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Biomaterial Biotechnology Using Self-Assembled Lipid Microstructures

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Abstract Lipids are a class of molecules which self-assemble into a variety of phase-dependent morphologies. We have employed self-assembled lipid microstructures in the development of a number of biomedical material applications. The blood substitute, liposome encapsulated hemoglobin, is being investigated for the in vivo delivery of hemoglobin without many of the inherent toxicities associated with the delivery of free hemoglobin. This investigation is currently focused on demonstrations of efficacy in stressed animal models and on the safety of administering this material in models of sepsis. The synthetic modification of phospholipids to include photopolymerizable moieties such as diacetylenes has resulted in the spontaneous self-assembly of a hollow microcylinder which we are investigating for the controlled release of growth factors in soft tissue regeneration. Self-assembled monolayers are also being explored for the ability to surface modify biomaterials for improved cell adhesion. Photolithographic techniques have been combined with monolayer deposition to fabricate coplanar patterns of cell adhesion and inhibiting moieties. This results in the ability to spatially control the adhesion of cells to biomaterial surfaces. These cell patterns can form the basis for understanding two- and three-dimensional cellular events on the biomaterial surface and for the fabrication of improved cell-based biocompatible surfaces. The spontaneous self-assembly of lipids to form structures of biotechnological interest presents a unique opportunity to exploit this class of molecules for biomaterial applications.

Key words: self-assembly, lipids, blood substitutes, controlled release, monolayer formation

Biological lipids are lyotropic amphiphiles which display a number of phase-dependent properties which can be exploited for biomaterial-based biotechnology. The propensity of lipids to self-assemble based on the van der Walls interactions of the fatty acyl chains and the free energy gained by sequestering these regions from high dielectric solvents such as water (termed the entropic-based "hydrophobic force") [see Tanford, 1980], results in a smectic or layered ordering of the lipid molecules. These assemblies can be manifest as micelles, monolayers, or bilayers. Perhaps the most exploited lipid assembly for technological application was elucidated by Alec Bangham in the 1960s. This assembly is a spherical structure which encompasses an aqueous compartment, or liposome [an excellent overview of this field is found in Bangham, 1983]. A liposome in circulation can be considered a circulating particulate biomaterial with many of the interactions at the liposome surface similar to sessile blood contacting materials. The basic understanding of the dynamics of liposome bilayers (e.g., molecular order–disorder, permeability) and interliposome behavior (e.g., bilayer fusion, aggregation) have been an active basic research field. These studies have also led to applications of liposomes for drug delivery formulated with a number of therapeutic and diagnostic agents [Ostro, 1987; Ostro and Cullis, 1989]. The development of liposomes for these applications has involved in vivo and in vitro studies of host cell interactions, biocompatibility, metabolic disposition, immunological response, toxicology, shelf-life, and large scale manufacturing methods.

The rich diversity in natural lipid structure and the synthetic modification of lipids has led to the discovery of other lipid phases and physicochemical characterizations of their structures. Some examples of such phases include cubic phases (three-dimensional nanoporous sponges), lipid microcylinders, and lipid-based microemulsions. Each of these assemblies are being examined for their fundamental physico-

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chemical properties as well as potential medical and nonmedical applications.

Our laboratory, as well as others, has been involved with the technological development of a number of lipid self-assemblies for biomaterial biotechnology. These studies have involved understanding the phase behavior of these assemblies as well as studying potential biomaterial applications.

CURRENT STATUS OF RESEARCH

Liposome Encapsulated Hemoglobin: An Artificial Oxygen Carrying Fluid

One lipid based self-assembly which has been investigated is liposome encapsulated hemoglobin (LEH), an artificial oxygen carrying resuscitative fluid (Fig. 1a). Conceptually, the encapsulation of a drug or biologic reduces the toxicity and increases the circulation persistence of that agent. For hemoglobin, encapsulation is designed to reduce the well documented nephrotoxicity and vasoactivity of hemoglobin, and increase the short retention time of free hemoglobin. There is clear evidence that the biodistribution of LEH is significantly different than cell free hemoglobin with no significant accumulation of LEH in the kidney. LEH, like most liposome preparations, is cleared by the monocyte phagocyte system, principally the liver and spleen, over the course of 24 h [Rudolph et al., 1991a]. Histopathology evidence shows clearance through these organs over the course of one week (Rudolph et al., submitted). The circulation half-life of radiolabeled LEH is 15–20 h. The clearance of LEH is most likely mediated by the adherence of serum proteins to the surface of LEH. Thus, circulating liposomes may provide a useful model for understanding recruitment of proteins to a biomaterial surface and how this protein layer mediates a specific in vivo immune response. Early liposome studies have examined ex vivo liposomes to evaluate the recruitment of serum proteins and the relation to circulation persistence [Juliano and Lin, 1980]. We have extended these initial studies to address the immune response to LEH. These studies have focused on the interactions of LEH with tissue resident macrophages. Isolated tissue resident macrophages are not stimulated to express the inflammatory cytokine, tumor necrosis factor (TNF), when presented with LEH. The ability of these macrophages to express TNF in response to an endotoxin challenge following exposure to LEH is diminished in a dose- and time-dependent manner. This could have important consequences for macrophage ability to mount a secondary response following LEH application.

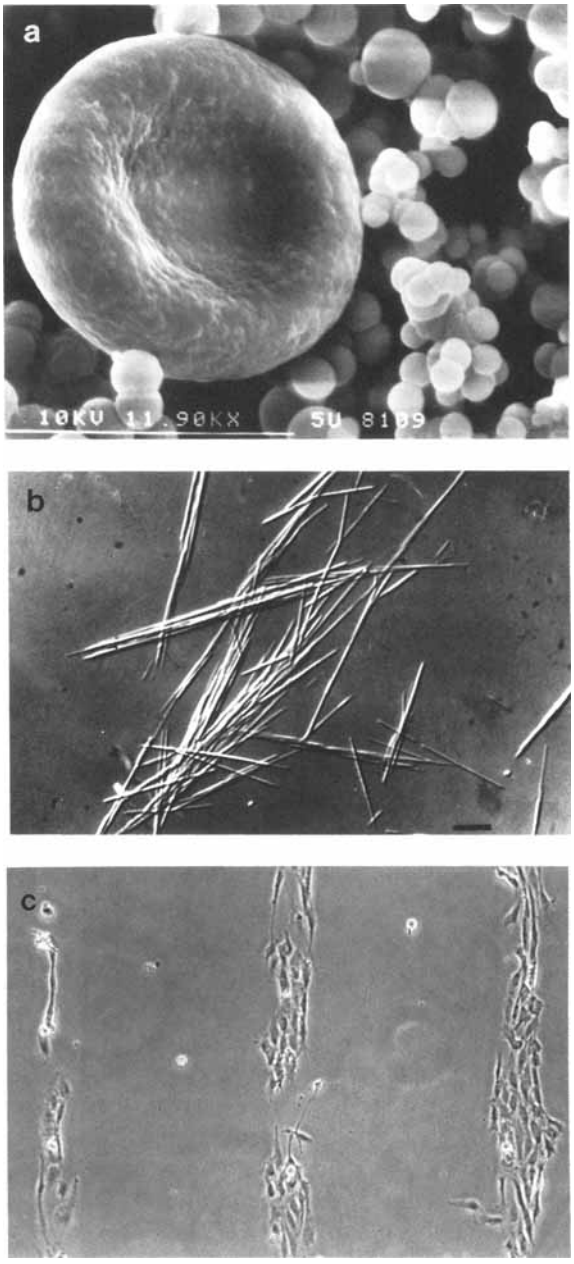

Fig. 1. **a:** Liposome encapsulated hemoglobin surrounding a human red blood cell. The average particle diameter of the liposomes is 0.5 microns. **b:** Diacetylenic phospholipids organized into hollow microcylinders used to deliver growth factors. Scale bar = 5 μm. **c:** Coplanar self-assembled monolayers of aminosilane and fluorosilane created with photolithographic mask showing preferential adhesion of human umbilical endothelial cells to the 50 μm line of aminosilane and no cell attachment to the fluorosilane region.

Efficacy of LEH in terms of oxygen delivery to tissues has been demonstrated in compromised animal models of 70% hemorrhagic shock [Rabinovici et al., 1993]. We have also developed large scale production methods to successfully lyophilize LEH with the hope that the lyophilized material could be deployed to far forward locations such as ambulances and field aid stations [Rudolph, 1988].

The encapsulation of hemoglobin may be the only safe method of delivering this vasoactive and nephrotoxic molecule. The development of a safe and efficacious blood substitute would have major implications for trauma medicine and could alleviate many problems faced by the current blood supply (e.g., shortage, tranfusion reaction, transmission of disease).

Controlled Release Using Diacetylenic Lipid-Based Microcylinders

The synthetic modification of lipids has led to the ability to alter the lipid molecule and study the effect on the phase behavior of the lipid. The synthetic modification of lecithins to include photopolymerizable moieties such as diacetylenes was motivated by the desire to decrease the lability of the lipid assembly by introducing a chemical moiety which, when appropriately stimulated, would result in the formation of a new covalently linked lipid polymer. The result would be a laterally cross-linked lipid polymer which is cross-linked following lipid assembly. Perhaps more importantly, the introduction of diacetylenic groups into double-chain phospholipids resulted in a significant change in the phase properties of the lipid with the spontaneous formation of a highly ordered, hollow microcylinder (Fig. 1b) [Yager and Schoen, 1984]. This finding is unusual in that most double-chain phospholipids prefer to assemble into closed spherical structures. The microcylinder morphology is comprised of helically wrapped bilayers. There has been considerable documentation of microcylinder physicochemical characteristics, but the driving force for their formation still remains largely unknown. This lipid forms another unusual morphology in its phase properties with the formation of a large net of phospholipid filaments [Rudolph et al., 1991b].

In spite of this, the clear advantage of having a lipid-based microcylinder with transport properties for large molecules largely mediated out of the ends of the cylinder (which can be 50–500 microns in length and 0.5 to 1.0 microns in diameter) is being exploited in controlled release applications. One application utilizes the microcylinders as templates for the deposition of metal [Schnur et al., 1987; Rudolph et al., 1989]. This results in the formation of a metal microcylinder which can then be filled with a resin containing antibiotic for use in an antifouling paint for the bottom of boats or ships [Price and Patchan, 1993]. The paint composite has been shown to release antibiotics over the course of months. Our laboratory has also explored the use of the lipid microcylinder for the release of growth factors and cytokines for soft tissue regeneration. The release of radiolabeled transforming growth factor beta (TGF-b) from lipid microcylinders has been shown to be zero order with 1 ng/ml/day released over the course of 10 days [Spargo et al., 1992]. This release can be modified by temperature as the transition temperature of the lipid microcylinder is 42°C. The release can be "pulsed" by increasing the temperature above the transition temperature which results in the melting of the microcylinder. The interaction of the microcylinders with cells of the immune system has also been defined [Rudolph et al., 1992]. We have examined the bioactivity of the released protein and have demonstrated that the released TGF-b is biologically active (Spargo et al., manuscript submitted). We are also currently evaluating the TGF-b microcylinder in a full thickness punch biopsy wound model.

The advantage of the microcylinder may be the long-term release observed from the ends of the microstructure. Thus, the ability to control the morphology through synthethic modification of the self-assembling amphiphile could have important implications for the control of release rates in novel release vehicles.

Surface Modification of Biomaterials With Self-Assembled Monolayers

The self-assembly of lipids and other anisotropic amphiphiles into monolayers has been used to study the physicochemical properties of lipids as well as form the basis for a number of applications based on multilayer langmuir blodgett technology. The self-assembly of monolayers also presents an opportunity to construct a regularly ordered array of organic molecules on a biomaterial surface. Toward this goal, self-assembled monolayers (SAMs) have been used to modify surfaces to include cell adhesion promoters and inhibitors [Kleinfeld et al., 1988].

These SAMs have been fabricated on a variety of surfaces using organosilanes containing chemical moieties which mediate cell adhesion or inhibition of adhesion. Applications of this work include the ability to improve the adhesion of cells to a desired surface. We have examined the interaction of endothelial cells with SAMs of aminosilanes. Lithographic methods can be used to create two-dimensional patterns of organosilanes and result in the patterning of endothelial cells on a surface (Fig. 1c). We have been examining such patterns in order to understand the differentiation of endothelial cells from two-dimensional patterns as well as transferring such patterning technology to vascular graft materials. The latter effort is designed not only to improve endothelial cell seeding to such graft materials, but direct the differentiation of endothelial cells on the surface of a vascular implant material.

PROSPECTS

Liposome technology has matured over the last decade as antifungal and antibiotic liposome preparations are now in late-stage clinical trials in the U.S. (some of these products are already for sale in Europe). As these products have matured, second generation liposome preparations have been developed. These new products hope to utilize liposome surface modifications to prolong circulation persistence and improve the ability to target specific sites within the body. Toward this goal, the use of lipids with coordinated polyethyleneglycol moieties have shown promise in extending circulation persistence [Allen and Papahadjopoulos, 1992]. The low encapsulation efficiency of solutes in liposomes has also led to the development of new lipid-based encapsulation systems based on the formation of three-dimensional cubic phases which form a nanoporous, lipid sponge [Luzatti et al., 1968; Lindblom and Rilfors, 1989]. Other alternative lipid microstructures such as lipid microcylinders offer the possibility of long-term release of solutes.

With the revolution in cytokine biology, the biological effects of lipid microstructure application can now be defined in the context of specific cellular responses of the reticuloendothelial system. In particular, as tissue resident macrophages are responsible for identifying and removing lipid microstructures, cytokine response of macrophages will provide new insights into potential liposome-based therapies and immune response to liposome application.

The use of self-assembled monolayer modification of biomaterial surfaces has important implications for directing molecular absorption to such surfaces. This is turn may have significant effects on more complex processes such as cell adhesion. The ability to create highly defined spatial regions with specific functionality could result in spatially directing such events. Thus, one could envision spatially directing the growth cone of a neuron or the assembly of endothelial cells into microvasculature. Future efforts may focus on combining monolayer modification with materials that can release factors which promote specific cellular events.

Finally, the attraction of using lipids for many biomaterial applications is the spontaneous self-assembly of lipids with astounding regularity. Lipids also can be synthetically modified to include specific biochemical moieties either at the polar headgroup or in the apolar fatty acyl chains. Fabrication of such lipid microstructures is currently, in many cases, done in large scale and offers tremendous future possibilities for a number of biomaterial biotechnological applications.

ROADBLOCKS

Regardless of the regulatory path, many of these systems share the same technical issues for their successful development. For bilayer assemblies, the large scale controlled fabrication remains a challenge. The processing of these structures is often formulation dependent, which requires significant research and development before scale-up can be attempted. For liposomes, issues such as encapsulation efficiency, particle size, and sterilization methods are important for large scale production. The particle size also dictates important parameters such as circulation persistence and organ biodistribution. Thus, production issues must be closely developed with safety and efficacy considerations. The quality control of lipid microstructures also requires documentation of lipid source and purity as natural lipids (from soybean or eggs) are often used to reduce cost.

For new lipid structures such as microcylinders, the development of these systems will require a complete characterization of the structure, its molecular interactions with the solute to be released, and release profile in different environments. Cell and tissue response to these structures will also be an important component

of defining the efficacy of this structure. In addition, further physicochemical characterization will be useful in optimizing their utility.

Finally, the use of self-assembled monolayers for biotechnological biomaterial application will require careful surface chemical analysis of the assembled monolayer on the biomaterial surface. Currently, the chemistry required to form the monolayer on the surface requires the presence of a regular array of surface hydroxyls, as these are the sites for organosilanes monolayer formation. The characterization of monolayer surface coverage is an important determinant of the utility of this strategy. Determining the stability of the SAM and potential effect on cell adhesion, proliferation, and differentiation will be important for their application.

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