong sign, positive instead of negative, and could not contribute to the negative entropy change in the development of entropic elastic force.

Therefore, one concludes that a change in solvent entropy does not contribute directly to the entropic component of elastic force. Even at the outset, it would seem apparent that the continuous backbone of a polymer chain sustains force, not the surrounding water molecules [1,3]. The change in entropy of water, however, is central to hydrophobic association that determines polymer structure and function, as occurs in energy conversion [1,11], and the hydrophobic association of certain chain segments results in the extension of interconnecting chain segments that do sustain the force [11].

3. Decrease in Entropy on Extension Due to the Damping of Internal Chain Dynamics

Development of a molecular structure for $(GVGVP)_n$ represented in Fig. 3 utilized the physical methods of proton and carbon-13 nuclear magnetic resonance, of Raman, infrared, circular dichroism, and ultraviolet absorption spectroscopies; of transmission electron microscopy with negative staining and optical diffraction; and of x-ray diffraction of cyclic analogs, and utilized molecular mechanics calculations constrained by allowed ranges of torsion angles and hydrogen bonding determined from NMR [23,24].

The molecular structure represented in Fig. 3E immediately suggested an entropic elastic mechanism of the damping of internal chain dynamics on extension [6,23] and just as abruptly pointed to a set of chemical tests in which the Gly residues were replaced by D-alanine and L-alanine residues [25–28]; to physical tests involving NMR relaxation and dielectric relaxation methodologies [29–33]; and to computational tests [6,7] of the mechanism. Furthermore, using molecular mechanics to calculate the net dipole moment of the (VPGVG) permutation of the pentamer and of a D-alanine analog of the pentamer reproduced the dielectric relaxation results [34]. Thus, the concept of the damping of internal chain dynamics on extension was placed on a solid experimental and theoretical foundation. Nonetheless, so entrenched was the random chain network theory that it took the AFM single-chain force-extension results and the acoustic absorption data on the elastic protein-based polymers in direct comparison with random chain elastomers in combination with thermodynamic and statistical mechanical analyses [8,9] to be convincing to many parties. In addition, one hopes recent analyses of isometric contractions [1], reviewed above, will put to rest the proposition that the solvent entropy change makes a direct contribution to entropic elastic force.

B. The Comprehensive Hydrophobic Effect: Process of Hydrophobic Association

1. Thermodynamics of Solubility of Hydrophobic Groups in Water

The remarkable feature of the solubility of hydrophobic groups is that their dissolution in water is a favorable exothermic process [17]. In particular, Butler measured the heat of dissolution in

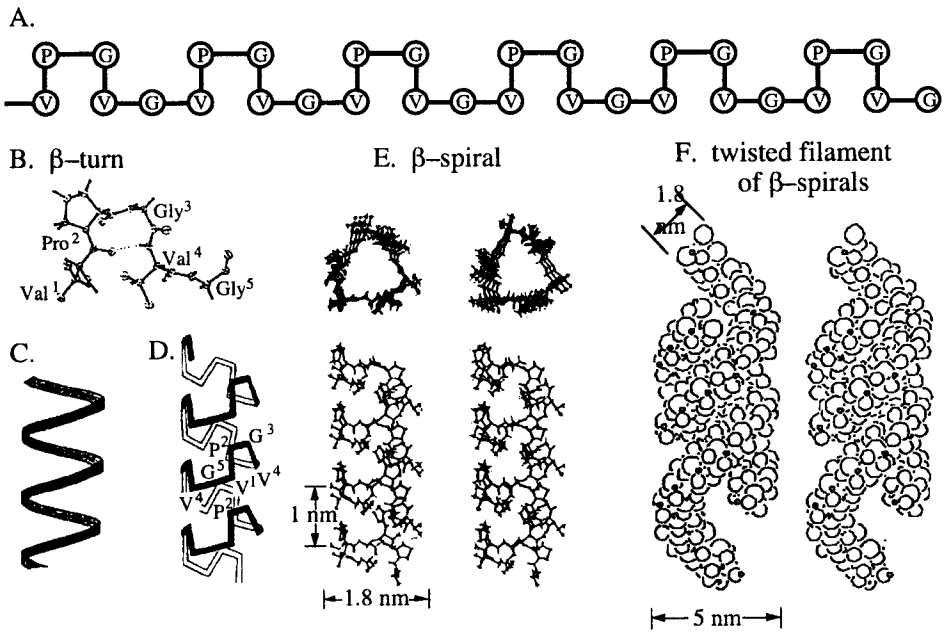


Figure 3 Molecular structure of poly(GVGVP), also referred to as poly(VPGVG). (A) Schematic representation as a series of β-turns inserted by the Pro-Gly sequence with interconnecting Val-Gly-Val sequences. (B) Detailed structure of the β-turn as determined from the crystal structure of the cyclopentadecapeptide cyclo(GVGVP)$_3$, showing the single secondary structural feature of a ten-atom hydrogen-bonded ring involving the Val^1 $-$CO ●●● HN-Val^4 residues. The residues are so numbered as to enclose and identify the β-turn structure. (C) Schematic of helical structure that forms on raising the temperature with optimization of intramolecular hydrophobic contacts. (D) Schematic of helical structure showing the series of β-turns to function as hydrophobic spacers between turns of what is called a β-spiral. (E) Stereo views (side and axial) of detailed computed structure of the β-spiral derived using NMR structural constraints of coupling constants and nuclear Overhauser effects to limit allowed torsion angle ranges and of the Val^1 $-$CO ●●● HN-Val^4 hydrogen bond and molecular mechanics calculations. (F) Association of β-spirals to form twisted filaments as observed in the transmission electron micrographs of negatively stained incipient aggregates undergoing a phase separation.

water of the methanol to n-pentanol series of linear alcohols, and determined the average heat released on addition of each CH_2 to be a favorable $\Delta H = -1.4$ kcal/mol-CH_2, whereas the average change in free energy due to formation of structured hydrophobic hydration from bulk water for the series was $(-T\Delta S) = +1.7$ kcal/mol-CH_2 [17]. As the change in Gibbs free energy for solubility, $\Delta G(\text{solubility}) = \Delta H - T\Delta S$, the ΔG(solubility) of methanol is a large negative number that shifts positively by 0.3 kcal/mol for each of the four added CH_2 groups as the series progresses from methanol to n-pentanol.

Even though the heat released on dissolution increases with each CH_2 group added, solubility is entirely lost once seven CH_2 groups have been added to methanol, that is, n-octanol is insoluble at 25°C. Obviously, the solubility of n-hexanol, for example, would decrease with increases in temperature, because the $(-T\Delta S)$ term would become more positive by the magnitude of the increase in temperature. This is the thermodynamic basis for the inverse temperature transition of hydrophobic association on raising the temperature.

2. Phase Diagrams for Inverse Temperature Transitions of Elastic Protein-Based Polymers

The traditional way to characterize phase transitions is by phase diagrams that plot temperature of the transition as a function of polymer concentration in a solvent. Figure 4 gives the phase diagrams in water for two elastic protein-based polymer compositions, poly(GVGVP) and poly(GGVP), as well as partial plots for additional compositions, e.g., poly(GVGIP), poly[0.8(GVGVP),0.2(GEGVP)]. Interestingly, the curvatures of the plots are inverted from those of conventional petroleum-based polymers dissolved in organic solvents. The solubilities are also inverted, that is, the elastic protein-based polymers are soluble below the binodal or coexistence line and insoluble above, rather than the usual increase in solubility with increase in temperature.

Characterization of the inverse temperature transition of hydrophobic association involves determination of the onset temperature for the phase separation as the temperature is raised (see Fig. 5). The onset temperature for the inverse temperature transition has been designated as T_t, which is equivalent to temperature of the binodal or coexistence line. For discussion of inverse temperature transitions, therefore, we refer to the binodal or coexistence line as the T_t-divide, because it is the line that divides the lower temperature soluble state from the higher temperature insoluble state.

3. The Change in Free Energy of Hydrophobic Association, ΔG_{HA}

The change in Gibbs free energy of hydrophobic association resulting from any variable χ that changes the value of T_t has been derived as [1]

$$\Delta G_{HA}(\chi) = [T_t(\chi) - T_t(\text{reference})]\Delta S_t(\text{reference}) \quad (1)$$

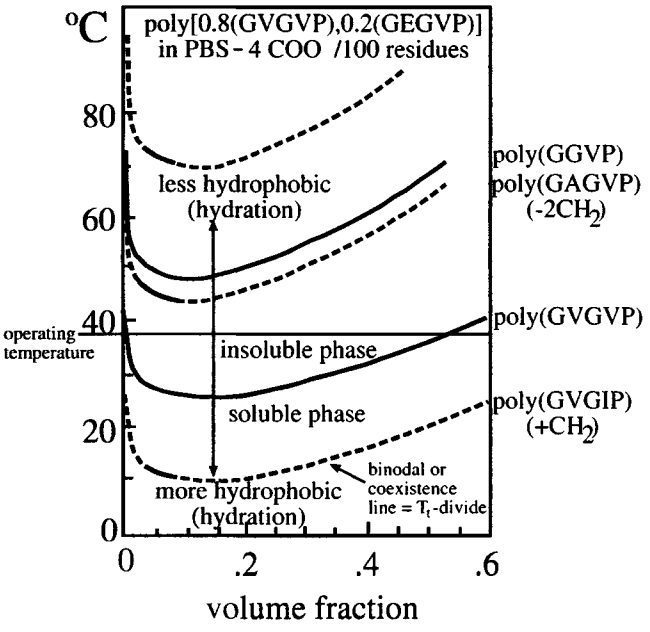


Figure 4 Phase diagrams of poly(GVGVP) and poly(GGVP) showing an inverted curvature with respect to the x-axis and an inverted solubility with the soluble state below the binodal or coexistence line (called the T_t-divide). The inverted curvature and inverted solubility, when compared to the more common phase diagrams of petroleum-based polymers, is part of the reason why the phase transition for these elastic protein–based polymers is called an inverse temperature transition. (Adapted from Ref. 19.)

ΔS_t(reference) is the entropy change for the transition before introduction of the variable, χ, where ΔS_t(reference) is obtained from the differential scanning calorimetry data by dividing the increment of heat absorbed over an incremental change in temperature and summed over all of the increments of the transition. Thus, the change in the onset temperature for the transition due to a perturbation $\Delta T_t(\chi)$ times a constant for the reference polymer provides an approximate measure of the change in Gibbs free energy of hydrophobic association for these elastic protein-based polymers. Since the energy of deformation on stretching the chain is less than 20% of the heat of the hydrophobic hydration that attends the extension [3], neglecting ΔH_t(chain) gives one aspect of the approximation. The derivation also takes the chemical potential of the hydrophobically associated state at the T_t-divide to be equal to the chemical potential of the disassociated state. This approximation becomes less satisfactory as the width of the transition increases.

4. The Competition for Hydration Between Hydrophobic and Charged Moieties

From Fig. 4 the presence of four carboxylates, COO^-, of the glutamate (Glu, E) residue per 100 residues in poly[0.8(GVGVP),0.2(GEGVP)], increases the value of the T_t-divide by 45°C in the presence of phosphate-buffered saline (PBS), 0.15 N NaCl, and 0.01 M phosphate. In the absence of PBS, the T_t-divide for poly(GVGVP) increases by no more than 2 to 3°C, whereas the T_t-divide for poly[0.8(GVGVP),0.2(GEGVP)] extrapolates toward 200°C. The ion pairing COO^- Na^+ dramatically lowers the T_t-divide to 70°C.

From the above discussion of ΔG(solubility), the fact that the carboxylate in the absence of cations raised the T_t-divide so high suggested that the carboxylate did so by destructuring hydrophobic hydration in the process of obtaining its own hydration. Indeed using microwave dielectric relaxation methods, the loss of hydrophobic hydration on formation of less than two carboxylates per 100 residues has been directly observed and quantified [11,18]. Thus, ion paring allows reconstitution of hydrophobic hydration with the lowering of the T_t-divide. This lowering of the T_t-divide on ion pairing is used to follow the loading profile of positively charged polymers with negatively charged drugs, as seen below in Figs. 6A and B.

5. Apolar–Polar Repulsive Free Energy of Hydration, ΔG_{ap}

The other side of the coin of the competition for hydration between hydrophobic and charged moieties is the increase in free energy of the charged state as seen in hydrophobic-induced pKa shifts. Prior to the work on these protein-based polymers it was thought that any significant pKa shift arose either due to charge–charge repulsion or due to a conformation that forced the ionizable function into a medium of low dielectric constant. We reject the latter explanation and show that charge–charge repulsion can be a few tenths of a pH unit with one charged residue every fifth residue in the absence of PBS, but even that is relaxed in the presence of 0.15 N NaCl [35,36,37]. On the other hand hydrophobic-induced pKa shifts as much as 6 pH units have been observed [38].

Thus, we have that the change in Gibbs free energy due to the apolar–polar repulsive free energy of hydration, ΔG_{ap}, can be written as [11]

$$\Delta G_{ap} = 2.3\,RT\Delta pKa \tag{2}$$

When charge–charge repulsion is negligible and the competition for hydration dominates, $\Delta G_{ap} \approx \Delta G_{HA}$. This provides an understanding of the energetics involved in the loading and release of drugs by appropriately designed elastic protein-based polymers.

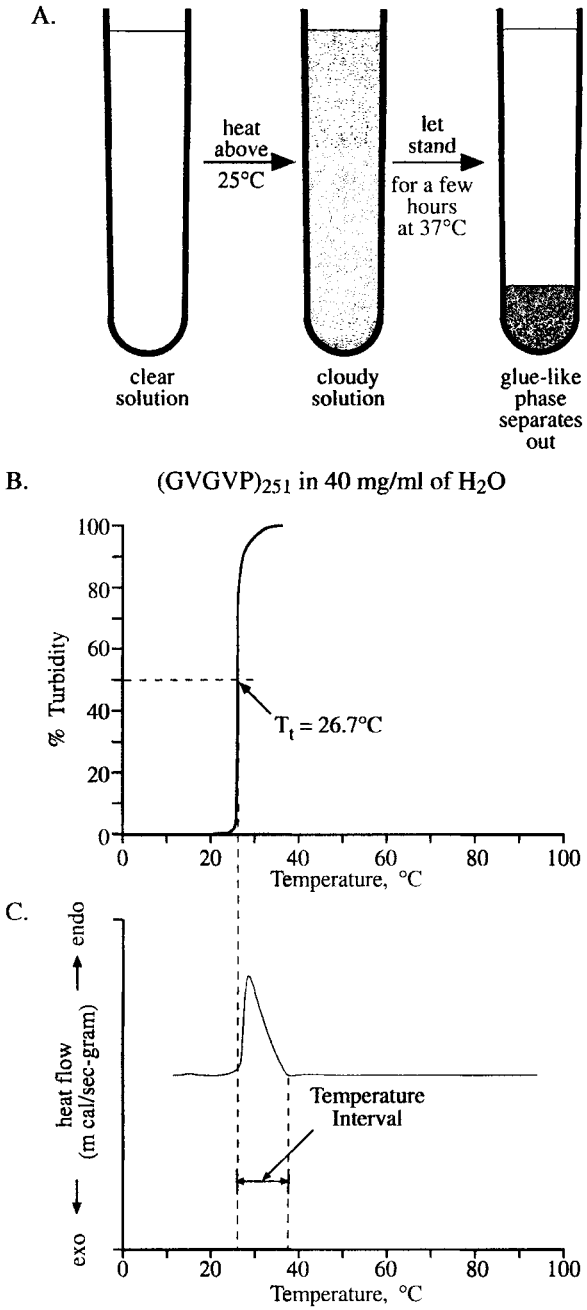


Figure 5 Characterization of the phase transition of $(GVGVP)_{251}$ on raising the temperature. (A) Tubes containing $(GVGVP)_{251}$ and water showing a clear solution turn cloudy due to aggregation on raising the temperature above 25°C. On standing the aggregates settle out and form a phase-separated state. (B) Following the temperature elicited aggregation by light scattering with the development of tubidity being used to define the value of T_t. (C) Differential scanning calorimeter curve of $(GVGVP)_{251}$ showing the relationship between T_t and the onset and breadth of the phase transition. Note that the curve has been inverted from the plots of Fig. 4. (Adapted from Ref. 11.)

III. ELEMENTS OF THE ION-PAIRED CONTROLLED RELEASE DEVICE

A. Design of Elastic Protein-Based Polymers for Loading and Release of Anionic Drugs

When considering the design of elastic protein-based polymers for controlled release of anionic drugs with the above-presented background information, a series of lysine-containing polymers with a range of hydrophobic-induced pKa shifts become an obvious choice. The following family of polymers were designed, prepared by means of recombinant DNA technology, and purified by means of the inverse temperature transition [39]:

Polymer i′ : (GVGVP GVGVP G**K**GVP GVGVP GVGVP GVGVP)$_{22}$(GVGVP) K/0F
Polymer ii′ : (GVGVP GVG**F**P G**K**G**F**P GVGVP GVGVP GVGVP)$_{22}$(GVGVP) K/2F
Polymer iii′ ': (GVGVP GVGVP G**K**GVP GVGVP GVG**F**P G**F**G**F**P)$_{22}$(GVGVP) K/3F
Polymer iv′ : (GVGVP GVG**F**P G**K**G**F**P GVGVP GVG**F**P GVG**F**P)$_{21}$(GVGVP) K/4F
Polymer v′ : (GVGVP GVG**F**P G**K**G**F**P GVGVP GVG**F**P G**F**G**F**P)$_{21}$(GVGVP) K/5F

The pKa values for the above polymers i′ through v′ are 10.0 (K/0F), 9.8 (K/2F), 9.5 (K/3F), 9.0 (K/4F), and 8.5 (K/5F) (unpublished results). These elastic protein-based polymers were designed for systematic nonlinear hydrophobic-induced pKa shifts. The expectation is that affinity for anionic drug will parallel the pKa shifts and that release rates will be inversely related to the to the pKa shifts. Larger hydrophobic-induced pKa shifts equate to lower release rates.

B. Profiles for Loading Anionic Drugs into Cationic Elastic Protein-Based Polymer Release Devices

1. Determination of T_t Values for Elastic Protein-Based Polymers

As shown in Fig. 5A, the elastic protein-based polymer (GVGVP)$_{251}$ is completely soluble in water at temperatures below 25°C. On raising the temperature above 25°C, the solution becomes cloudy, and on standing phase separation occurs. Our standard determination of the T_t value utilizes a solution of 40 mg/mL polymer with a molecular weight of approximately 100 kDa. At a sufficiently low temperature the polymer is completely dissolved as a clear solution exhibiting no scattering of light. The temperature is raised at a rate of 30°C per hour while monitoring light scattering at 300 to 400 nm. There occurs an abrupt development of light scattering that soon reaches a maximum. The temperature at 50% turbidity is defined as the T_t for the sample, as indicated in Fig. 5B. In Fig. 5C the differential scanning calorimetry curve for an equivalent sample is reported with the onset of the endothermic reaction for the destructuring of hydrophobic hydration corresponding to the value of T_t. The T_t values for the five polymers, polymers i′ through v′, listed above are plotted at 0 on the x axes of Fig. 6A and B for a 0.01 M phosphate solution at pH 7.5.

2. Determination of the Profile for Loading Drug into a Phase-Separated State

The profile for loading drug into the release device again uses a 40-mg/mL solution of polymer at pH 7.5, which is approximately 16 mM in lysine ε-amino (—NH_3^+) side chains. When a less than equimolar quantity of dexamethasone phosphate (DMP) or betamethasone phosphate (BMP) is dissolved with the particular polymer at a sufficiently low temperature for complete dissolution and the temperature is increased, the value of T_t is plotted as a function of the ratio of [DMP]/2[Lys residues], as the phosphate of the DMP is expected to bridge between two lysine ε-amino (—NH_3^+) groups.

The drug loading profiles are given in Fig. 6A and B. The set of loading profiles are essentially identical for the two drugs, as might be expected since they differ only in the orientation of the methyl at position 16 in the D ring of the steroid nucleus, being 16 α-methyl for dexamethasone and 16 β-methyl for betamethasone.

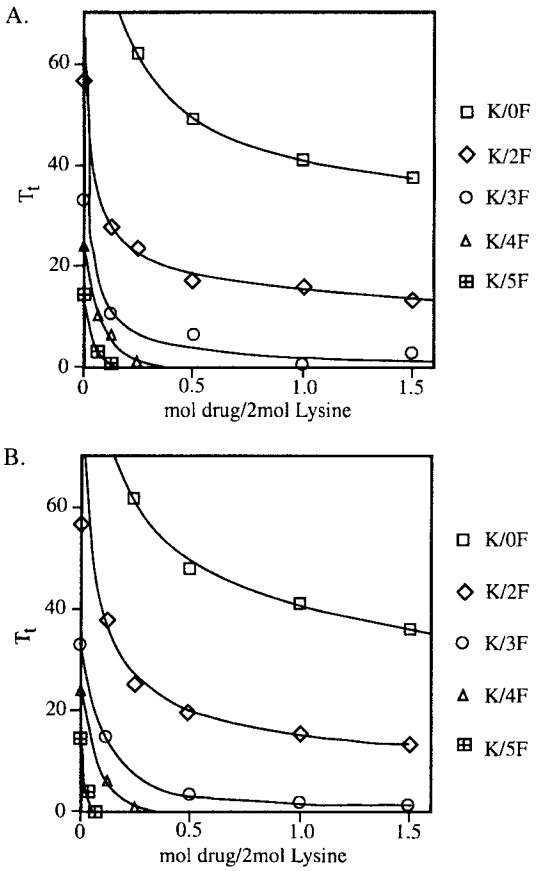


Figure 6 Loading profiles for dexamethasone phosphate (A) and betamethasone phosphate (B) followed by the decrease in the value of T_t on formation of an ion-pair between anionic drug and cationic polymers with one lysine (Lys, K) residue per thirty residues but with 0, 2, 3, 4, and 5 Phe (F) residues having replaced Val (V) residues in each 30 mer. The affinity for ion-pairing formation increases in parallel with the hydrophobic-induced pKa shifts. The two steroids differ only by the orientation of a methyl substituent at position 16 in the D-ring. Because the basis for the affinity is the relaxation of the apolar-polar repulsive free energy of hydration on ion-pairing and does not involve the selective binding of the steroid, the loading profiles are essentially indistinguishable.

At a temperature above T_t the drug is in the phase-separated state of the polymer. The drug-containing phase-separated state becomes the drug delivery device. In fact, the drug provides the chemical energy to drive phase separation for its own packaging. In particular, if the polymer were cross-linked into a swollen elastic matrix suspending a weight, the addition of drug would drive contraction with performance of the mechanical work of lifting the weight.

C. Release Profiles of Anionic Drug Release from Cationic Protein-Based Polymeric Devices

The drug release profiles for dexamethasone phosphate (DMP) and betamethasone phosphate are given in Figs. 7A and B, respectively, for the polymers indicated above as polymers i′ through v′ . Also the release data are tabulated in Tables 1 and 2.

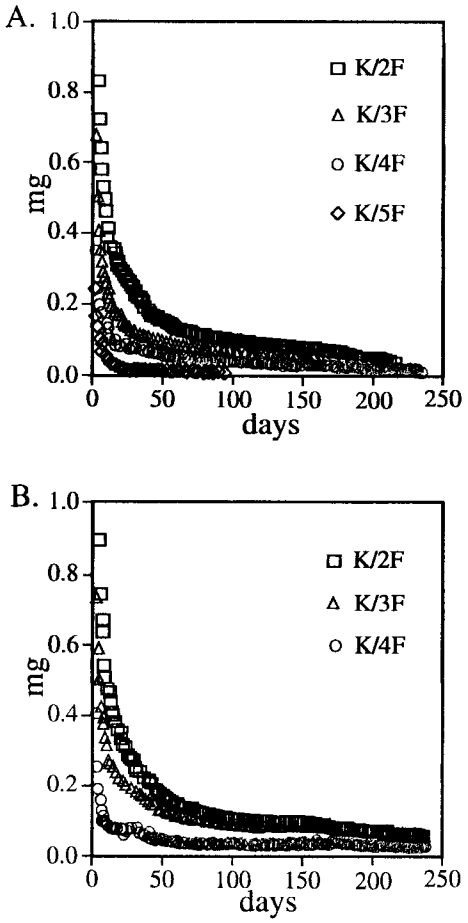


Figure 7 Release profiles for dexamethasone phosphate (A) and betamethasone phosphate (B) from a constant surface area of phase separated state formed due to ion-pairing of anionic drug with cationic polymer. Again the profiles of the two steroids are essentially identical. Interestingly, the release profile for betamethasone phosphate phase-separated on ion-pairing with (GVGVP GVGFP GKGFP GVGVP GVGFP GVGFP)$_{21}$(GVGVP) appears to exhibit zero order release kinetics for a period of over 100 days.

Table 1 Dexamethasone Release

Polymer	mg loaded	mg released	Days of release	Days remaining	Last release,
K/2F	35.3	31.4	216	111	.035
K/3F	34.5	17.4	169	444	.039
K/4F	18.2	13.7	235	277	.016
K/5F	13.7	3.4	94	953	.011

Table 2 Betamethasone Release

Polymer	mg loaded	mg released	Days of release	Days remaining	Last release,
K/2F	41.4	37.6	236	70	.0487
K/3F	33.5	28.8	236	83	.0566
K/4F	18.2	10.5	236	314	.0245

Clearly the principle is established for lysine-containing elastic protein-based polymers that the extent of hydrophobic-induced pKa shifts can be used to control the relative rates of release. Because of the phosphate in the medium and its capacity to exchange for the drug used in this case, however, not all compositions result in a zero order release profile. The K/4F composition, polymer iv′ , does appear to give a near zero order release profile for both drugs.

D. Release Profiles of Cationic Drug Release from Anionic Protein-Based Polymeric Devices

Zero order release profiles are possible when using a cationic drug with an anionic, carboxylate-containing, protein-based polymer. Of course, the level of release can be controlled by design of the desired hydrophobic-induced pKa shift. This is demonstrated in Fig. 8 for Leu-enkephalin amide and a series of glutamate-containing elastic protein-based polymers [16]. In this case we have loaded with a 50% excess of drug over ion-paired binding sites, such that there is a burst release. This would not occur if the device began with an exact equivalency of drug to binding site. Also, as indicated in Fig. 8B, the drug delivery device disperses with the drug.

The length of time that the constant, zero order release profiles last depends on the size of the depot, and the release remains zero order for a constant surface area. The release rates for a constant surface area of 0.55 cm^2 are seen to vary from 4.7 to 1.85 μM per day, with the constant release in the latter case demonstrated for three months.

IV. NANOPARTICLE OF ELASTIC PROTEIN-BASED POLYMERS

A. Formation of Nanoparticles from Elastic Protein-Based Polymers

Nanoparticles of the protein-based polymers, $(GVGVP)_{251}$ and [(GVGVP GVGFP GEGFP GVGVP GVGFP GFGFP)$_{32}$ (GVGVP)], have been successfully prepared. Polymer solutions of $(GVGVP)_{251}$ (25 mg/mL) and of [(GVGVP GVGFP GEGFP GVGVP GVGFP GFGFP)$_{32}$(GVGVP)] (7.2 mg/mL) were cross-linked at above 40 and 50 °C, respectively, by cobalt-60 γ-radiation with an exposure rate of 1.5 Mrad per hour. The transmission electron micrographs of Figs. 9A and B show the size and relative homogeneity of the nanoparticles obtained from $(GVGVP)_{251}$ and from [(GVGVP GVGFP GEGFP GVGVP GVGFP GFGFP)$_{32}$ (GVGVP)]. Particle sizes with diameters in the range of 50 to 200 nm were achieved for $(GVGVP)_{251}$, whereas a narrower range of particle sizes with diameters of 50 to 100 nm were obtained for [(GVGVP GVGFP GEGFP GVGVP GVGFP GFGFP)$_{32}$ (GVGVP)]. It may be noted that the distribution of nanoparticles of (GVGVP GVGFP GEGFP GVGVP GVGFP GFGFP)$_n$ containing Glu and Phe residues, being more hydrophobic and controllable by pH, are much more homogeneous than those of $(GVGVP)_{251}$.

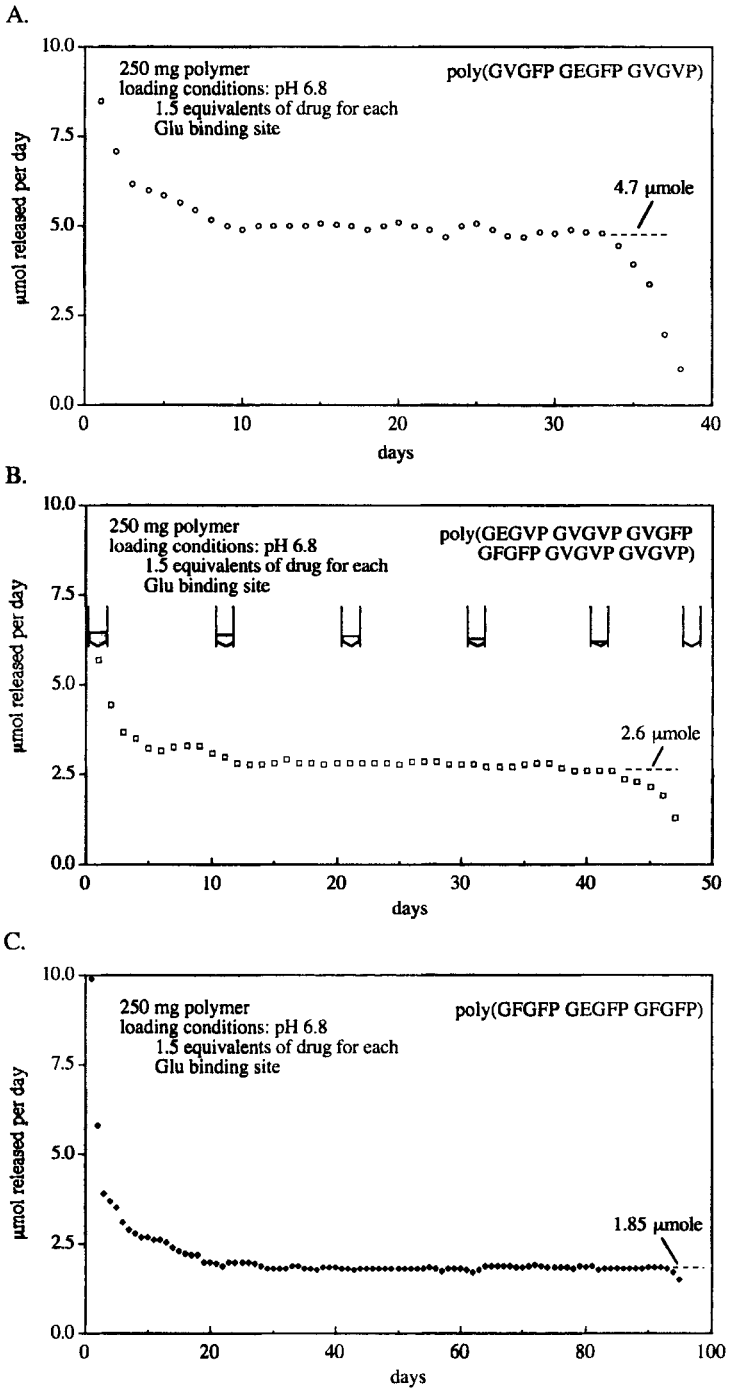
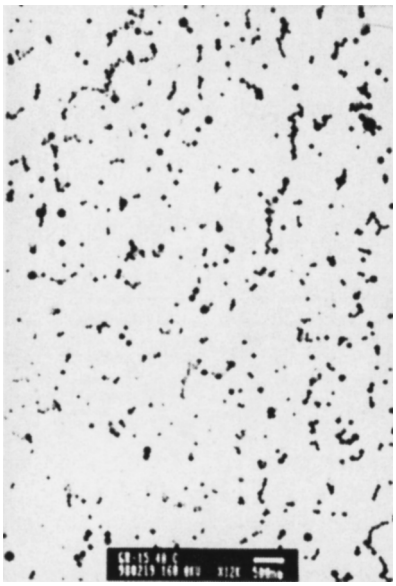


Figure 8 Release profiles for cationic Leu-enkephalin amide ion-paired with anionic, carboxylate-containing protein-based polymers. For this configuration of cationic drug and anionic polymer zero order release profiles are obtained once the excess drug is released. The release levels are inversely proportional to the hydrophobic-induced pKa shifts; they differ by a factor of 2.5, and remain at constant release levels for one to three months. (From Ref. 16.)

A.



B.

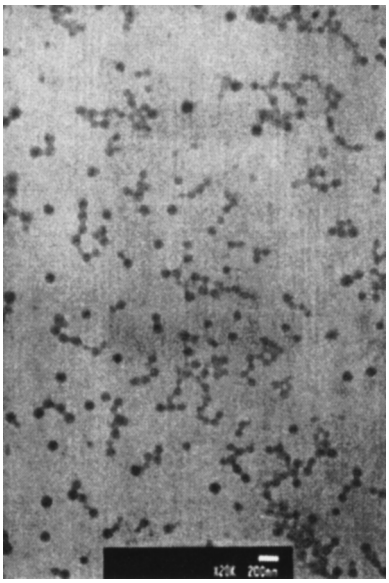


Figure 9 Nanoparticles of elastic protein-based polymers fixed by cross-linking with 20 Mrads of γ-irradiation. (A) Composed of $(GVGVP)_{251}$. (B) Composed of [(GVGVP GVGFP GEGFP GVGVP GVGFP GFGFP)$_{32}$(GVGVP)].

B. Advantage of Nanoparticles Made from Elastic Protein-Based Polymers

Nanoparticles made from elastic protein-based polymers have the usual advantages of being able to escape from the circulation into diseased tissues and be taken up by the liver; aspirated into the lungs; and injected, as desired, intramuscularly, subcutaneously, intrathecally, etc. Being transductional, however, nanoparticles made from elastic protein-based polymers exhibit a number of special advantages. They can be designed for a range of different release rates, as seen in Figs. 7 and 8, and as they are depleted of ion-paired drug they swell and become proteolytically degradable. In addition, it becomes a simple matter to introduce into the protein-based polymer desirable biologically active peptide sequences such as cell attachment sites, proteolytic cleavage sites, selective kinase sites, etc. Another specific advantage of elastic protein-based polymers is the basic biocompatibility of these dynamic polymeric structures from which they derive their entropic elastic property.

V. BASIS OF BIOCOMPATIBILITY OF ELASTIC PROTEIN-BASED POLYMERS

The preceding reviews our understanding of the mechanism of elasticity and the comprehensive hydrophobic effect, which enable the use of elastic protein-based polymers as biomaterials. Also, the particular way in which they achieve their entropic elasticity provides insight into their remarkable biocompatibility. Information establishing the outstanding biocompatibility is briefly reviewed followed by the proposed entropic explanation of the unique biocompatibility.

A. Studies Establishing Biocompatibility of the Basic Elastic Sequences

1. Standard Battery of Eleven Biocompatibility Tests on (GVGVP) and (GGVP)

The full battery of 11 biocompatibility tests performed at North American Science Associates (NAmSA) have been obtained on the basic elastic protein-based polymer sequences poly(GVGVP) and poly(GGAP) [15,40] and their γ-irradiation cross-linked matrices. A listing of the tests, their description, the test system, and the results is given in Table 3 for poly(GVGVP) and its crosslinked matrix. The results are the same for poly(GGAP).

2. In Vitro Assay for the Response of Human Macrophage (Monocyte) Cell Lines to Biomaterials

Macrophages fulfill the role of identifying and destroying foreign materials in the body. On identification of a foreign object they give off bursts of superoxide and hydrogen peroxide that oxidize and thereby begin the degradation and disposal of materials recognized as foreign. Grace Picciolo and coworkers [41] at the Center for Devices and Radiological Health of the Food and Drug Administration (FDA) developed an instrument to evaluate the reaction of human monocytes to biomaterials. The usual biomaterials elicit substantial oxidative bursts. They determined, however, that poly(GVGVP) and the related polymer-containing cell attachment sequences, poly[40(GVGVP),(GRGDSP)], slightly reduce the background oxidative activity of the monocytes. Simply stated these elastic protein-based polymers are not seen as foreign by human macrophage cell lines.

3. Efforts to Obtain Monoclonal Antibodies

Perhaps most remarkably, using sensitive and sensitized Balb/c mice, a primary immunization using 200 μg of poly(GVGVP) in emulsion with complete Freund's adjuvant, followed by a

Table 3 Summary of Biological Test Results for Poly(GVGVP) and Its γ-irradiation Cross-Linked Matrix

Test	Description	Test System	Results
(1) Ames (Mutagenicity)	Determine reversion rate to wild type of histidine-dependent mutants	Salmonella typhimurium	non-mutagenic
(2) Cytoxicity	Agarose overlay determine cell death and zone of lysis	L-929 mouse fibroblast	non-toxic
(3) Systemic Toxicity	Evaluate acute systemic toxicity from an I.V. or I.P. injection	Mice	non-toxic
(4) Intracutaneous Toxicity	Evaluate local dermal irritant or toxic effects by injection	Rabbit	non-toxic
(5) Muscle Implantation	Effect on living muscle tissue	Rabbit	favorable
(6) I.P. Implantation	Evaluate potential systemic toxicity	Rat	favorable
(7) Systemic Antigenicity (BPAT)	Evaluate general toxicology	Guinea Pigs	non-antigenic
(8) Sensitization (Kligman Test)	Dermal sensitization potential	Guinea Pigs	non-sensitizing
(9) Pyrogenicity	Determine febrile reaction	Rabbit	non-pyrogenic
(10) Clotting Study	Whole blood clotting times	Dog	normal clotting time
(11) Hemolysis	Level of hemolysis in the blood	Rabbit blood	non-hemolytic

* Reports from North American Science Associates (NAmSA)

secondary immunization using 200 μg of poly(GVGVP) in emulsified incomplete Freund's adjuvant, and finally boosted with a third immunization repeating the conditions of the second failed to produce antigen-specific hybridomas to poly(GVGVP). The use of two additional strains of mice (DBA and CB6 F1) similarly failed. As stated in the report of the results [16], "The preceding results suggest that certain protein-based (bioelastic) polymers, such as the elastic poly(GVGVP), appear to be incapable of eliciting a significant immune response."

4. Guinea Pig Subcutaneous Injection Model for Evaluation of Inflammatory and Pyrogenic Responses

A substantial body of work established chemically synthesized elastic protein-based polymers to have remarkable biocompatibility. On developing recombinant DNA technology to produce the elastic protein-based polymers in E. coli [39], a strong inflammatory response was obtained in the guinea pig subcutaneous injection model on using several cycles of phase separation for purification. This occurred even though by the Western immunoblot technique impurities were less than 1 ppm [42]. This is in spite of the fact that a level of less than 10 ppm impurities was the standard for use of insulin produced by E. coli. The difference is that a drug such as insulin is commonly used in microgram quantities or less. On the other hand the use of an elastic protein-based polymer as a biomaterial involves quantities of one thousand or even one million times greater. In particular in the guinea pig subcutaneous injection model 30 mg of $(GVGVP)_{251}$ was injected.

The gold standard for biocompatibility would then become the subcutaneous injection in the guinea pig of 30 mg of an elastic protein-based polymer that would release all of its impurities within a few days time without eliciting a significant inflammatory response. This was achieved

with the microbially produced elastic protein-based polymer (GVGVP GVGVP G**E**GVP GVGVP GVGVP GVGVP)$_{32}$(GVGVP). As seen in Fig. 10, at 2 weeks after subcutaneous injection of 30 mg of this polymer there was no evidence of the polymer having been present. Out of five test sites three showed traces of no more than a half dozen inflammatory cells [42]. Accordingly, the desired level of purification was achieved, and most remarkably in some sites despite a thorough search no tissue reaction whatever was discernible.

5. Glu- and Phe-Containing Polymers Also Biocompatible in Guinea Pig Subcutaneous Injection Model

It had been our expectation, once the elastic protein-based polymer contained a polar group such as a carboxylate in combination with more hydrophobic groups as in (GVGVP GVG**F**P G**E**G**F**P GVGVP GVG**F**P G**F**G**F**P)$_n$(GVGVP) and (GVGVP GVGVP G**E**GVP GVGVP GVG**F**P G**F**G**F**P)$_n$(GVGVP) that significant epitopes would be present and that a much more significant inflammatory response would be found. From the anecdotal evidence to date using the guinea pig subcutaneous injection model, the expected increase has not been seen.

The question becomes, therefore, whether there might be a fundamental reason why these elastic protein-based polymers and the mammalian elastic fiber itself elicit such meager immuno-

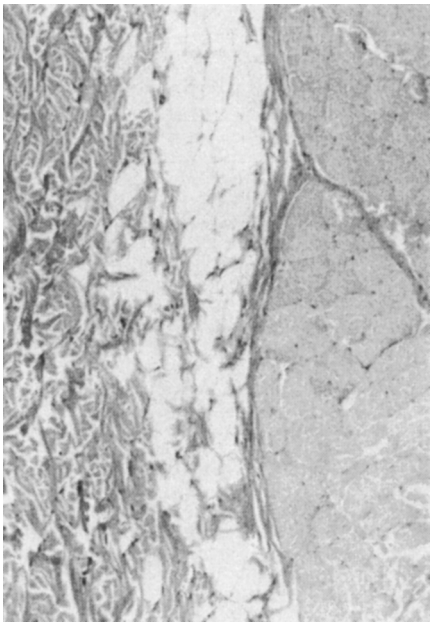


Figure 10 One of four subcutaneous sites in the guinea pig for injection of 30 mg of {GVGVP GVGVP G**E**GVP GVGVP GVGVP GVGVP}$_{36}$(GVGVP). At two weeks no trace of the polymer was observed at any of the four sites. On thoroughly searching through all of the slides of the four sites, two of the four sites (one of which is represented here) exhibited no evidence of any kind that this large injection of the elastic protein-based material had ever occured. In two sites there was only a single trace at one spot one spot in each of a half dozen to a dozen inflammatory cells. This provides the ultimate test for biocompatibility and for purification of E. coli-produced elastic protein-based polymer. (From Ref. 42.)

genic responses. An answer emerges below when considering further the biological implications of the mechanical resonances indicated by Fig. 1.

B. Proposed Basis for the Remarkable Biocompatibility of Elastic Protein-Based Polymers

1. The Presence of Mechanical Resonances.

As seen in Figs. 1A and B, on raising the temperature from below to above the inverse temperature transition observed in the dielectric relaxation experiment, noticeable are the development of strong relaxations localized near 3 kHz and again near 5 MHz. Therefore, during the hydrophobic association transition the pentamers fold into a regularly repeating dynamic structure. If there were no regular structure, the responsible rotation about bonds would occur throughout the frequency spectrum. On the contrary for this family of elastic protein-based polymers, more than 90% of the relaxation can be fit by a single relaxation frequency [29]. This means that the relaxations may be considered as describing mechanical resonance where the entire pentamer moves at the frequency of the relaxation. As argued below, such low frequency motions make large contributions to lowering the free energy of the structure, and an interaction that would interfere with or stop such motion would occur only on overcoming the stabilization that the motion represents.

2. The Dynamic Entropic Shield Against Identification as Foreign

Insight into the energetics that the coordinated oscillations about backbone bonds indicated by the mechanical resonances at 3 kHz and 5 MHz comes from the harmonic oscillator model approximation for the relevant partition function. At the top of Fig. 10A is the expression for the entropy of a harmonic oscillator as a function of the frequency of the oscillation. Using this expression, a plot of the entropy as a function of log(frequency) is given in Fig. 11A. The salient feature is that as the frequency decreases, the entropy increases. On the right-hand ordinate is plotted the TS_i term. For a frequency of 5 MHz the contribution, based on this idealized model, to lowering the free energy would be about 9 kcal/mol-pentamer, whereas the mechanical resonance near 3 kHz would calculate to lower the free energy by some 14 kcal/mol-pentamer. One should not take the harmonic oscillator approximation as quantitative in any way. Nonetheless, it is informative and allows the perspective that presence of such resonances very significantly lowers the free energy of the hydrophobically associated phase-separated state.

The next point to consider is that the barrier to such motions is very low, as demonstrated in Fig. 11B. This means that an oscillating electric field is not required to set the resonances in motion, nor for that matter is an acoustic wave required to producem motion, as the nominal 3 kHz resonance is in the acoustic frequency range. It would seem that the low frequency motions that lower the free energy of the phase-separated, β-spiral–containing state would be present under physiological conditions.

Now in order to identify elastic protein-based polymers exhibiting these low frequency motions would require that the elements that constitute an epitope would have to be stopped. Just as the motions lower the free energy of the β-spiral–containing state, stopping the motions to identify an epitope would require raising the free energy of the elastic protein-based polymer. The need to raise the free energy in order to identify an elastic protein-based polymer as foreign constitutes an entropic shield that would give the impression that the elastic protein-based material was being ignored by the host.

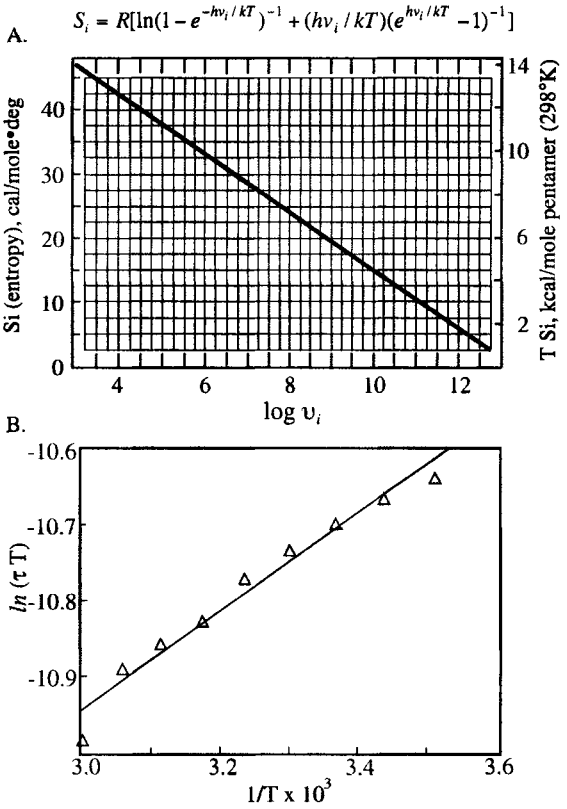


Figure 11 (A) Plot of the contribution to the entropy of a harmonic oscillator as a function of frequency from 10^{13} to 10^3 Hz(cycles/sec). The relaxation frequencies of the two mechanical resonances of Fig. 1 were approximately 3×10^3 Hz (3 kHz) and 5×10^6 Hz (5 MHz). By this approximation these mechanical resonances would contribute 9 and 14 kcal/mol-pentamer to stabilization of the regular, but dynamic, structure of the elastic protein-based polymer. Of course, plotting the entropy calculated using the harmonic oscillator partition function to such low frequencies cannot be considered numerically accurate, but it does show the trend and the trend is to increasing stabilization with decreasing frequency. (B) Plot of the temperature dependence of the 5 MHz mechanical resonance to determine the barrier to mobility. The barrier is 1.2 to 1.3 kcal/mol-pentamer, which means that the motion occurs without excitation by either an oscillating electric field as in Fig. 1, or an acoustic wave as would be relevant to the 3 kHz relaxation. See text for the implication with respect to biocompatibility. (From Ref. 33.)

To the extent that this is a significant factor in the remarkable biocompatibility, it would be unwise and self-defeating to compound this family of elastic protein-based polymers to other protein-based materials without such beneficial properties.

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3

Noninvasive Measurement of the Transient Adhesion of Cells to Blood-Contacting Surfaces

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I. INTRODUCTION

Contact with synthetic polymer and metal surfaces of blood-contacting equipment (pumpoxygenators, dialyzers, ventricular assist devices, vascular prostheses, etc.) activates host defenses, including the coagulation and inflammatory pathways, and the fibrinolytic cascade [1]. In large-surface contact situations, especially with cardiopulmonary bypass (CPB) equipment, the end result of these reactions includes reduced platelet function and survival and increased postperfusion bleeding times [2]. The resulting organ dysfunction may be viewed as a direct complication of CPB. The generalized inflammatory response is multifactorial and includes activation of complement, the initiation of fibrinolytic, kallikrein, kinin, and coagulation cascades within the body. It also includes the activation of platelets and neutrophils. The activation of platelets may render them less effective for hemostasis, leading to postoperative bleeding problems. The activation of neutrophils may lead to sequestration of neutrophils within the pulmonary vasculature, contributing to postoperative complications, including capillary leak syndrome and microvascular lung injury.

Oxygenator membranes are believed to represent the greatest challenge to host inflammatory systems during CPB, due to the large blood-contacting surface area (0.6–2 m^2) and relatively slow blood flow (<3 cm/s). How to improve the biocompatibility of CPB circuits becomes one of the critical factors that affect the performance of CPB circuits. Underlying the response of the organism to the CPB equipment is the initial protein adsorption and subsequent cell interactions at the foreign material–blood interface. Numerous studies have shown these interactions to be transient events, which are analyzed in detail in numerous laboratories. However, the focus on these interactions is generally lost when one moves to performance evaluation of full-scale clinical devices in vivo or ex vivo. Most works report at best the changes in formed elements between the inlet and outlet of the device under study. These changes represent a small difference of two large numbers, e.g., inlet and outlet whole blood platelet count. The large variances in each of these numbers preclude the accurate measurement of the transient uptake and release of cells from the device surface. These transient measurements are theoretically feasible by

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noninvasive gamma scintigraphy. In fact, platelet responses to devices have been studied by various applications of noninvasive gamma counting, ranging from simple detectors to scintigraphic imaging systems [3–6]. However, these methods have not discriminated between those cells passing through the device without surface interaction and those cells that attach to the surface. We have modified a scintigraphy system and developed an algorithm to permit this discrimination, including the visualization of the kinetics of blood cell distribution shifts in both the oxygenator circuits and internal organs. We present it here in the context of CPB system analysis of platelet and neutrophil adhesion and release from the device surfaces. This analysis is used to demonstrate the quite different responses of these cells to different surface treatments of the oxygenators. The regional distribution of platelets and neutrophils in internal organs, during CPB and postperfusion, is also feasible with this system, although better characterized with a tomographic system, rather than a 2D system. Inspection of the simpler 2D application presented herein will provide the reader with the knowledge necessary for the tomographic 3D system application.

The quantitative measurement system is composed of gamma scintigraphy combined with circulating cell counting. Additional measurements of cell activity, e.g., by flow cytometry or whole blood platelet or neutrophil impedance aggregometry, could be used if assessment of cell function were to be combined with that of cell adhesion and release. However, the focus of this chapter will be the modified gamma scintigraphy and analysis system. Dual-channel images of ^{111}In-labeled platelets and ^{99m}Tc-labeled neutrophils were acquired periodically by a GE 400T gamma camera in a 90-min CPB focusing on oxygenator circuits studies, followed by a 120-min post-CPB focusing on organ studies. The images were digitized, analyzed, and carefully corrected for energy overlap; blood pool subtraction; gamma decay; attenuation from plastic phantom, tissue, and distance; ratio change of labeled to total cells; and isotope concentration change in plasma.

Platelet and neutrophil distributions were determined on the microporous polypropylene membrane surfaces of two different types of oxygenators, the Cobe sheet membrane oxygenator and the Medtronic hollow fiber membrane oxygenator. Furthermore, these systems were evaluated with different types of surface treatments. First, that of a surface modifying additive (SMA^{TM}) coating, which is composed of polycaprolactone (PCL) and polydimethylsiloxane (PDMS) functional blocks, aiming to lower interfacial free energy, is one of the polymeric surfactant techniques. SMA coating on CPB circuits may reduce activation of cellular and protein blood components. SMA is licensed for coatings on Cobe DUO^{TM} oxygenator circuits by Thoratec Laboratories [7]. Second we evaluated a popular heparin coating technique, using the Carmeda^{TM} process: end-point attachment of heparin by covalent binding developed by Larm et al. [8] as applied to the Medtronic Maxima^{TM} oxygenator circuit. For this report we also present another promising method which does not involve surface treatment, the infusion of trace amounts of nitric oxide (NO) in the oxygenator inlet gas port.

II. CELL SEQUESTRATION AND ISOTOPE LABELING

A. Platelet Labeling

The ^{111}In-oxine method proposed by Thakur et al. [9] made it possible to label and quantify platelets for studies of acute and chronic phase thrombosis, and atherosclerosis and thrombosis. The interest in the use of ^{111}In stemmed from its efficient incorporation and the ability of cells to retain the label after they have been introduced back into the subject. The relatively long half-life (2.8 days) allows studies to be performed for up to 5 days and the efficient gamma photons (173 keV 84% and 247 keV 94%) permit excellent images to be obtained with an

administered dose of less than 500 μCi [10]. Chelates are used as isotope carriers. The two most commonly used carriers are oxine (8-hydroxyquinoline) and tropolone [11,12]. Other chelates have been compared with these two agents. Tropolone and mercaptopyridine-N-oxide resulted in a high labeling efficiency in a comparison of oxine, oxine-sulfate, tropolone, and mercaptopyridine-N-oxide in human and rabbit platelets [11]. Labeling efficiency was not significantly different for any of these when a labeling medium free of plasma was used. The platelet life span was significantly longer when labeling was performed with tropolone in plasma compared with oxine in ACD-saline. In a similar study, however, labeling of human platelets with either ^{111}In oxine in saline or ^{111}In tropolone in plasma did not result in any difference in platelet kinetics [12].

^{111}In-labeled platelet imaging, with its ability to noninvasively localize and quantitate platelet uptake, has considerably expanded the evidence supporting a platelet role in the thrombotic response to prosthetic materials. Multiple studies have demonstrated consistent ^{111}In platelet accumulation on prosthetic surface in vivo. The experimental types of platelet–prosthetic material interactions that have been assessed using labeled platelets in animal and in human studies include arterial and venous prosthetic materials, autologous vein coronary bypass grafts, vascular catheters, mechanical heart valves, acutely placed grafts, long-term implanted grafts (>1 month), drug effects on graft platelet deposition, cardiopulmonary bypass, etc. [13]. Hope et al. [14] studied the kinetics and sites of sequestration of ^{111}In-labeled platelets during CPB in patients. They found that the mean loss of ^{111}In platelets was 13 ± 4%, with 10.8 ± 1.3% of administered platelets lost in the pump and oxygenator. The survival of the remaining platelets was markedly shortened to 58 ± 8 h. In the 48 hr following surgery most of the senescent platelets localized in the liver.

To obtain platelets in as viable a condition as possible (not influenced by anesthesia), platelet harvesting is generally done from awake pigs, which are calmed by gentle handling and small amounts of food. Platelet activation must be inhibited during separation procedures, either by acidification of the labeling medium or addition of inhibitory prostaglandins. Platelet aggregation as measured by aggregometry was unaltered in the labeled platelet suspension compared with platelet aggregation in the unlabeled PRP. The aggregometry curve was similar, with the same aggregation velocity and aggregation maximum, which is an indication of intact platelet function [15]. When reinjected, labeled platelets retained radioactivity throughout their life spans of 158 ± 25 h.

B. Neutrophil Labeling

In evaluating a neutrophil-labeling technique, the most important criteria are satisfactory migration and recovery of the labeled cells in the circulation after reinjection. Diisopropyl fluorophosphonate (DFP-32), ^{111}In-oxine, and ^{99m}Tc are commonly used isotopes for neutrophil labeling. The gamma emission recovery for ^{111}In-oxine (or ^{111}In-tropolone) [16], and ^{99m}Tc [17] in the circulating granulocyte pool is 35 and 10%, respectively. The labeling efficiency with ^{111}In for neutrophil labeling is 80 to 90% for tropolone [18], but only 36% for oxine. The labeling efficiency with ^{99m}Tc by classical phagocytosis procedure is about 30 to 40%, compared to about 90% for human and canine blood with revised method [19]. The 6-h half-life of ^{99m}Tc was clearly too short for platelet survival and long-term imaging studies; however, it is fine for short-term studies like cardiopulmonary bypass. As a commonly accepted conclusion, ^{99m}Tc is inferior to ^{111}In when considered for neutrophil labeling. The half-life of ^{111}In-labeled neutrophils reported is 5 to 12 h [16,20]. The half-life for ^{99m}Tc-labeled neutrophils is not available.

Dual radiotracer methods, with platelets labeled with ^{111}In and red blood cells labeled with ^{99m}Tc, were introduced by Powers et al. [21]. We used ^{111}In-labeled platelets and ^{99m}Tc-