Professor David L. Kaplan

OVERVIEW - Research Program in the Kaplan Laboratory

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**Biopolymer Engineering - Structure-Function Relationships and Supramolecular Assembly** - The interface between biology and biopolymer science and engineering is a focus of our research program. Our efforts are aimed at understanding biological synthesis and processing of polymers within the context of polymer/materials science. This understanding is relevant to molecular recognition, self-assembly and the formation of materials with well-defined architectures, as well as biomaterials and tissue engineering utility. We utilize a variety of experimental strategies to gain insight into these areas: (1) genetic engineering - exploration of the molecular genetics of biosynthesis pathways for biopolymers, (2) whole cell physiology - manipulation of the cell environment to regulate the chemical and physical features of the biopolymer synthesized, and (3) enzymatic - polymer synthesis or modification using enzymes \textit{in vitro} in novel environments. In all cases, our aim is to manipulate the structure of biopolymers as a route to understand and control assembly, molecular recognition and biological interactions. In addition, since the synthesis, modifications and processing of these polymers are carried out within a biological context, issues related to green chemistry and environmentally-compatible processes are fostered.

**Biomaterials & Tissue Engineering** - The impact of selective environmental factors (e.g., growth factors, mechanical stress) on stem cell differentiation, the relationship between biomaterial structure
(supramolecular assembly) and stem cell responses, and the role for complex bioreactor designs to study these interactions are current areas of inquiry. We are particularly interested in how environmental cues (biomaterial structure/architecture, mechanical forces) influence stem cell processes and tissue engineering outcomes using both in vitro and in vivo studies.

Key words: bioengineering, biomaterials, biopolymer engineering, tissue engineering, self-assembly, supramolecular assembly, fibrous proteins, polysaccharides, genetic engineering, biopolymers

Research Summaries

Protein-based Biomaterials [silks, collagens]
Fibrous Protein Structure, Assembly & Processing [self fabricating, silk, collagen]
Polysaccharide-based Biomaterials [emulsan, cellulose]
Tissue Engineering [ligament, bone, stem cells]
Polymers via Enzymatic Catalysis

PROTEIN-BASED BIOMATERIALS – Bioengineering, Biocompatibility, Bioprocessing
Silk Films for Bone Formation - Silk-based biomaterials are used for the study of osteoblast behavior and bone formation. Silk proteins are selected due to their unique mechanical properties, biocompatibility and tailorable at the genetic and protein-polymer levels. Silk films were formed from cocoon silk fibroin from the Bombyx mori silkworm and characterized by contact angle, FTIR, ESEM, and AFM, indicating that these surfaces were fairly flat and moderately hydrophobic. Several peptides were covalently coupled to the silk surface using EDC/NHS coupling chemistry to explore osteoblast adhesion and bone formation, the two primary peptides being RGD (arginine-glycine-aspartic acid) and PTH (parathyroid hormone, PTH 1-34). For determination of bone formation, SAOS-2 cells were cultured for 2 and 4 weeks on the various substrates. After 4 weeks of culture, calcium content, assayed biochemically, was 1.4, 1.6 and 3.4-fold greater on the RGD-coupled surface than on unmodified silk, RAD, and plastic surfaces, respectively. The RGD surfaces showed significantly greater nodule area after 2 and 4 weeks in comparison to the other treated and control surfaces. These results indicate that silk proteins with coupled RGD have a significant potential to induce bone growth in vitro versus PTH-coupled surfaces, untreated silk surfaces, and the control surfaces. These RGD-coupled silk materials, combined with the unique mechanical and self-assembling properties of silks, suggest that these materials may be useful systems with which to explore bone formation in vitro and in vivo.

Silk – Biocompatibility - The properties of silk fibers and films make them promising candidates for tissue engineering scaffold, particularly where high mechanical loads or tensile forces are applied or in cases where low rates of degradation are desirable. A critical issue for biomaterial scaffolds is biocompatibility. The direct inflammatory potential of intact silk fibers as well as extracts was studied in an in vitro system. The results indicate that silk fibers are largely immunologically inert in short- and long-term culture with RAW 264.7 murine macrophage cells while insoluble fibroin particles induced significant TNF release. Soluble sericin proteins extracted from native silk fibers did not induce significant macrophage activation. While sericin did not activate macrophages by itself, a synergistic effect was demonstrated with bacterial lipopolysaccharide. The low level of inflammatory potential of silk fibers makes them promising candidates in future biomedical applications.

Silk– Electrospinning - Electrospinning offers an alternative approach to protein fiber formation that can potentially generate nanometer scale diameter fibers. This would be a useful feature in some biomedical and tissue engineering applications. Interest in the use of reprocessed silks such as fibroin in biotechnological materials and in biomedical applications derives from the unique mechanical properties of these fibers and their biocompatibility and biodegradability. We have reported the formation of electrospun scaffolds from aqueous silkworm silk (Bombyx mori) solutions with poly(ethylene oxide) (PEO). Fiber sizes ranged from 500 nm to 1 µm. Mineralized electrospun fibers were characterized by Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS), and surface morphology was observed by scanning electron microscopy (SEM). X-ray diffraction (XRD) of electrospun silk fibroin fiber and the calcium carbonate coating was also determined. The results of this study suggest new biomaterial utility for electrospun silk proteins as matrices for mineralized scaffolds such as for more mechanically robust systems.

Biomaterial Blends - Phase separation into controllable patterned microstructures was observed for Bombyx mori silkworm silk and poly(ethylene oxide) (PEO) (900,000 g/mol) blends cast from solution. The evolution of the microstructures with increasing PEO volume fraction is strikingly similar to the progression of phases and microstructures observed in surfactants. The chemically patterned materials obtained provide engineerable biomaterial surfaces with predictable microscale features which can be used to create topographically patterned or chemically functionalized biomaterials. Solution blending was used to incorporate water-soluble into silk to enhance the elasticity and hydrophilicity of silk. The presence of the PEO on the blend film surface was verified by contact angle analysis and XPS. Controllable phase separation was found that depended on the ratio of PEO to silk. Optical microscopy and SEM analysis confirmed the micro phase separation between the PEO and silk, forming the unusual globule shape inside of blend films.
Furthermore the PEO can be easily extracted from the films to generate silk with definable porosity and enhanced surface roughness. These new blend films formed from two biocompatible polymers provide new potential tissue engineering scaffolds and controlled release drug delivery materials.

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FIBROUS PROTEIN STRUCTURE & ASSEMBLY

Self-Fabricating Fibrous Protein-Based Biomaterials - By re-designing native proteins with specialized features, novel self-fabricated materials result. The concept is based on biomimetics, borrowing from Nature and using these design principles in new ways to make the materials. The key design feature was the formation of miniblock designs so that solubilizing ‘end blocks’ bracketing central ‘folding blocks’ permit high concentrations of the protein in solution to be formed, leading to the unique behavior observed. The core ‘folding blocks’ are initially based on silk-like or collagen-like sequences, as these are well-defined protein sequences with predictable solution behavior. The assembly-self-fabrication behavior is essentially a replication of what happens in Nature as biopolymers form more complex materials, via liquid crystalline intermediary steps.

Assembly of Silks - Recent studies involved the genetic modification of spider silk proteins to incorporate protein-based ‘triggers’ into the native consensus sequence to control structure, assembly and solubility. Two modes were developed, chemical and enzymatic. Methionines for chemical oxidation or reduction, and recognition sites for enzymatic phosphorylation/dephosphorylation, were incorporated flanking the polyalanine, β-sheet forming regions in the protein. The genes were constructed, cloned and expressed in E. coli. The methionines were amendable to selective chemical oxidation and reduction, altering the bulkiness and charge of the sulfhydryl-groups and in turn, the assembly of the polyalanine domains that crystallize to form β-sheets. The addition of the methionines into the consensus spider silk sequence did not disrupt the normal macromolecular assembly behavior of the protein. In the oxidized state (phenacyl bromide) the protein did not form β-sheet crystals and appeared morphologically featureless based on Transmission Electron Microscopy (TEM). To further confirm changes in assembly behavior observed for the recombinant protein containing the methionines, a model peptide with the same repeat amino acid sequence present in the recombinant protein was synthesized and characterized in the reduced and oxidized states. The expected shifts in molecular weight were observed by MALDI, and corresponding changes in crystallinity were determined by electron diffraction. A significant increase in protein solubility was also observed upon activation of the trigger. Similar changes were observed with the enzymatically activated phosphorylation/dephosphorylation trigger. These results support the use of redox or phosphorylation triggers as useful control elements with which to regulate and interrogate the assembly of β-sheet forming proteins (i.e., silks, prions, amyloids).
Assembly of Collagen - An understanding of macromolecular assembly is a cornerstone in polymer science, since the ability to design polymers that can 'assemble themselves', starting from the molecular scale, offers exciting opportunities in future materials design and fabrication. Collagen is a critical component in almost all tissues in the human body. Collagens provide surfaces upon which cell growth and development occurs and are involved in many disease states that can severely impact physiological function. Despite these essential roles for collagens in our body, very little is known about how complex collagen structures form in tissue. While there is good understanding of how collagen proteins become organized into triple helices, the formation of more complex organized structures that form the basis of bone, ligaments, skin and many other tissues is not understood. Our goal is to develop fundamental insight into the relationship between collagen primary sequence and the formation of higher-order collagen structures. Model peptides and recombinant proteins are studied in aqueous and solvent environments to understand how environmental changes influence assembly from the molecular to macromolecular scale. Initially, cholesteric-like arrangements of model collagen peptides have been studied in the glassy and semicrystalline solid state. In order to better understand liquid crystallinity in proteins the period of the rotation of rods in the solid materials (believed to be solid cholesterics) is compared to experimentally accessible molecular features such as conformation and crystal packing. A correlation between the asymmetry in interhelical packing and the period of the "cholesteric" is observed. Peptides and genetically engineered variants of collagens are under study to explore these issues. We have also recently used dip pen nanolithography to self-assemble native collagen and collagen peptides with nanoscale features. This AFM writing technique is being explored for further control of structure and function with these materials.

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POLYSACCHARIDE BIOMATERIALS – Bioengineering, Pharmacology

**Pharmacologically and Environmentally Relevant Bioengineered Polysaccharides** - A system currently under study is the microbial synthesis of a family of emulsifiers. Strategies to modulate structural features in order to explore function in a systematic way is the goal. To achieve this aim, the structures of a class of microbial amphiphiles, emulsans, lipoheteropolysaccharides secreted by *Acinetobacter calcoaceticus*, are being explored. The changes in structure are altered through changes in the physiology of whole cells and genetic modifications. A family of structurally-related analogs has been generated and used to explore solution properties and biological interactions, including CMC, immunomodulation *in vivo*, and cytotoxicity and stimulation of cytokine release *in vitro*. The opportunity to match biological response to structural features offers a new window into pharmaceutical discovery as well as issues related to controlled release applications. Transposon mutants of *A. calcoaceticus* were generated using a modified Tn*10* transposon and screened for deficiencies in fatty acid metabolism and characterized by Southern hybridization. These emulsans were characterized for fatty acid content, polysaccharide molecular weight and emulsification properties. Studies were carried out in vitro to determine the influence of emulsan structural features on macrophage activation as measured by the release of the proinflammatory cytokine, TNF. A dose-dependent response, the higher the extent of fatty acid substitution on the polysaccharide of emulsan the higher the level of TNF released from macrophages, was determined. Controls, such as the polysaccharide backbone alone did not result in macrophage activation, while *E. coli* LPS resulted in high levels of activation. In subsequent studies in vivo, the emulsan had an immunomodulation influence in mice using a classic haptan carrier protocol.

**New Vaccine Adjuvants from Emulsans** - The need for effective, nontoxic and versatile adjuvants remains despite 75 years of research effort to identify replacements for alum. The complex acylated polysaccharide, emulsan, secreted from *Acinetobacter calcoaceticus*, represents a new structurally and functionally tailor able class of emulsifying candidate adjuvants. The efficacy and lack of toxicity of one member of this family of biopolymers in an experimental Lyme disease model was demonstrated. Emulsan and OspA-immunized animals demonstrated high antibody titers, and lack of pathology. Moreover, this adjuvant activity was dependent on a functional toll-like-4 receptor.
Fluorinated Polysaccharides – The incorporation of fluorinated fatty acids into emulsan was demonstrated using $^{19}$F-NMR and GC-MS analysis of the fatty acids hydrolyzed from the secreted and purified polymers. The percentage of fluoro fatty acids detected was 8.5 mole % and 5 mole %, respectively, of the total fatty acids present in emulsan for 2-poly(perfluoro propanoxy)-methyl perfluoro propanoate and 2-[(carboxy-difluoromethoxy)-poly(perfluoro methoxy)]-poly(perfluoro ethanol)-difluoro acetic acid. The solution behavior of these modified polymers was evaluated by emulsification assay and found to be significantly higher when evaluated against short chain alkanes and lower against long chain alkanes, when compared with native emulsan. The results suggest new polymer properties can be engineered by incorporating nonnative fatty acid substituents into this family of amphiphilic polymers.

Bioengineered Bacterial Cellulose – Cellulose membranes generated by the bacterium, Acetobacter xylinum, are under study as a unique biological synthesis and processing system. Submicron diameter cellulose fibrils are formed by the organism directly into membranes. The genetics of the biosynthesis and the processing variables involved in membrane formation are currently under investigation.

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Tissue Engineering

Tissue Engineered Ligaments - Complex mechanical forces are clearly involved in cell and tissue development and repair in vivo, with impact on tissue structure and function. To begin to understand relationships between these forces and tissue outcomes, we have focused our recent studies on the design and implementation of novel bioreactors that can impart complex mechanical stimuli under controlled conditions in 3D environments. We have studied the impact of these stimuli on mesenchymal stem cell responses leading to specific tissue types: ligament and bone. These studies demonstrate the importance of this environmental factor in directing progenitor cells toward desired tissue outcomes. The bioreactor system used to engineer anterior cruciate ligaments starting from human bone-marrow derived mesenchymal stem cells (MSCs) and imparted mechanical loading designed to mimic ligament-like multi-dimensional mechanical strains (translational and rotational strain) over 21 days with 90° rotational and 2 mm translational deformations at 0.0167 Hz. The application of mechanical stress to MSC-seeded 3D collagen gels up-regulated ligament fibroblast markers, including collagen types I and III and tenascin-C, fostered statistically significant cell alignment and density as compared to unloaded controls, and resulted in the formation of oriented collagen fibers, all features characteristic of ligament cells. At the same time, no up-regulation of bone or cartilage-specific cell markers was observed. A novel silk protein matrix was also designed that is biocompatible, mechanically robust and biodegradable over the long-term in vivo.

Tissue Engineered Bone - Bone is a dynamic tissue which is able to sense and adapt to mechanical stimuli by modulating its mass, geometry, and strength. Bone marrow stromal cells (BMSCs) are known to play an integral part in bone formation by providing an osteoprogenitor cell source capable of differentiating into mature osteoblasts in response to mechanical stresses. Characteristics of the in vivo bone environment including the 3-D lacuno-canalicular structure and extracellular matrix composition have previously been
shown to play major roles in influencing mechanotransduction processes within bone cells. To more accurately model this phenomenon \textit{in vitro}, we cultured human BMSCs on 3-D partially demineralized bone scaffolds in the presence of four point bending loads within a specially designed bioreactor. The effect of mechanical loading and dexamethasone concentration on BMSC osteogenic differentiation and mineralized matrix production was studied. Mechanical stimulation promoted osteogenic differentiation of BMSCs by significantly elevating alkaline phosphatase activity as well as alkaline phosphatase and osteopontin transcript levels over static controls. Mineralized matrix production also increased under these culture conditions. Dexamethasone concentration had a dramatic effect on the ability of mechanical stimulation to modulate these phenotypic and genotypic responses. These results provide increased insight in the role of mechanical stimulation on osteogenic differentiation of human BMSCs \textit{in vitro} and may lead to improved strategies in bone tissue engineering.

\textbf{Stem Cell Aging/Differentiation on Protein Biomaterials} – Adult bone marrow stromal stem cells (BMSCs) show a severe decline in differentiation potential with prolonged cultivation ex vivo, a phenomenon that can be regarded as one of manifestations of in vitro cellular aging. Recently, our results with primary human fibroblasts indicated that growth on denatured collagen at certain concentrations leads to the reduction of the rate of cellular aging. Growth of BMSCs on a denatured collagen matrix significantly reduced the rate of morphological changes, and resulted in a dramatic increase in the retention by ex-vivo expanded cells of the potential to express osteogenic-specific functions and markers upon treatment with osteogenic stimulants. BMSCs are a promising and increasingly important cell source for tissue engineering as well as cell and gene therapeutic strategies. For use of BMSCs in these applications, an ex-vivo expansion is necessary to obtain a sufficient, therapeutically useful, numbers of cells. It has been shown, however, that during ex vivo expansion under culture conditions currently in use, BMSCs progressively lose their ability to proliferate.
and differentiate into multiple mesenchymal lineages, and generate bone tissue in vivo. Findings that growth on certain matrices enhances proliferation capacity and preserves differentiation potential of BMSCs indicate a promising approach to address this problem.

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Enzymes in Polymer Science - The role of enzymes in the synthesis and modification of polymers in novel environments, such as solvents and at interfaces, is an approach to modulate structure. Enzymes offer many benefits in comparison to traditional synthetic approaches to polymer synthesis, including simplified stereochemical and regioselective control, modulation of molecular weight and polydispersity, and high yielding rapid reactions. A key focus is on extending reactions to nonnative substrates. The role of enzymes in the synthesis and modification of polymers is providing new routes to both ‘new’ and ‘old’ polymers. These enzymatic methods are founded in green chemistry and offer many benefits in comparison to traditional synthetic approaches to polymer synthesis. Examples of reactions under study from current research include polyester formation through ring-opening polymerizations using lipases to synthesize polyesters, vinyl polymerizations using peroxidases, peroxidase-catalyzed cross-linking reactions for hydrogel formation, and the use of multiple biocatalytic steps to generate selectively functionalized polymers. For example, smart hydrogel systems can be generated from polyamino acid polymers, such as polyaspartic acid, upon functionalization and enzymatic cross-linking. This approach leads to biodegradable protein-based gel systems that can be tailored in properties depending on cross-link density.

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