Defining Fluoropolymer Surfaces for Enhanced Nerve Cell Interaction

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Chemical Engineering and Applied Chemistry
University of Toronto

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Abstract

Defining Fluoropolymer Surfaces for Enhanced Nerve Cell Interaction

Doctor of Philosophy, 2000

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Fluoropolymers are modified in an attempt to enhance their interaction with nerve cells for the purpose of modelling biomaterial-cell interactions for eventual application in spinal cord injury repair. Poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) was modified with oxygen and nitrogen-containing functional groups and subsequently coupled with cell-adhesive peptides to mimic the laminin basal lamina environment of the peripheral nervous system. The surface chemistry of the modified surfaces were fully characterized. Hippocampal neurons were cultured on all the modified surfaces to study for neurite outgrowth and cell adhesion.

FEP surfaces were initially functionalized by reduction with sodium naphthalide followed by either hydroboration oxidation (to alcohol) or oxidation (to carboxylic acid). Functionalized surfaces were then coupled with individual peptide: YIGSR, IKVAV or RGD. The initial cell-material interaction study showed that hippocampal neurons responded to the peptide-modified surfaces in a quantitatively comparable manner to positive controls consisting of poly(L-lysine) (PLL) and laminin coated surfaces. However, qualitative differences in cell response were observed. To further study the neuron-surface interaction, a new method of surface modification was developed.
The novel mercat amination reaction was used to functionalize FEP surfaces by exposing films to a UV-activated mercury in ammonia system. Relative to hydroxyl and carboxylic acid chemistries, the mercat reaction is simpler, involving only one step and uses less reactive chemicals. The modified surfaces were defined to have amines, nitriles, hydroxyl, carboxylic acids, and carbon-carbon double bonds, which allowed for the coupling of cell-adhesive peptides. Initial cell culture studies of hippocampal neurons on these surfaces showed that the neuronal response was comparable to PLL/laminin surface even though the peptide concentrations were lower.

An in-depth study of cell-material interaction was developed to better distinguish the surfaces by neuronal response. Neurite number and length were investigated in conjunction with cell adhesion and viability. The results indicated that the hippocampal interaction was best on PLL/laminin surfaces, but was closely approximated by FEP surfaces coupled with both YIGSR and IKVAV. Surfaces with a single peptide showed significantly lesser response. The specificity of the cell-peptide interaction was demonstrated with a competitive assay. These well-defined fluoropolymer surfaces confirmed that nerve cell interaction was enhanced by the coupling of cell-adhesive peptides.
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Chapter 1. Introduction

1.1 Overview

1.1.1 Rationale

Spinal cord injury is a devastating disorder of the central nervous system for which there is no cure. The current therapy only aims to prevent further damage of spinal neurons, and hope for recovery of partial function. Recently, however, research has shown that damaged spinal cord neurons have the ability to regenerate. In the absence of inhibiting molecules, or in the presence of a permissive environment, severed or lesioned axons regrow and may reconnect with the target tissue.

In this thesis, a series of published papers will present a model system that will be engineered by designing a surface to promote the regeneration of axons. An introduction into the physiology of the nervous system and its functions will be given in the next section, followed by a discussion on the injuries to the nervous system. Particular focus on the differences between the central nervous system and the peripheral nervous system will illustrate how the intrinsic ability of injured neurons to recover is masked by environmental factors. Such barriers to regeneration include the release of myelin-associated glycoproteins, neurite inhibitor molecules, and the absence of laminin and nerve growth factors. An examination of the basal lamina extracellular matrix and its components will show that these molecules provide a suitable environment for neurons to grow. These molecules stimulate the integrin receptor on the cells to cause cell adhesion, neurite outgrowth and neurite extension.
An in-depth look into laminin will reveal certain peptide sequences of the molecule that are active and can be isolated for the regeneration of injured central nervous system neurons.

1.1.2 Biomaterials for Regeneration

The use of a biomaterial to provide a suitable environment for nerve regeneration has many advantages over autografts, allografts or xenografts. Ideally, biomaterials are easily obtainable, can be manufactured in large quantities, and will not suffer from adverse immune system response. These materials can also be designed to specifically elicit a response from the cells.

1.1.2.1 Engineering a Surface

The current generation of biomaterials are, unfortunately, not engineered based on a specific requirement. Instead, they are selected from commercially available polymers, metals or ceramics that are non-toxic to cells and do not cause unfavourable reactions. Some of the materials are not even biocompatible, which is defined as the ability of a material to perform with an appropriate host response in a specific application (Williams, 1991). Biocompatible material will not induce harm to the host and fulfills its intended function (Ratner, 1999). In this thesis, a biocompatible material would therefore be defined as a material that does not induce an inflammatory or cytotoxic response while supporting the growth and functions of neurons. The recent challenge, however, is to engineer or design a material for the required biomedical application, and specifically that of the material surface, as the interface is where reactions and processes with cells occur. Surface properties such as
surface chemistry and morphology can directly influence the cell-material interaction, and thus should be considered the critical criteria in the making of a biomaterial.

Herein, fluoropolymer surface chemistry is studied as a model system for the regeneration of central nervous system neurons. The advantages of using a fluoropolymer system will be illustrated. Surface modification techniques will be used to design a peptide surface for enhancing neuronal response. Well-known surface modification reactions will be compared to novel techniques, and the modified surfaces will be analyzed and characterized using various analytical methods. The engineered surface will be used for in vitro cultures of primary neuronal cells to study the cell-material interaction. Novel culture conditions and cell characterization methods will be developed to identify the responses of the cells on the different surfaces.

The cure for spinal cord injuries will not be presented in this thesis, but the following sections and chapters will hopefully present a viable model for the development of a device that can be made and used in the near future.
1.2 Hypothesis

It is hypothesized that CNS neuron adhesion and neurite extension can be enhanced and controlled by introducing peptide sequences on to fluoropolymer surfaces via peptide concentration, type and method.

1.3 Objectives

To test the hypothesis above, the main objectives of this thesis are:

(a) To functionalize the fluoropolymer FEP surface with oxygen or nitrogen functional groups;

(b) To covalently attach three different cell adhesive peptides to functionalized FEP film surfaces;

(c) To study the neuronal cell-material interaction in terms of neurite number, neurite length, number of neurons, and neuron viability.
1.4 Approach

To achieve the objectives of the thesis, the following approaches were taken:

(a) FEP film surfaces were modified to introduce either hydroxyl, carboxylic acid or amine functional groups which then served as reactive handles for the covalent coupling of laminin-derived adhesive peptides;

(b) The peptides, YIGSR, IKVAV and/or RGD, were coupled to modified FEP film surfaces at different concentrations to determine their effects on neuron-surface interaction;

(c) Cell-material interaction was studied by investigating neurite outgrowth on all the modified FEP film surfaces via neurite length and number, neuronal viability and neuron adhesion.

1.5 The Nervous System

1.5.1 Nervous System Physiology

The human nervous system is separated anatomically into two regions, the central nervous system (CNS) and the peripheral nervous system (PNS) (Figure 1.1). The distinct separation between the two systems can also be seen functionally and physiologically.
Figure 1.1 The major anatomical parts of the nervous system. The CNS is illustrated on the right and the spinal nerves are illustrated on the left (Guyton, 1987).

1.5.1.1 Central Nervous System

The CNS consists of the brain and the spinal cord. The brain itself is divided into several regions: the medulla oblongata, the pons, the cerebellum, the midbrain, the diencephalons and the cerebrum. The brain mainly functions in an integrative manner, controlling many sensory and motor functions, storing memory, generating emotions etc. It is
the most complex and unknown organ in the body. The spinal cord (Figure 1.2) is the most caudal part of the CNS (Kandel, 1991). It functions not just as a conduit for many nervous pathways to and from the brain, but also to coordinate many subconscious activities (Guyton, 1987). The spinal cord thus serves as the main link connecting the body with the brain. It is composed of white matter on the surface, surrounding the gray matter where the neuronal cell bodies lie (See Table 1.1 for functions of the gray and white matter). Peripheral spinal nerves connect to the spinal cord through the dorsal and ventral roots (Figure 1.3).

![Diagram of the spinal cord vertebral and spinal nerve roots entering the spinal cord segments](image)

Figure 1.2 A diagram of the spinal cord vertebrae and the spinal nerve roots entering the spinal cord segments (Gilman, 1996).
The CNS is an immuno-privileged site, where a barrier of three cellular membranes separates brain tissue and cerebrospinal fluid from direct contact with blood. This blood-brain barrier (BBB) is formed from astrocytic projections, called glial end-feet, together with endothelial cells of brain capillaries. This prevents the normal diffusion of ions to and from the blood, and also of non-lipid soluble molecules and immune system cells. The BBB serves to prevent sudden fluctuations in extracellular ion concentration, and the influx of growth
promoting or inhibiting factors and neurally active compounds, which might cause CNS neurons to die or malfunction.

| Table 1.1 The function of the spinal cord structures (Guyton, 1991). |
|------------------------|----------------------------------|
| **Structure**          | **Function**                     |
| **Gray Matter**        |                                  |
| Dorsal horns           | Loci of sensory input neurons     |
| Lateral horns          | Loci of preganglionic autonomic neurons |
| Ventral horns          | Loci of motor neurons for skeletal muscles |
| **White Matter**       |                                  |
| Propriospinal tracts  | Signals between cord segments     |
| Long motor tracts      |                                  |
| 1. Lateral corticospinal | Motor signals from cortex to spinal cord |
| 2. Ventral corticospinal | Same                             |
| 3. Rubrospinal         | Motor signals from brain stem to spinal cord; most are excitatory, a few are inhibitory |
| 4. Reticulospinal      |                                  |
| 5. Olivospinal         |                                  |
| 6. Vestibulospinal     |                                  |
| 7. Tectospinal         |                                  |
| Long sensory tracts    |                                  |
| 1. Fasciculus gracilis and fasciculus cuneatus | Discriminatory sensory signals to gracile and cuneate nuclei, thence to thalamus in medial lemnisci |
| 2. Ventral and lateral spinothalamic | Crude touch, pain, and temperature signals to brain stem and thalamus |
| 3. Ventral and dorsal spinocerebellar | Proprioceptor sensory signals to cerebellum |
| 4. Spino-olivary       | Cord signals to inferior olivary nuclei, then relayed to cerebellum |
| **Spinal Nerve Roots** |                                  |
| Dorsal                 | Sensory input                     |
| Ventral                | Motor output to muscles and preganglionic output to autonomic nervous system |

1.5.1.2 Peripheral Nervous System

The PNS consists of all the nerves connecting every tissue in the body to either the spinal cord, or to the brain. It has two divisions: (a) the somatic division that includes sensory neurons from the body to the dorsal root of the spinal cord and motor neurons from the ventral root of the spinal cord projecting to skeletal muscles; and (b) the autonomic division that is the motor neurons for the smooth muscles and exocrine glands of the body (Kandel,
The PNS is not immuno-privileged, unlike the CNS, and has direct contact with blood vessels.

1.5.1.3 Nerve Cells

As seen in Figure 1.4, a typical neuron can be divided into 4 morphological regions (Kandel, 1991): the cell body or soma, the dendrites, the axon and the presynaptic terminals. The cell body generally is the major metabolic centre of the neuron, where the nucleus and perikaryon resides, generating proteins and neurotransmitters for the whole cell. The processes extending from the cell body are the dendrites and the axon. Several branches of dendrites can arise at several different locations in the soma, but only one axon can be defined. The dendrites function to receive signal inputs from other neurons while the axon is the main conducting channel for conveying action potentials to other neurons. The presynaptic terminals at the transmitting ends of axons are classified into a separate region as the axon branches into specialized swellings for transmitting information across the synapse to the nearby post-synaptic terminals. In certain neurons, the presynaptic terminal contains Golgi apparatus for synthesizing neurotransmitters.
Figure 1.4 The main structures of a typical vertebrate neuron, with its 4 morphological regions (Kandel, 1991).
Nerve cells can be distinguished from one another by function, location and shape in a non-exclusive manner. The shape of the neuron is affected by the number and form of the processes extending from the cell, giving rise to the classification of unipolar, bipolar or multipolar cells (Figure 1.5). Classifying neurons by function is done in three ways: afferent or sensory neurons, motor neurons, and interneurons. Sensory neurons carry information from the afferens into the nervous system, while motor neurons carry commands to muscles or glands. Interneurons are the major neuronal type in the CNS; they process information or convey signals to other neurons.
1.5.1.4 Glial Cells

Glial cells are the other type of cells in the CNS, surrounding the nerve cells at a population of 10 to 50 times higher. They mainly serve as support cells for neurons, providing structure and separation. They also act as scavengers and as immune cells, removing cell debris or antigens. Glial cells can buffer ionic concentrations and remove released neurotransmitters from synaptic junctions, thereby stabilizing neuronal function. Two types of glial cells, Schwann cells (in the PNS) and oligodendrocytes, also provide the insulating myelin layer of major axons. Other functions of glial cells include serving as guides for neuronal migration and axonal outgrowth, and providing nutritional support for neurons. The principal types of glial cells are oligodendrocytes, astrocytes and microglia in the CNS, and Schwann cells in the PNS. Unlike nerve cells, glial cells are reactive and proliferative, especially when subjected to stresses such as injury and disease (Kandel, 1991).

1.5.2 Nervous System Injuries

Physical injuries to the nervous system, such as a crush or transection, represent one of the major challenges for the medical field. As neurons are non-proliferative, injury to the cell body leads to the death of the neuron and permanent loss of function. However, injuries to the processes only do not necessarily lead to cell death, as the neuron is capable of regenerating the axon in a similar manner to the developmental cell growth (Kandel, 1991). The response of the neuron depends on the environment of cell immediately following the injury.
1.5.2.1 Peripheral Nerve Injuries

Injuries to the PNS by the severing of major nerve trunks normally cause a total loss of function in the muscles innervated by that nerve. However, damage to peripheral nerves is often reversible. The severed processes spontaneously regenerate by extending axons through nerve bundles from the proximal end to the distal end that have degenerated. Several factors present in the PNS promote this regeneration. The presence of neurotrophic factors from the blood stream prevents neurons from degenerating and preganglionic cells from withdrawing their presynaptic terminals. The ensheathing Schwann cells on the peripheral axons also play a role by releasing extracellular proteins such as laminin while proliferating, and expressing adhesion molecules on their surface which act as “contact guidance” (Skene, 1983). The Schwann cells also increase their synthesis of nerve growth factor (NGF) due to the presence of macrophages releasing the cytokine interleukin-1 (Kandel, 1991).

A major problem to full recovery of function is the misdirection of the regenerating axon to its proper target (Lundborg, 1987). To ensure an accurate direction of growth, severed ends are normally sutured together. If the lesion is large, however, a graft is often used to prevent tension of the nerves and increased scar formation (Millesi, 1981). The most common grafts are nerve autografts (Aguayo 1976, Millesi, 1984) as they not only provide a mechanical guide but also the optimal microenvironment similar to the undamaged nerve (Figure 1.6). Other materials used include collagen, tantalum, nylon, Teflon, etc. (Danielsen, 1988, Lundborg, 1982, Suematsu, 1989).
Figure 1.6 Example of a graft sutured onto the severed nerve ends to facilitate regeneration.

1.5.2.2 Spinal Cord Injuries

Spinal cord injuries (SCI), being a CNS trauma, are viewed as severe and irreversible as opposed to PNS injuries which are at least partially curable. Axons in the CNS do not spontaneously regenerate due to inhibitory factors present in the CNS. Due to this fact, SCI epidemiology and trauma management has been well studied (Berkowitz, 1998, Capen, 1998, Petersen, 1995). Over 10,600 persons suffer from SCI each year, with total cost exceeding $9.76 billion (Table 1.2) for trauma management and care (Berkowitz, 1998). Causes of SCI include motor vehicle accidents, violence, accidental falls, and sports injuries (Petersen, 1995). Because there is no cure currently available, predictions for SCI recovery are not possible, and SCI can only be classified by severity for eventual medical care (Capen, 1998).
Table 1.2 Spinal Cord Injury costs (Berkowitz, 1998).

<table>
<thead>
<tr>
<th>Costs (Millions $)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIRST YEAR COSTS</td>
</tr>
<tr>
<td>First year medical and related costs</td>
</tr>
<tr>
<td>Initial home modifications</td>
</tr>
<tr>
<td><strong>Total First Year Costs</strong></td>
</tr>
<tr>
<td>ANNUAL COSTS</td>
</tr>
<tr>
<td>Medical care cost (recurring)</td>
</tr>
<tr>
<td>Medications and supplies</td>
</tr>
<tr>
<td>Vehicle modifications</td>
</tr>
<tr>
<td>Home modifications (recurring)</td>
</tr>
<tr>
<td>Wheelchairs</td>
</tr>
<tr>
<td>Personal assistance</td>
</tr>
<tr>
<td><strong>Total Annual Costs</strong></td>
</tr>
<tr>
<td>INDIRECT COSTS</td>
</tr>
<tr>
<td><strong>TOTAL OF ALL COSTS</strong></td>
</tr>
</tbody>
</table>

Depending on the location of injury on the spinal cord (or the level of injury), different motor functions are disabled. The loss of motor function to the upper and lower extremities bilaterally and to the trunk is termed quadriplegia, and often occurs with injury to the higher cervical areas (C1 to C4). Injury to the distal cervical segments and thoracic or lower segments (C8 to S5) normally causes loss of lower extremity function while preserving the upper extremities and is termed paraplegia (Petersen, 1995).

The concept of CNS trauma however extends beyond the primary injury cause, as secondary processes often cause greater damage to the spinal cord. Local ischemia and edema formation lead to necrosis and inflammation, releasing membrane breakdown products (leukotrienes, thromboxane etc.), cations, excitatory amino acids, monoamines, and neuropeptides (Schwab, 1996). This secondary cascade of reactions lead to scar formation and further neuronal death, in addition to preventing axonal regeneration.
1.5.2.3 Current Therapies

The knowledge of secondary injury processes has led to many of the current treatments for acute SCI (Young, 1994). These include using opiate receptor antagonists, antioxidants, glutamate receptor blockers, anti-inflammatory drugs, hypothermia, cyclosporin etc. Antioxidants such as tirilazad mesylate are used to reduce free radicals and Ca$^{2+}$ activated phospholipase breakdown of lipid membranes (Braughler, 1985). Anti-inflammatory drugs such as methylprednisolone sodium succinate are used to prevent inflammatory mediators from causing tissue damage (Hall, 1986). Glutamate and opiate receptor blockers such as naloxone are used to prevent the release of excitatory neurotransmitters causing excitatory cell death (Bakshi, 1990). These therapies only aim to reduce tissue damage and improve neurologic recovery and not to cure the primary injury or promote axonal regeneration.

Non-regenerative approaches to SCI, based on theories of redundant spinal pathways and remyelination of spinal axons, are also currently being used (Young, 1993). Recovery and treatment are aimed specifically on the neurons/axons that survive, and not the neurons that are lost.

1.5.2.4 Functional Recovery

The end goal of therapy for SCI is recovery of locomotory function in the extremities. It has been shown that survival of 5-10% of the normal myelinated axonal population is sufficient for somatosensory evoked potentials and return of locomotory ability (Blight, 1990). The implication is that complete regeneration of all axons is not necessary for functional recovery. Since complete spinal cord transections are rare, and even the most
severely injured SCI patients have surviving neurons, recovery should be possible. After achieving axonal regeneration, sensory reeducation will be necessary (Lundborg, 1987) as the formation of new afferent fibre projections will force the brain to adapt.

1.5.3 CNS Nerve Regeneration

The intrinsic ability of all nerve cells to regenerate may involve a similar mechanism during developmental growth. It is the environmental factors surrounding the neurons that affect regenerative capability. Therein lie the fundamental differences in spontaneous regeneration capacity of injured axons in the PNS and CNS (Ramon Y Cajal, 1928). These differences can be observed between adult and immature mammalian CNS neurons, the latter of which possesses some capacity for self-repair (Nicholls, 1996).

1.5.3.1 Barriers to Regeneration

The observation of the glial scar as one of the barriers to axonal sprouting (Windle, 1954) has led to the discovery of various molecules that inhibit axonal outgrowth. One type of CNS glial cell, astrocytes, becomes reactive after injury and proliferates, producing many interweaving processes filled with extracellular matrix, sealing off the injured area and preventing spread of damage. These reactive astrocytes release molecules such as chondroitin and keratan sulphate proteoglycans and tenascins that have axon-growth inhibitory properties (Fawcett, 1997, Fitch, 1997). Another type of CNS glial cell, oligodendrocytes, produces CNS myelin, functioning like the Schwann cells of the PNS. However, the CNS myelin has membrane-bound proteins (NI-35/250) that causes the collapse of growth cones, preventing even the growth of peripheral sensory neurites (Schwab, 1985). Another of the membrane-
bound proteins. myelin-associated glycoprotein (MAG), also demonstrates neurite growth inhibitory effects (Mukhopadhyay, 1994). Other neurite growth inhibitory molecules not associated with glial cells include collapsin (Luo, 1993), fasciclin IV (Pushel, 1995), netrin (Colamarino, 1995) among others. The other barrier to CNS axonal regeneration is the glial scar itself, a physically impenetrable barrier.

1.5.3.2 Regeneration into Bridging Grafts

Attempts to overcome the regeneration barriers have used bridging grafts, an idea similar to the peripheral nerve grafts mentioned in section 1.2.2.1. The use of peripheral nerve grafts, having an optimal microenvironment for stimulating peripheral axon growth, in CNS lesions showed that CNS neurons can regenerate fibres. However, the number of fibres entering the graft is limited and axon growth does not proceed past the graft to reenter the CNS (Schwab, 1996). Implants of foetal CNS tissue as a graft into spinal-cord lesions overcame the limitations of the peripheral nerve grafts. The CNS fibres grew into and through the embryonic graft, producing structural and functional recovery (Bregman, 1993, Iwashita, 1994). The use of foetal tissue, however, raises many ethical and moral issues that limit its applications.

Attempts have been made to recreate these permissive axonal outgrowth environments, or to exclude the normal inhibitory CNS environment, using other materials. For example, collagen, hydrogels, laminin-coated nitrocellulose fibres etc. have been used as grafts, producing mixed results (Schreyer, 1987, Schnell, 1994a, Paino, 1994). This indicates that specific environmental conditions must be met, particularly the material interfacial properties and the biocompatibility of the biomaterial.
1.5.3.3 Regeneration with Growth Stimulating Molecules

Growth stimulating molecules that can be found in the developing nervous system are usually absent in the mature CNS. These molecules include (a) neurotrophic factors like NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and fibroblast growth factor (FGF); and (b) extracellular matrix molecules like fibronectin, laminin and vitronectin. Injection of NGF, BDNF and NT-3 into the lesioned spinal cord increased the spontaneous sprouting of neurites, while a combination of NT-3 with NI-250 antibodies (IN-1) produced elongation of nerve fibres (Schnell, 1994b). The role of extracellular matrix molecules for regeneration is less established and more complex, and will be discussed in the following section.

1.5.4 Extracellular Matrix Molecules

Axonal outgrowth during development and regeneration depends on guidance cues that are either substrate bound or diffusible, and either inhibitory or attractive. Substrate bound guidance cues consists mostly of extracellular matrix molecules. The extracellular matrix (ECM) is defined as the acellular material (the basal lamina) filling up intercellular space and encompassing secreted molecules from cells that are immobilized (Reichardt, 1991). These insoluble molecules include collagens, non-collagenous glycoproteins, proteoglycans, hyaluronic acid and elastin. As all cells have contact with the ECM, cellular behaviour and interaction are greatly influenced by the various molecules (Ruoslhti, 1987). In the nervous system, the ECM affects neural cell migration during development, and neurite growth during regeneration. The main ECM components affecting neurons are laminin, fibronectin, vitronectin, tenascin and thrombospondin (Letourneau, 1994). The
receptor on cell surface that recognizes the molecules are a family of structurally related membrane proteins called integrins.

1.5.4.1 Laminin

Laminin is one of the first ECM molecules discovered in the nervous system. It is a large, multidomained, cross-shaped glycoprotein, with a molecular weight of 850 000 (Luckenbill-Edds, 1997). It is composed of three polypeptide chains (Figure 1.7): a 400 kDa α chain, a 210 kDa β chain, and a 200 kDa γ chain, bound together by disulfide bridges. There are various domain structures in the molecule due to the tertiary polypeptide conformations, and these are associated with the distinct functions of laminin. For example, the IKVAV and LQVQLSIR sequence of the α chain promote cell attachment and neurite outgrowth (Tashiro, 1989), while the YIGSR sequence in the β chain promotes cell attachment (Graf, 1987), and the RKRLQVQLSIRT and KNRLTIELEVRT sequence from the α chain are active for attachment and neurite outgrowth for only some types of neurons (Richard, 1996).
There are many cell-surface receptors and binding proteins mediating the interaction between laminin and a cell (Mecham, 1991). The intracellular signalling pathways between laminin, its receptor and cellular cytoskeleton affects cell behaviour such as adhesion, spreading, and neurite outgrowth. The types of laminin receptors include: (a) integrins, which are important for cell adhesion and neurite outgrowth (Venstrom, 1995); (b) non-integrin binding proteins; and (c) carbohydrate-binding moieties.

The effects of laminin on neuronal outgrowth and neural development are well known (Luckenbill-Edds, 1996, Manthorpe, 1983, Sanes, 1989). It affects the velocity and direction of growth cones on neurites. It acts as a permissive substrate for neurons, however, in a concentration-independent manner (Buettner, 1991). The β2 chain of laminin specifically guides motor neurons to form synapses during regeneration and during presynaptic
differentiation (Sanes, 1990). In embryonic development, laminin affects neuroblast migration and nerve fibre tract growth (Hunter, 1992).

1.5.4.2 Fibronectin and Other ECM

Fibronectin is another ECM, cell-binding protein composed of two disulfide-linked subunits (Figure 1.8). It often shows comparable cell attachment activity to laminin with many cells (Timpl, 1986), having many binding sites for cells, other ECM proteins and proteoglycans (Kandel, 1991). Other than laminin, it is the other major ECM that has a role in neural cell adhesion and neurite outgrowth. Other less common ECM with similar properties are tenascin and thrombospondin.

![Fibronectin structure](image)

Figure 1.8 The structure of fibronectin, showing the dimer with its various subunits (Kandel, 1991).
Studies of embryonic neuronal cell migration have shown that integrin receptors recognize the majority of ECM proteins, suggesting that the integrin receptor is the main cellular factor affecting migratory process (Bronner-Fraser, 1986).

1.5.4.3 The Integrin Receptor

The integrin receptor is made of non-covalently linked heterodimeric subunits, with 15 different α subtypes and 8 different β subtypes, for a total of at least 20 discovered members of the integrin family (Luckenbill-Edds, 1997). It mediates cell adhesive interactions and affects the cellular functions of many cells, not just neurons. Its transmembrane structure transmits signalling from extracellular cues to intracellular cytoskeletal complexes and actin filaments (Clark, 1995). The extracellular binding sites with ECM are located near the amino-terminals of the globular head (Figure 1.9) where Ca$^{2+}$ binding occurs. Different ECM molecules have been found to bind to the same integrin, indicating that the same peptide recognition sequence for the receptor exists on the different ECM (Tomaselli, 1988). Blocking studies with antibodies for the receptors showed that both α and β subunits are involved in laminin-mediated neurite outgrowth (Venstrom, 1995), while antibodies for peptide sequences on the ECM showed that there exist specific peptide domains (called cell adhesive peptides) on the ECM that bind to integrins (Pierschbacher, 1984).
Figure 1.9 The structure of integrins showing its two membrane-spanning subunits (Kandel, 1991).

1.5.4.4 Cell Adhesive Peptides

As mentioned in sections 1.2.4.1 and 1.2.4.3, specific peptide sequences in the ECM molecules form integrin recognition domains. Isolated peptides of these sequences have been found to be active by themselves in promoting cell adhesion, thus the label of cell adhesive peptides. However, certain sequences also evoke other cell responses such as cell migration and neurite outgrowth (Graf, 1987, Tashiro, 1988). The most well known of these cell adhesive peptides is a simple tripeptide, arginine-glycine-aspartic acid (RGD). Over 400 different proteins contain the RGD sequence, including ECM molecules such as fibronectin (where it is found 18 times), tenascin (7 times), thrombospondin (6 times), and collagen among others (Ruoslalti, 1987). Other peptide sequences well known for neuronal reactivity
are isoleucine-lysine-valine-alanine-valine (IKVAV) and tyrosine-isoleucine-glycine-serine-arginine (YIGSR). Immobilization of these peptides onto surfaces by themselves has shown increased cell adhesion, spreading and neurite outgrowth (Bellamkonda, 1995, Massia, 1993, Pierschbacher, 1987). Therein lies the idea of mimicking the effects of a neuronal permissive substrate by creating an artificial material with a surface containing cell-adhesive peptides.

1.6 Fluoropolymers

The use of an artificial material for medical applications, such as prostheses, implants or drug delivery, defines a biomaterial. Until recently, commonly used biomaterials were selected from commercially available materials such as silicone rubber, titanium, collagen, etc (Table 1.3). The prevailing idea was to choose a material based mainly on its mechanical properties, and hope that it will not rejected by the body. The bypassing of designing or engineering a biocompatible material for a specific application has brought about sluggish kinetics, nonspecificity, and a broad spectrum of simultaneously occurring processes, which are not all wanted (Ratner, 1993). The engineering approach to the design of a suitable biomaterial couples the knowledge of specific cell recognition and interactions (i.e. cell biology) with the knowledge of synthesizing materials with suitable physical and chemical properties (i.e. material science and chemical engineering).
Table 1.3 Commonly used biomaterials and their applications (Ratner, 1993).

<table>
<thead>
<tr>
<th>Material</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicone rubber</td>
<td>Catheters, tubing</td>
</tr>
<tr>
<td>Dacron</td>
<td>Vascular grafts</td>
</tr>
<tr>
<td>Teflon</td>
<td>Catheter, vascular grafts</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Dialysis membranes</td>
</tr>
<tr>
<td>Poly(methyl methacrylate)</td>
<td>Intracocular lenses, bone cement</td>
</tr>
<tr>
<td>Polyurethanes</td>
<td>Catheters, pacemakers</td>
</tr>
<tr>
<td>Hydrogels</td>
<td>Ophthalmologic devices</td>
</tr>
<tr>
<td>Carbon</td>
<td>Heart valves</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>Orthopedic devices</td>
</tr>
<tr>
<td>Titanium</td>
<td>Orthopedic and dental devices</td>
</tr>
<tr>
<td>Alumina</td>
<td>Orthopedic and dental devices</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>Orthopedic and dental devices</td>
</tr>
<tr>
<td>Collagen (reprocessed)</td>
<td>Ophthalmologic and burns</td>
</tr>
</tbody>
</table>

1.6.1 Fluoropolymers as Biomaterials

Fluoropolymers are a family of plastics that were discovered in 1937. Fluoropolymers have at least one fluorine atom in the backbone of the monomer unit, giving it unique properties. The family includes poly(tetrafluoroethylene) (PTFE), poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP), poly(chlorotrifluoroethylene) (PCTFE), poly(vinylidene fluoride) (PVDF), poly(perfluoropropyl vinyl ether) (PPVE), poly(vinyl fluoride) among others. These polymers have been used commercially in membranes, coatings and as thermoplastics, mainly as a result of their unique properties (Feiring, 1994). It is for the same reason fluoroplastics have also been used for implants and biomaterials since 1980. For example, expanded PTFE (ePTFE) has been used in vascular grafts (Boyce, 1982) and as peripheral nerve conduits (Danielsen, 1988).

1.6.1.1 Synthesis

Fluoropolymers are generally synthesized by radical polymerization, either in aqueous emulsions or in fluorinated organic solvents. The monomer units are highly reactive.
as the fluorovinyl molecules are unstable due to the electronegative fluorine atoms. Polymerizations are exothermic and an extremely high molecular weight molecule is rapidly formed. Long chain branches are not formed as the strong carbon-fluorine (C-F) bond prevents chain transfer, thus the polymer is highly linear. The choice of polymerization technique produces different particle sizes (Feiring, 1994). Suspension polymerization with little dispersion agent produces coagulated granular resin, while dispersion polymerization with emulsifying agent makes very small particles (0.2 μm). The particle size affects subsequent processing steps of the polymer into shaped objects. For example, granular resins are used for preformed mouldings, fine powder resins are used in paste extrusion, and dispersed particles are used in coatings and fibres.

1.6.1.2 Physical Properties

As a family of polymers, the various perfluorinated plastics have very similar physical and chemical properties. Due to the highly electronegative fluorine atoms in the polymer chain, fluoroplastics have a very low surface and interfacial energy, making them very hydrophobic and repellant to many materials. They are not miscible with hydrocarbon polymers, thus copolymerization is preferred over polymer blends. Due to the high molecular weight chains normally formed, the polymers show excellent toughness and flexibility and high elongation (Feiring, 1994). Due to the stability of the C-F bond of the backbone, fluoropolymers have high melting points and glass transition temperatures, high dielectric strength (i.e. a low dielectric constant), and have high electrical insulating properties. This also gives the polymer thermal stability and prevents environmental degradation. A summary of the physical property data of the three most common fluoropolymers is given in table 1.4.
Table 1.4 Physical properties of PTFE, FEP and poly(tetrafluoroethylene-co-perfluoro(propyl vinyl ether)) (PFA) (Feiring, 1994).

<table>
<thead>
<tr>
<th></th>
<th>PTFE</th>
<th>FEP</th>
<th>PFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting points (°C)</td>
<td>327</td>
<td>260</td>
<td>305</td>
</tr>
<tr>
<td>Upper use temperature (°C)</td>
<td>260</td>
<td>200</td>
<td>260</td>
</tr>
<tr>
<td>Relative density</td>
<td>2.18</td>
<td>2.14-2.17</td>
<td>2.13-2.17</td>
</tr>
<tr>
<td>Tensile strength (MPa)</td>
<td>7-28</td>
<td>20-31</td>
<td>28-31</td>
</tr>
<tr>
<td>Elongation (%)</td>
<td>100-600</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Flexural modulus (MPa)</td>
<td>280-630</td>
<td>580-660</td>
<td>650-690</td>
</tr>
<tr>
<td>Impact strength (J m⁻¹)</td>
<td>160</td>
<td>no break</td>
<td>no break</td>
</tr>
<tr>
<td>Dielectric constant (1 MHz)</td>
<td>2.1</td>
<td>2.02</td>
<td>2.06</td>
</tr>
<tr>
<td>Dissipation factor (1 MHz)</td>
<td>0.003</td>
<td>0.008</td>
<td>0.0008</td>
</tr>
<tr>
<td>Refractive index</td>
<td>1.376</td>
<td>1.345</td>
<td>1.350</td>
</tr>
<tr>
<td>Critical surface tension (mN m⁻¹)</td>
<td>18.5</td>
<td>17.8</td>
<td>17.8</td>
</tr>
</tbody>
</table>

1.6.1.3 Chemical Properties

The strong C-F bond and high molecular weight of fluoropolymers that impart the unique physical properties also impart chemical inertness to the polymers. Even at elevated temperatures and pressures, fluoropolymers will not react with most chemicals and will not dissolve in most solvents. Only fluorinated organic solvents such as perfluoro(propyl vinyl ether) can dissolve or swell these polymers. Only strong reducing agents such as alkali metals and elemental fluorine have been known to defluorinate the polymer. Harsh bombardment of the polymer with plasma or high energy radiation is required to overcome the high energy barrier to reaction. The chemistry of modifying fluoropolymers will be discussed in section 1.4.
1.6.1.4 Biocompatibility

Due to the chemical inertness of the material, fluoropolymers are non-reactive with biological enzymes or proteins, rendering them virtually non-biodegradable. The hydrophobic nature of the surface prevents cells from attaching directly to the surface. Due to the hydrophobic interactions of protein adsorption (Van Oss, 1986, Wojciechowski, 1996), extracellular proteins readily adsorb on fluoropolymer surfaces, reducing their interfacial free energy (Shoichet, 1991a). Formation of a layer of ECM proteins surrounding the polymer causes non-specific cell response and triggers a foreign-body response. The recruitment of monocytes and fibroblasts leads to fibrosis and scar tissue, which is acceptable by the classical definition of biomaterial biocompatibility (Ratner, 1993). However, this non-engineered tissue response is normally undesired and not in agreement with recent definitions of biocompatibility (cf. p. 2), that is to “specifically exploit cells and proteins to meet a specific goal” (Ratner, 1993).

1.6.1.5 Applications

Fluoroplastics are used in a wide variety of applications, with wiring insulation being the largest due to its excellent insulating properties and low flammability. The low surface energy imparts low adhesion (or non-sticky) properties for use in gaskets, seals and bearings, and also for cookware surface coatings. Their stability and low-degradability make them useful for coating outdoor appliances such as solar collectors. The chemical inertness makes one of the few materials capable of handling corrosive liquids such as acids and gases (Feiring, 1994).
1.6.2 Teflon

Fluoropolymers are made by a few producers in the world. However, the trade name of Teflon® by Du Pont (USA) is the most well known and is used interchangeably with generic fluoropolymers. Teflon PTFE is the largest volume commercial fluoropolymer, with over 33 million kg produced in 1988 (Feiring, 1994), while about 6 millions kg of Teflon® FEP was made.

1.6.2.1 Poly(tetrafluoroethylene)

PTFE is chemically the simplest fluoropolymer (Figure 1.10). The monomer tetrafluoroethylene (TFE) is a highly reactive gas produced by gas-phase pyrolysis of chlorodifluoromethane (Downing, 1956). It is normally stored with radical inhibitors to prevent auto-polymerization, violent disproportionation to CF₄ and carbon, and formation of explosive peroxides (Pajaczkowski, 1964).

\[
\begin{pmatrix}
\text{F} & \text{F} \\
\text{C} & \text{C} \\
\text{F} & \text{F}
\end{pmatrix}_x
\]

Figure 1.10 Chemical structure of poly(tetrafluoroethylene).

Radical polymerization of TFE is usually initiated with a water-soluble initiator such as ammonium persulfate. Suspension or dispersion polymerization produces extremely high molecular weight end particles with over 90% crystallinity. It can be easily fibrillated to form a porous material, ePTFE or generically known as Gore-Tex, which is used for vascular grafts (Boyce, 1982).
1.6.2.2 Poly(tetrafluoroethylene-co-hexafluoropropylene)

FEP is a copolymer of TFE with hexafluoropropylene (HFP) (Figure 1.11). About 10-12 wt% of HFP is added as comonomer to increase the processability of PTFE. It also reduces the crystallinity of PTFE from 90% to 50-75% due to the fluoromethyl side group, disrupting the helical PTFE polymer chains. However, this also reduces the melting point and thermal stability of FEP relative to PTFE.

![Chemical structure of Poly(tetrafluoroethylene-co-hexafluoropropylene).](image)

HFP and TFE are copolymerized by aqueous or non-aqueous radical polymerization methods. Aqueous polymerization using ammonium persulfate as the initiator requires fluorinated surfactants (Khan, 1983). Non-aqueous polymerization uses fluorinated solvents with organic initiators such as propionyl peroxide, but with a lower polymerization temperature (Wachi, 1988). The processing of FEP can be done without the exotic methods used for PTFE due to its lower viscosity and melting point. Nevertheless, processing is more difficult than conventional thermoplastics, and solution processing is practically impossible.
1.7 Surface Modification

As mentioned in section 1.3.1.4, virgin fluoropolymers used as biomaterials are not rejected by the body but they elicit an unwanted foreign-body response. Even so, the bulk physical and chemical properties of fluoropolymers make it attractive for biomaterial applications. Therefore, modification of the surface to render it biocompatible is necessary.

1.7.1 Surface Properties in Biomaterial Applications

The surface properties of any biomaterials are the most important factor in any cell-material interaction as it is at the material surface where the cells contact. The biomaterial surface governs the biological response of cells through receptor binding to surface ligands. Cell adhesion, differentiation, growth, and neurite extension are all affected, by the particular surface chemistry and morphology.

1.7.1.1 Surface Chemistry

The binding interactions of a receptor to its ligand are generally through intermolecular forces, either through hydrogen bonds or weak van der Waal’s forces or ionic forces. A surface can be designed to manipulate these binding chemistries by using various chemical functional groups. An example, as mentioned in section 1.2.4.4, is by covalently bonding RGD peptides onto a surface to increase cell adhesion through activation of the cell integrin receptor. Another example is the coating of a surface with monoamines to enhance cell adhesion through the integrin receptor (Ranieri, 1993). Therefore, surface chemistry can be engineered using specific functional groups, peptides, or glycoproteins. In the reverse
case, where cellular or biological response of any kind is unwanted, a surface can be
designed to be non-reactive or non-adhesive by incorporating poly(ethylene glycol), which
has been shown to inhibit protein adsorption and cell adhesion (Harris, 1992).

1.7.1.2 Surface Morphology

The surface topography, or morphology, of a material also influences cellular
response. It has been found that there are microscopic morphologies in the basal lamina of
tissue, where cells adhere. These pores, or complex geometric forms, elicit a cellular
response via the internal cytoskeletal structures, and not through cell-surface receptors. For
example, human gingival fibroblast morphology, orientation and proliferation can be
controlled by surface topography, which disrupts assembly of focal adhesion sites (Kononen,
1992). Migration of endothelial cells can also be controlled using microgrooves carved onto
tissue-culture dishes (Matsuda, 1996).

1.7.1.3 Eliciting Specific Cell-Material Interactions

The “pinnacle” of engineering a biomaterial is to design a surface capable of
demonstrating precise and rapid reactions with proteins and cells (Ratner, 1993). Knowledge
of molecular cell biology is required for specific cellular responses, while chemistry and
material science is needed for mimicking biological ligands on an artificial matrix. This
thesis focuses on a cross disciplinary approach of modifying FEP to elicit neuronal adhesion
and neurite outgrowth by introducing cell-adhesive peptides to the surface.
1.7.2 Wet Chemical Techniques

Fluoropolymers are notoriously unreactive to many chemicals, thereby complicating the means to attach peptides to their surface. The general approach is to first introduce a reactive handle layer of functional groups before covalently coupling the peptides. Due to the inertness of fluoropolymers however, reactions are restricted the surface layer, thus maintaining the bulk properties of the fluoropolymer. The few techniques used to introduce functional groups onto fluoropolymer surfaces include wet chemical techniques, plasma modification techniques, and novel approaches. Wet chemical techniques are generally useful because specific modification can be predicted and applied to produce the required surface chemistry. However, the nature of the highly reactive reactions produces surface morphologies that are difficult to control.

1.7.2.1 Sodium Naphthalide Reduction

The reduction of FEP using reactive alkali metals originated from a method using a solution of sodium metal in liquid anhydrous ammonia to make Teflon more adhesive (Purvis, 1957). The reactivity of alkali metals were high enough to defluorinate the polymers by forming strong ionic alkali fluorides, leaving behind a carbonaceous layer. Sodium naphthalide was used to reduce FEP (Dwight, 1974), introducing unsaturated carbon-carbon double bonds and oxygen functionalities. A detailed study of the surface functional group density and depth of modification has shown that the reduction can be controlled (Bening, 1990). The reduced carbonaceous FEP layer was proposed to consist of unsaturated frameworks of sp$^2$ or sp hybridized carbon atoms that have higher conductivity than
unmodified FEP (Hung, 1995). The metallized layer also has a higher surface energy and is more chemically reactive.

![Chemical reaction diagram](image)

**Figure 1.12 Proposed skeletal electron transfer mechanism for reduction of FEP with sodium naphthalide (Bening, 1990).**

Further modifications of the carbonaceous layer can introduce a seemingly uniform surface with hydroxyl, carboxylic acid, or ethylene oxide groups (Shoichet, 1991b). The advantages of this method to introduce surface functionalities are: (a) the reaction is a simple two step reaction, (b) the reaction is surface sensitive, (c) a submonolayer or a monolayer coverage of only one functional group can be achieved.

### 1.7.2.2 Benzophenone and Sodium Hydride Reduction

Another alkali metal reduction of fluoropolymer has been recently used to modify PTFE surfaces for biomaterial applications (Noh, 1997). Benzophenone reacting with sodium hydride is photoexcited to form benzhydrol anion via a diphenyl ketyl radical anion intermediate (Caubere, 1983). The reaction produces a defluorinated surface with oxygen functional groups that is hydrophilic. As the reaction is less harsh than other alkali metal reduction, the morphological damage was limited.
1.7.2.3 Metal Deposition and Removal

A different method of modifying PTFE and FEP surfaces without using alkali metals is by depositing a layer of aluminum on the polymer surface and subsequently removing it (Ryan, 1972, Roberts, 1976). Active hydroxyl groups can be selectively introduced using this method without the formation of a carbonaceous layer (McKeown, 1991). Surface functional group density can be controlled by the number of aluminum deposition and removal steps. This reaction also limits the modification to the very surface of the polymer (3 nm) and does not affect surface morphology in any manner.

1.7.3 Plasma Modification

Plasma techniques are used industrially to modify the surfaces of a wide array of materials, e.g. in thin film deposition, reactive ion etching and plasma cleaning (Sodhi, 1996). Plasma is a gas-phase mixture of ions, electrons, free radicals, gaseous atoms and molecules that are excited by external energy sources. Control of plasma composition can be achieved by changing: (a) the energy source to either radio frequency (RF) or microwave (MW) or electrical discharge, (b) gas type, (c) gas flow rate, (d) reactor geometry. This subsequently affects the modification of the surface to achieve a variety of surface chemistry. The disadvantage of using plasma techniques is the complex nature of the plasma itself and the unpredictability of the surface composition (Clark, 1978). It also requires a vacuum to produce the plasma discharge, and therefore is not applicable for large-scale productions. However, this technique is very reproducible and does not affect the surface morphology. The creation of patterns of modified regions is also possible by using a mask.
1.7.3.1 Radio Frequency Plasma

RF plasma systems are the most common techniques used for polymer modification (Ratner, 1986). They are less powerful than MW plasma systems, thus producing a lower density plasma at the same energy which only affects the density of surface modification. The position of the polymer within the plasma stream affects the surface chemistry and concentration profiles. RF plasma of hydrogen and methanol has been used to defluorinate FEP and to introduce hydroxyl functional groups with minimal changes in surface morphology (Vargo, 1995).

1.7.3.2 Microwave Plasma

MW plasma systems are not as well studied as RF systems. The difference between the systems, other than the energy source, is that MW does not involve the participation of the plasma in downstream modification. Ultra-violet (UV) radiation produced by the higher energy MW is the main modification reaction downstream (Eggito, 1990). Plasma of $O_2$ and $O_2/N_2$ does not produce any reaction while plasma treatment with $NH_3$ produces a defluorinated PTFE surface and incorporation of nitrogen and oxygen functionalities. Plasma of $CO_2$ as the gas phase produces intense UV radiation that forms radicals on the surface of PTFE (Vasilets, 1997). Oxygen species reacting with the radicals produces oxygen functionalities that can be used for further modification.
1.7.4 Novel Techniques

Plasma modification techniques and wet chemistry modification of fluoropolymers are both well-studied methods that have distinct advantages and disadvantages. However, new techniques for modifying the surface of fluoropolymers are required where the surface chemistry can be produced with specificity and control, and where the surface morphology is not affected. Several of the new methods have recently been discovered include using excimer laser to induce chemical modification and radiation bombardment with X-rays or gamma rays.

1.7.4.1 Excimer Laser

Laser modification of fluoropolymers was first reported in 1989 when PTFE that was subjected to high intensity ArF laser in humid air incorporated oxygen functional groups (Chitaib, 1989). Later, ArF excimer laser was used to irradiate PTFE under a hydrazine atmosphere to enhance adhesion (Niino, 1997). The laser photolyzes hydrazine, which abstracts fluorine atoms from the fluoropolymer and incorporates amino groups into the PTFE chain. The excimer laser modification is a simple procedure and is done under mild conditions, producing a monofunctional surface. Other advantages of using laser processing are that spatial controllability (i.e. patterning) is high, and facility in operation is easy.

1.7.4.2 Irradiation Techniques

Irradiation of polymers with x-rays, low energy electrons or energetic ions is well known for inducing cross-linking, fragmentation and degradation. Bombardment of PTFE with high electron dosages under vacuum defluorinates the surface atomic layer (Duraud,
1986). Irradiation of FEP with accelerated electrons in air also leads to surface defluorination and incorporation of carboxylic acid groups (Burger, 1993). The advantages of this irradiation method are that no solvents or chemicals are used, a convenient two-step procedure produces a surface-selective carboxylic acid layer, and the procedure is done in air.

1.7.4.3 Mercat Modification

Photoexcited mercury atoms in the presence of gas-phase ammonia have been used to abstract hydrogen atoms from alkanes (Crabtree, 1995) and fluorine atoms from perfluoroalkanes (Burdeniuc, 1995), both notoriously unreactive compounds. This electron transfer reaction occurs when excited state mercury ($^3\text{P}_1$) binds to gaseous ammonia to form an exciplex which undergoes N-H bond breaking to form H and NH$_2$ radicals (Krajnik, 1993). The NH$_2$ radical is a powerful F and H radical abstractor, capable of partial oxidation of alkanes, fluoroalkanes and even fluoropolymers (Figure 1.13). Subsequent reaction of the unsaturated carbon-carbon with ammonia via a series of nucleophilic addition reactions of NH$_3$, followed by loss of HF and readdition of NH$_3$ leads to formation of nitriles and amino groups.

\[ \text{F}_3\text{C}\begin{array}{c} \text{CF} \\text{CF}_2\text{CF}_2\text{CF}_3 \end{array} \xrightarrow{\text{Hg/NH}_3} \text{UV light} \text{NC}\begin{array}{c} \text{NH}_2 \\text{NC} \\text{CF}_2\text{CF}_3 \end{array} \]

\[ \xrightarrow{\text{NH}_3} \text{F}_3\text{C}\begin{array}{c} \text{CF} \\text{CF}_2\text{CF}_3 \end{array} \]

**Figure 1.13** Reaction scheme for the defluorination of a fluorocarbon using photosensitized mercury in ammonia (Burdeniuc, 1995).
The photosensitized mercury in ammonia system (or the Mercat reaction) is surface-selective as the mercury exciplex cannot penetrate deep within a solid polymer. The reaction is one-step, and requires only mercury and ammonia to introduce nitrogen functionalities to fluoropolymers. As it is a mild gas-phase reaction, the morphology of the fluoropolymers is also not affected.

1.7.5 Surface Characterization

Methods to characterize a surface after modification are required to determine the actual surface properties such as chemical composition, morphology or topography, hydrophobicity/hydrophilicity, and physical properties. Many modern techniques are now available to provide detailed information on just the surface and not the bulk (Ratner, 1992). However, it is often necessary to use a combination of different methods to get a comprehensive overview as each technique provides complementary information.

1.7.5.1 Contact Angle

The measurement of a material hydrophobicity/hydrophilicity is usually achieved by the contact angle technique (Feast, 1987). This classical surface science technique measures the solid-liquid interfacial energy indirectly though the angle created between the tangent and the horizon at the solid-liquid-air interface (Figure 1.14). The Young’s equation (1) correlates the angle created to the surface free energy as follows:

\[ \gamma_{LV} \cos \theta = \gamma_{SV} - \gamma_{SL} \] (1)
Figure 1.14 Contact angle measurement of a solid-liquid-air interface to determine the solid-liquid interfacial energy and surface hydrophobicity/hydrophilicity (Shoichet, 1995).

By measuring the dynamic angle of the solid-liquid-air interface while withdrawing or adding liquid, advancing and receding angles can be measured. The difference in advancing and receding angles, or the hysteresis, provides an indication of surface roughness and chemical heterogeneity. For biomaterials, water is used as the liquid in the measurement. The reason is that the biomaterial interfaces with the body which is composed mainly of water.

1.7.5.2 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), is a popular method for determining the atomic composition of a surface to 100 Å in depth. The technique is based on Einstein’s photoelectron theory, whereby photons of sufficient energy impinge upon an atom, exciting or ejecting core electrons. The energy of the emitted electron is characteristic of the atom and the orbital layer from which it is ejected (Briggs, 1990, Ratner, 1992). The measurement of the energy of the electrons that escape from the material is used to identify the atoms present on the surface. As the chemical environment of the atom affects the orbital energies of electrons, the fine
differences in electron energy measured can also provide specific bonding information for the atom. The intensity or the number of the electrons detected correlates to the concentration of the atomic species. By factoring in instrumental effects and cross-sectional areas of specific atoms into a sensitivity factor, the relative concentration of each atom can be quantified.

Figure 1.15 Schematic diagram of a typical XPS instrument (Ratner, 1992).

XPS can be enhanced in many ways to provide additional information. Angle resolved XPS (ARXPS) is a method to increase the surface sensitivity of XPS by tilting the sample at various angles from the detector. The path length for ejected electrons increases at lower angles such that only electrons ejected from atoms closer to the surface can escape and be detected. Quantitative determination of specific functional groups can also be done with the XPS by chemical derivitization methods, where the functional groups are labelled with a
unique and XPS-sensitive atom. XPS can also be used for spatial imaging of atomic composition, or in cold-stage applications.

1.7.5.3 Attenuated Total Reflectance Fourier Transform Infra-Red Spectroscopy

Attenuated total reflectance Fourier transform infra-red spectroscopy (ATR-FTIR) is a classical spectroscopic method adapted to measure surface chemistry. Infra-red spectroscopy measures vibrational absorption of chemical bonds, and in attenuated total reflectance mode, the incidental IR source creates an evanescent wave that is only absorbed by the outer surface of the sample. Detection depth, up to 1 μm for some materials, depends on the IR transparency of the sample and the wavelength. The energy of the photon absorbed is characteristic of the vibrational mode of a chemical bond and thus, an absorption spectra provides qualitative and quantitative chemical information.

1.7.5.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is an imaging technique to view surface morphology at very high resolutions. Whereas normal light microscopy uses photon reflection as the detection source, SEM uses focused electron beams and detects either the primary reflected electrons, or the backscattered electrons for imaging. SEM can also use the secondary emitted electrons from collisions with the electron source to determine atomic composition of a surface at depths of 15Å and at resolutions of 40Å. Because of the high energy of the electron beam required, sample damage often occurs.
1.7.5.5 Atomic Force Microscopy

Atomic force microscopy (AFM), a type of scanning probe microscopy, is one of the latest imaging techniques that can produce images of surface topography on the atomic scale. By using a probe with a tip size of one atom to scan across a surface, the interatomic forces caused by changes in surface topography will deflect the probe which is measured as a height change. In tapping mode, where the probe is lightly tapped onto the surface as it scans, AFM can image biological materials and soft surfaces.

1.7.5.6 Other Methods

Other surface analytical techniques that are used include: (a) secondary ion mass spectroscopy (SIMS) which detects the mass of molecules ejected from the surface by the bombardment of argon ions, (b) auger electron spectroscopy, a technique similar to XPS that measures the auger electrons, i.e. the secondary emitted electrons, instead of the primary photoelectrons, (c) ellipsometry, which measures the refractive index of incidental light deflections on a surface layer.

1.8 Cell-Material Interaction

The study of cell-material interactions is an important aspect of engineering and evaluating a biomaterial. The application of molecular cell biology to surface chemistry can only be tested by examining how each type of cell responds to the material. In vitro cell culture and in vivo experiments of the material will prove if the specific response is elicited by the surface as designed. In this section, the culture and identification of CNS neurons will be discussed, followed by methods of quantifying neuronal response on biomaterials.
1.8.1 Central Nervous System Cells

Although neurons can be classified either functionally or shapewise as discussed in section 1.2.1.3, there exist a vast number of different neuronal types that differ at the molecular level. This means that although some neurons may appear similar, they differ in their biomolecular response mechanism, such as neurotransmitter release, receptor signalling pathways, etc. This fact makes it difficult to specifically design any biomaterial application that can be used for all neurons. However, it is possible to make a material that specifically elicits a response from a particular cell type. In this thesis, the goal of promoting neurite outgrowth and neuron survival applies to all cell types even though the focus is on spinal cord neurons such as motor and sensory neurons. This is because the response mechanism for neurite outgrowth does not differ radically from a brain cell such as hippocampal neuron to a spinal motor neuron.

Hippocampal neurons are chosen as the model system for neurite outgrowth because: (a) the cells are easily obtained as a relatively uniform population of a single cell type, (b) cultures of the neurons are relatively long and stable, (c) there are many characterization and identification methods for the neuron, (d) it has been used for a variety of studies, ranging from new culture media to electrophysiology to synapse formation (Brewer, 1993, Valiante, 1997).

1.8.1.1 Primary Cells

Primary cells are the cells derived or harvested directly from the body for tissue culture. They can be prepared in three methods for the different degree of tissue organization required (Nelson, 1975): (a) culture of entire organs, which maintain physical anatomy and
function to enable the examination of intercellular interactions; (b) tissue explant cultures, which only remove parts of the organ that is required for analysis, thus simplifying the culture conditions and enabling a more detailed cellular assessment; and (c) dissociated cell cultures, which are the most popular technique of using primary cells as they are easily obtained and accessed. Very detailed cellular morphological and physiological studies can be easily done on dissociated cell cultures. Their disadvantage is that reliability and reproducibility are low, thus requiring many repeats to eliminate uncertainties. Some different dissociated neuronal cell cultures include cerebral cortex cells (Romijn, 1984), hippocampal neurons (Brewer, 1997) and spinal ganglion neurons (Luduena, 1973).

1.8.1.2 Cell Lines

Unlike primary cells which have a limited life span, cell lines constitute a continuously dividing cell population that have been transformed from primary cells (Nelson, 1975). A large variety of cell lines has been produced for all cell types by cloning primary cells either with tumour cells or with blastomas. There are some advantages of using cell lines over primary cell culture. The cells are capable of cell division thus the allowing for clonal analysis and neuronal differentiation. Also, the reproducibility and stability of cell lines are high as all the cells are the same genetically. The primary drawback of using cell lines for this thesis is that the response of primary cells to extracellular stimulation differs greatly from that of cell lines. For example, the prediction of cell-adhesive peptide effect on neurite outgrowth cannot be approximated by using cell lines. The possibility of mutations after several generations of culture is another drawback of using cell lines. Examples of neuronal cell lines include PC12 cells (pheochromocytoma cell), a clone of kidney cells and
tumour cells (Tomaselli, 1990) and NG108 cells (neuroglia cell), a neuroblastoma and glioma hybrid (Chueh, 1994).

1.8.2  *In Vitro* Tissue Culture

The culture of neuronal cells *in vitro* is used because it offers a very simple but powerful tool to analyze cell-material interactions. The biomaterial produced can be used as a culture substrate in tissue culture dishes or plates, onto which dissociated cells can be easily plated. Thereafter, the neuronal response can be examined by using various different techniques, as described in sections 1.5.3 and 1.5.4.

1.8.2.1 Dissociated Cells

Harvested CNS cells from either rat or mouse are normally dissociated into individual cells to study the process of neurite development with high resolution (Nelson, 1975). However, there are some technical problems that occur (Brewer, 1997): (a) dissociation of the neuron from its network of axonal and dendritic connections might cause severe damage, with neurites being severed and surface receptors detached; (b) achieving a homogenous population of a single cell type is not easily done, especially in light of the close association between neuronal cells with glial cells; and (c) choosing a suitable medium for nutrient supply while not affecting the experimental outcome is difficult. The use of embryonic neuronal cells in dissociated cultures alleviates some of these problems as the neurons are relatively immature and not fully differentiated. Axonal connections in embryonic brains are not established until very late in gestation (Kandel, 1991), and the embryonic CNS cells have the capacity to repair themselves (Nicholls, 1996). Also, the use of established dissociation
procedures involving mechanical trituration and proteases such as papain and DNase minimizes any unwanted damage to cell surface receptors (Banker, 1977).

1.8.2.2 Culture Conditions

Successful culturing of neurons are greatly affected by culture conditions such as media type, oxygen and carbon dioxide concentration, and temperature fluctuations (Brewer, 1997). Many formulations of media have been used, with those containing serum having the best neuronal survival rate. However, serum-free media is used when non-specific protein adsorption onto a surface interferes with the experimental factors (Romijn, 1988). In the design of a biomaterial where specific cell-ligand interaction is under investigation, the adsorption of serum proteins may mask the surface-attached ligands. The earliest serum free media of Bottenstein and Sato for neuronal cell-lines have been adapted to successfully culture hippocampal neurons (Brewer, 1993) up to more than 4 weeks in vitro.

The cell density at plating is also an important factor for in vitro cell culture. Concentrations of 160 neurons/mm² (Brewer, 1993) or 150,000 cells/mL (Banker, 1977) are necessary for long-term neuronal survival and optimal neurite extension.

1.8.3 Cell Characterization

Because a 100% pure culture of a single cell type is generally not available when culturing primary cells, it is necessary to identify and differentiate between the various cells before analysis can be done. There are two general methods of characterizing all cell types, but with neurons, a third method of using their electrical signalling function is also used.
1.8.3.1 Physical Identification

The identification of a cell type by its morphology and shape is a rough but quick method. This physical identification is normally used when chemical markers are unavailable or would affect cellular response. The morphology of neurons is very different from that of glial cells where astrocytes and oligodendrocytes have distinct cell body shapes (Kandel, 1991). The differentiation between various neuronal types, however, is more difficult. Neuronal classification into three types based on morphology (section 1.2.1.3) distinguishes most neurons but there exist several neuronal types within the same class. For example, hippocampal cells are multipolar pyramidal cells that can easily be distinguished from the bipolar retinal ganglion cells. However, spinal motor neurons are also bipolar and quite similar in shape to the retinal ganglion cells. Thus, the only way to clearly distinguish between neuron types is by chemical markers.

1.8.3.2 Chemical Markers

The use of chemical markers, of which immunocytochemistry is a subtype, provides the most distinct method of identifying a cell. Specific molecules that are present only in one cell type and not another can be fluorescently labelled with antibodies to that molecule, or chemical probes. Astrocytes can be distinguished by the presence of glial-fibrillary acidic protein (GFAP), which can be labelled with an anti-GFAP. Similarly, an antibody against galactocerebroside can be used to identify oligodendrocytes, and antibodies against neurofilaments such as NR4 and NE14 identify all neuronal axons (Kandel, 1991). Neurons are distinguished from each other generally by the different neurotransmitters or receptor subtypes using fluorescent probes such as Lucifer yellow (Zimmerman, 1986).
1.8.3.3 Electrophysiology

Quite simply, neurons transmit electrical signals from one cell to another. The electrophysiology of neuronal types have been studied by measuring the action potentials travelling from one cell to another, or from the cell body to the axon, using micropipette electrodes (Kandel, 1991). Because some neurons have very specific action potential shapes and pulses, they can be differentiated by recording the signal transmissions (Offenhauser, 1997). However, for these measurements, differentiated neurons must have formed synapses.

1.8.4 Quantifying Nerve Cell Growth

The assessment of cell-material interaction can be achieved by measuring: (a) the number and length of neurites extended from the cell body; (b) the number of adherent cells, and (c) the viability of the cells on the surface.

1.8.4.1 Neurite Outgrowth

The quantitative assessment of neurite outgrowth includes measurement of neurite length, the number of neurites per cell, and the ratio of neurite area to the total cell area. These are the most important measurements of neuronal response as the function of neurons is to transmit signals through the neurites. There exists specialized computer software that can process digital images of neurons and the neurites to analyze for the outgrowth automatically (Gunderson, 1987, Venstrom, 1995).
1.8.4.2 Cell Adhesion

Cell attachment or adhesion is a measure of how well the cells interact with the surface versus each other. For most anchorage-dependent cells, attachment to a surface promotes higher survival and differentiation. Dynamic assessment of adhesion strength can be achieved by applied shear flow to the culture to measure how many cells detach (Tempelman, 1994). Static adhesion strength can be measured by applying a short, high shear shaking and measuring the number of cells still attached (Hammarback, 1985).

1.8.4.3 Cell Viability

Neuronal viability can be affected by many things, including media type, dissociation method and culture substrate. Generally, neurons survive longer on surfaces where they attach well (Stenger, 1993). A simple live/dead assay stain uses calcein AM to label live cells and ethidium homodimer to label dead cells (Kaneshiro, 1993). Live cells are counted as a percentage of the total number of cells in the culture under a fluorescent microscope to determine the cell viability.

1.8.4.4 Qualitative Analysis

Images of cells taken under normal light microscopy can provide a quick qualitative assessment of neuronal health. Normal healthy cells can be easily distinguished from cells that are dying through the presence of disintegrating neurites, or visible lipids within the cytoplasm (Kandel, 1991). A rough qualitative assessment of neurite outgrowth can also be evaluated from micrographs of the neurite network and synaptic junctions.
1.9 References


Tong, Y.W. and Shoichet, M.S. “Enhancing the interaction of central nervous system neurons with poly(tetrafluoroethylene-co-hexafluoropropylene) via a novel surface amine-


Chapter 2. Peptide Surface Modification of Poly(tetrafluoroethylene-co-hexafluoropropylene) Enhances its Interaction with Central Nervous System Neurons

2.1 Overview

The goals and objectives of this thesis, as stated in Chapter 1, were to develop a model surface to study CNS axonal regeneration. This chapter describes the chemical modification of FEP by a two-step method to introduce hydroxyl and carboxylic acid functional groups which were used to couple cell-adhesive peptides. An initial study of hippocampal neuronal response to the peptide-coupled surface was used to test the hypothesis that neuronal interaction was enhanced by the peptides, and whether neurite outgrowth was affected by varying the types and concentration of peptides used.

2.2 Abstract

Poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) film surfaces were chemically surface-modified to introduce one of three laminin adhesive peptides: GYIGSR, GRGDS or SIKVAV. FEP film surfaces were first reduced, with sodium naphthalide, to introduce surface carbon-carbon double bonds at two reaction conditions, 20 min at -78°C, and 3 h at 25°C. Scanning electron microscopy and atomic force microscopy indicated that surface topography was unaffected by the reaction conditions. Reduced FEP film surfaces were further modified to introduce hydroxyl groups via hydroboration/oxidation or carboxylic acid groups via oxidation. The hydroxyl (FEP-CH$_2$OH) and carboxylic acid (FEP-COOH) functionalized surfaces provided reactive handles for peptide coupling using tresyl chloride. Surface elemental composition data, determined from X-ray photoelectron spectroscopy, indicated that equivalent amounts of GYIGSR, GRGDS and SIKVAV were introduced. Two additional coupling reagents, SMCC and TSU, were compared to tresyl chloride for the coupling of radiolabeled tyrosine of GYIGSR. Between 8 and 150 fmol/cm$^2$ of peptide was introduced to the hydroxyl and carboxylic acid functionalized surfaces, with the tresyl coupling reagent showing the greatest amount of peptide incorporated. The tresyl-coupled peptide-modified surfaces were compared in terms of the response of primary, embryonic hippocampal neurons plated from serum-free medium for 4 days. The number and length of neurites extending from the cell bodies were averaged over 50 cells after 1 and 4 days. FEP-CH$_2$O-Peptide surfaces had either a greater or equivalent hippocampal neuron interaction than the corresponding FEP-COO-Peptide surfaces. All peptide-functionalized surfaces had a greater hippocampal neuron interaction than the corresponding FEP-CH$_2$OH, FEP-COOH, and FEP controls after 4 days underlying the importance of the peptides over
hydrophilic or hydrophobic surfaces. After 4 days differences in neurite extension were evident among the peptide-functionalized surfaces, with the longest neurites observed on SIKVAV-functionalized surfaces.

2.3 Introduction

Central nervous system (CNS) neurons, unlike those of the peripheral nervous system, do not regenerate spontaneously following injury; however, CNS neurons can regenerate in a peripheral nerve graft (Cheng, 1996, David, 1981, Richardson, 1980, Richardson 1982) or in an environment that mimics that of the peripheral nerve (Bunge, 1994). A synthetic graft must provide the adhesion molecules that enhance cellular interaction and regeneration, such as those of the basal lamina of the peripheral nerve graft (Luckenbill-Edds, 1997).

Cell adhesion molecules, consisting of diverse cell-surface glycoproteins and extracellular matrices (ECMs), affect cell interactions during the development, maintenance and regeneration of the nervous system (Jucker, 1991). Specific cell-surface receptors (Brandley, 1988) (i.e. integrins) adhere to ECM proteins, such as laminin and fibronectin (Matsuda, 1989). Particular amino acid sequences of these naturally occurring ECM proteins have been identified as binding domains. For example, arginine-glycine-aspartic acid (RGD) has been found to be important for the binding of numerous cell types to fibronectin and laminin (Hubbell, 1992, Ito, 1991); tyrosine-isoleucine-glycine-serine-arginine (YIGSR), on the B1 chain of laminin, has been shown to be active in epithelial cell attachment (Bellamkonda, 1995) and in promoting neural cell adhesion and outgrowth (Vargo, 1995); and isoleucine-lysine-valine-alanine-valine (IKVAV), on the A chain of laminin, has been found to
promote neurite outgrowth of PC12 cells (Ranieri, 1995).

Several investigators have found that peptide-modified surfaces enhance cell adhesion. RGD promotes the interaction of fibroblasts and endothelial cells with, for example, poly(acrylamide) (Boyce, 1982), poly(vinyl alcohol) (Danielsen, 1988), polystyrene (Hickman, 1997), and glass beads (Dias, 1985). YIGSR, IKVAV and RGD enhance the interaction of neuronal cells with agarose (Shoichet, 1991), and YIGSR enhances the adhesion of neuronal cell lines with poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) that is pretreated with albumin (Vargo, 1995, Ranieri, 1995).

Fluoropolymers such as expanded poly(tetrafluoroethylene) (ePTFE) have a history of in vivo use, including those in vascular grafts (Edelman, 1985) and peripheral nerve repair (Hynes, 1987). Since fluoropolymers are inert in the CNS (Ruoslhti, 1988), peptide-modified fluoropolymers may lead to a cleaner delineation of the effect of immobilized oligopeptide bio-availability on axonal outgrowth than other substrates. Unlike plasma modification, which introduces a diverse set of functionalities, chemical modification of normally inert fluoropolymers allows discrete functional groups to be introduced to defined depths of reaction and topographies (Grant, 1989, Pierschbacher, 1984).

In this article, we describe the chemistry required to surface-modify FEP with laminin-derived adhesive peptides and the interaction of primary CNS neurons with the modified surfaces. By modifying the fluoropolymer surfaces with cell adhesive peptides (YIGSR, IKVAV or RGD), we aim to mimic the interaction normally observed between laminin and peripheral neurons, thereby preparing the optimum surface for enhanced cell interaction and process outgrowth in the CNS. To date, the interaction of primary CNS neurons with peptide-functionalized polymer surfaces has received limited attention. The
interaction of primary hippocampal neurons with three peptide-modified surfaces, which required no pre-treatment, is compared quantitatively in terms of neurite outgrowth and qualitatively in terms of cell adhesion and viability.

Figure 2.1 Poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) film samples are surface-modified to either hydroxyl or carboxylic acid groups which were in turn either labeled with trichloroacetyl chloride or thallous ethoxide, respectively, or coupled with cell adhesive peptides.

Herein, FEP film surfaces are chemically modified by a series of reactions to introduce either hydroxyl or carboxylic acid functional groups which are subsequently coupled to cell-adhesive peptides using one of three coupling agents. FEP film samples are surface-modified by sodium naphthalide reduction at several reaction conditions (20 min at -78°C, 0°C, 25°C and 3 h at -78°C, 0°C, 25°C) to determine whether surface chemistry can be modified independently from surface morphology. The two extremes (i.e., 20 min at -78°C vs. 3 h at 25°C) of the reduced films (FEP-C) are converted to either hydroxyl (FEP-CH₃OH) by hydroboration/oxidation or carboxylic acid (FEP-COOH) by oxidation. As shown in Figure 2.1, the hydroxyl and carboxylic acid groups provide two different synthetic routes to
introduce peptides, GYIGSR, GRGDS or SIKVAV, to the surface. The amount of peptide incorporated onto each surface is calculated by coupling radioactive, iodinated tyrosine of GYIGSR to the functionalized surfaces; the coupling efficiencies of three different reagents are compared as shown in Figure 2.2. The surfaces are characterized in terms of relative hydrophilicity, atomic composition, and morphology. The response of hippocampal neurons to the modified surfaces is compared quantitatively in terms of the number and relative length of extended neurites per cell.

Figure 2.2 Three coupling agents (tresyl chloride, SMCC, and TSU) were used to couple the GYIGSR peptide to functionalized FEP (FEP-X) film surfaces, yielding FEP-X-peptide. (Detailed mechanisms for the coupling chemistries are shown in Appendix A)
2.4 Materials and Methods

2.4.1 Materials

All chemicals were purchased from Aldrich (Milwaukee, WI) and used as received unless otherwise indicated. Tetrahydrofuran (THF, Fisher, Nepean, ON) was distilled from sodium benzophenone dianion and stored under nitrogen in Schlenk flasks. Hank's balanced salt solution (HBSS), neurobasal medium, B27 supplement and phosphate buffered saline (PBS) were sterile-filtered with 0.22 μm cellulose acetate filters (all from Gibco BRL, Burlington, ON). All peptides were purchased from Vetrogen (ON, Canada) and used as received. FEP films (5 mil thickness, received from DuPont and cut into 2 cm x 2 cm samples) were Soxhlet-extracted in THF for 24 h. All reactions were done under inert nitrogen atmosphere unless otherwise indicated. Deionized distilled water was obtained from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) and used at 18 MΩ resistance.

X-ray photoelectron spectroscopy (XPS) data were collected on a Leybold LH Max 200 using a polychromatic MgKα x-ray source at 15 kV and 20 mA emission current. An aperture size of 13 x 7 μm was used to collect data at takeoff angles of 20° and 90° between sample and detector for 1 spot per sample averaged over 3 samples. Advancing and receding water contact angles were obtained on a Ramé-Hart NRL telescopic goniometer. Values reported represent the average and standard deviation of 5 measurements. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were obtained on a Mattson Galaxy 5400 spectrometer using a germanium crystal (45°). Scanning electron microscopy (SEM) micrographs were taken on a Hitachi S4500 field emission microscope at 1 kV acceleration voltage. Atomic force microscopy (AFM) micrographs were obtained on a
Digital Instrument Nanoscope 3 using tapping mode imaging. Iodine-125 radiolabeled tyrosine of GYIGSR was quantified for 3 samples per film using a scintillation counter (LKB Wallac 1282-802 Universal γ-Counter) with a 2 x 2 cm sodium iodide detector well of 80% efficiency. Embryonic mouse (CD1 Type, Charles River, ON) hippocampal neurons that were cultured on all surfaces were photographed under normal light or filters for fluorescently-stained cells at 20x magnification under an Axiovert 150 microscope.

2.4.2 Reduction of FEP, FEP-C

FEP film surfaces were reduced as previously described (Shoichet, 1991, Bening, 1990) in 20 mL of a 0.16 M sodium naphthalide solution in THF for either 3 h at 25°C (referred to as 25°C from here forward) or 20 min at -78°C (referred to as -78°C from here forward). The films were then rinsed three times each with THF, dichloromethane and THF before drying under vacuum (pressure = 0.01 mmHg).

2.4.3 FEP Functionalization, FEP-X (X = -COOH, -CH₂OH)

FEP-COOH film samples were prepared as previously described (Shoichet, 1991) by oxidizing FEP-C films with 0.16 M potassium chlorate in sulfuric acid in a beaker for 2 h at room temperature (RT). Films were then rinsed five times each with water, methanol and dichloromethane prior to drying under vacuum. Unmodified FEP film samples were used as controls.

FEP-COO-Tl film samples were prepared as previously described (Shoichet, 1991) by immersing FEP-COOH film samples in 10 mL of thallous ethoxide for 60 s at RT and then rinsing six times with ethanol prior to drying under vacuum. Unmodified FEP film
samples were used as controls for this labeling reaction.

FEP-CH₄OH film samples were prepared as previously described (Bening, 1990) by hydroboration of FEP-C in 10 mL of a 0.5 M borane/THF solution for 18 h followed by oxidation in 10 mL of a 30% hydrogen peroxide solution and 20 mL of a 0.18 M aqueous sodium hydroxide solution for 3 h in a beaker. Films were then rinsed five times each with 20 mL dilute sodium hydroxide solution, dilute hydrochloric acid solution, water and THF prior to drying under vacuum.

FEP-CH₄O-C(O)CCl₃ films were prepared as previously described (Bening, 1990) by reacting FEP-CH₄OH film samples with 0.5 mL trichloroacetyl chloride in 19 mL THF and 1 mL pyridine for 12 h at RT. The film samples were rinsed three times each in THF, methanol and dichloromethane prior to drying under vacuum. Unmodified FEP film samples were used as controls for this labeling reaction.

2.4.4 Peptide Coupling to Functionalized FEP, FEP-X-peptide

Three peptides (GYIGSR, SIKVAV and GRGDS) were coupled to FEP-X film surfaces using the trifluoroethanesulfonyl chloride (tresyl chloride) coupling agent. In addition, GYIGSR was coupled to FEP-X film surfaces using one of three different coupling reagents as shown in Figure 2.2: tresyl chloride, O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TSU), or sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). Unmodified FEP film samples were used as controls for the coupling reactions.

FEP-X film samples were immersed in a solution containing 200 µL of tresyl chloride in 1 mL pyridine/19 mL THF for 20 min at RT and then added to a beaker containing 10 mL
of a 0.2 M, pH 10, sodium carbonate-buffered solution and 0.1 μg/mL of peptide (i.e. GYIGSR, GRGDS or SIKVAV) for 24 h (Nilsson, 1981). The films samples were rinsed five times each with the buffer solution, dilute hydrochloric acid, water, and THF prior to drying under vacuum.

FEP-X film samples were immersed in 20 mL of dimethyl formamide (DMF) containing 1.3 mM TSU and 1.3 mM diisopropylethylamine for 4 h at RT. Film samples were then added to a beaker containing 10 mL of a 0.2 M, pH 10, sodium carbonate-buffered solution containing 0.1 μg/mL of GYIGSR for 24 h (Drumheller, 1994). The film samples were rinsed five times each with the buffer solution, water and THF prior to drying under vacuum.

FEP-X were immersed in a beaker containing 30 mL of a 0.2 M sodium bicarbonate-buffered solution at pH 10 and containing 2 mg of SMCC. After 4 h, the activated film samples were immersed in a 0.2 M, pH 10, buffered sodium bicarbonate solution containing 0.1 μg/mL of cysteine-terminated CGYIGSR for 24 h at RT (Mattson, 1993). The film samples were rinsed five times each with the buffer solution, dilute hydrochloric acid, water and THF and then dried under vacuum.

2.4.5 Radioactive Labeling of Tyrosine

Tyrosine (Y) of GYIGSR was labeled as previously described with radioactive iodine (125I) (Drumheller, 1994). Briefly, 2 mg of CGYIGSR was dissolved in 5 mL of a pH 11 buffer containing 20 mM sodium phosphate and 0.15 M sodium chloride and then reacted with 1 mCi of carrier-free Na125I (ICN, Costa Mesa, CA) in the presence of Iodobeads (Pierce, Rockford, IL) for 15 min. Free iodide was removed by successive passes through
columns packed with anion-exchange resin (Dowex 1-X8, Aldrich). The labeled peptide was coupled to FEP-X as described above and FEP-X-peptide films were rinsed with 10 mM sodium iodide to desorb any trace $^{125}$I before counting by scintillation.

**2.4.6 Hippocampal Neuron-Fluoropolymer Interaction**

FEP-X-peptide films and controls (FEP, FEP-C, and FEP-X) were immersed in 70% ethanol for 1 h and then rinsed four times with sterile distilled water before air-drying. Positive control surfaces were prepared by coating glass coverslips with 1 mL of an aqueous 1 mg/mL solution of poly(L-lysine) (PLL; Sigma, MW = 37,000 g/mol) for 24 h at 37°C and then with 10 μL of a 1 mg/mL aqueous solution of laminin (Gibco) for 2 h at 37°C. PLL-laminin-coated glass coverslips were rinsed with sterile, distilled water and air-dried prior to plating the hippocampal neurons. Embryonic day 18 (E18) mouse hippocampal neurons were isolated as previously described (Brewer, 1993) by dissociation with papain (Worthington Biochemical Corporation) and DNase (Sigma) for 30 min and mechanical trituration in calcium-free HBSS. Then, 1 mL of hippocampal neurons was then plated at 1 x 10^6 cells/mL in serum-free medium (SFM) on each film sample (or 2.5 x 10^5 cells/cm²). The SFM was made with 2 mL B27 supplement, 100 mg chicken egg albumin (Sigma), 10 mg pyruvic acid (Sigma), 1 mL glutamine (Sigma), and 1 mL penicillin-streptomycin (Gibco, 10,000 unit/mL and 10,000 μg/mL, respectively) in 100 mL neurobasal medium. The antimitotic agent, fluorodeoxyuridine/uridine (Sigma), was added after 24 h. The cells were incubated at 37°C in 5% CO₂ for 4 days. The cell-material interaction was assessed after 1 and 4 days in terms of the number and length of extended neurites. At each time point, 50 cells were chosen at random and the number of neurites per cell and the number of cells having neurites longer
than one cell body were quantified. Phase contrast micrographs were taken after 1 and 4 days. To assess cell viability at 4 days, samples were incubated for 30 min at 37°C with 150 μL of stock viability assay solution (20 μL ethidium homodimer and 5 μL calcein AM in 10 mL PBS: Molecular Probes, Eugene, OR) and examined under fluorescent filters of the optical microscope.

2.4.7 Statistics

For every surface, on which 50 cells were evaluated, triplicate experimental data sets were subjected to statistical analysis using SAS (SAS Institute Inc., Cary, NC) on a SGI Challenge L Unix-based computer system. One-way analysis of variance (ANOVA) assuming a constant variance and a 95% confidence interval was used to determine statistical differences of various data sets. Results are reported as the mean ± the standard error of the mean, and statistically significantly differences are labeled with an asterix (*).

2.5 Results

2.5.1 Reduction of FEP to FEP-C and Subsequent Functionalization to FEP-X

FEP film samples were surface-modified to introduce hydroxyl (Bening, 1990) or carboxylic acid (Shoichet, 1991) functionalities and subsequently labeled with either trichloroacetyl chloride or thallous ethoxide, respectively, or coupled with cell adhesive peptides. GYIGSR, GRGDS and SIKVAV (cf. Figure 2.1). Tables 2.1 and 2.2 summarize the advancing (θA) and receding (θR) water contact angles and the XPS atomic composition data, respectively.
The FEP film samples were first reduced with sodium naphthalide in THF (to FEP-C) at the two reaction conditions (-78°C and 25°C) to determine if surface chemistry could be modified independently of surface morphology. Scanning electron micrographs for FEP and FEP-C were identical and smooth, having no topographical features. Since no differences in surface morphology were observed between FEP and FEP-C (at both times/temperatures) by either SEM or AFM (data not shown), we determined that surface chemistry alone accounted for the differences observed in the subsequent surface modifications and interactions with hippocampal neurons.
Table 2.2 XPS data showing atomic composition of FEP and surface modified films at a takeoff angle of 20° between sample and detector (n = 3 samples for film type).

<table>
<thead>
<tr>
<th>Film Type</th>
<th>Atomic Composition (%)</th>
<th>Atomic Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clean FEP</td>
<td>$C_{31.1}F_{68.9}$</td>
<td></td>
</tr>
<tr>
<td>FEP-C</td>
<td>$C_{91.7}F_{4.4}O_{3.8}$</td>
<td>$C_{84.7}F_{9.7}O_{5.2}$</td>
</tr>
<tr>
<td>Hydroxyl functionalized films:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEP-CH$_2$OH</td>
<td>$C_{59.7}F_{22.1}O_{18.2}$</td>
<td>$C_{74.5}F_{3.3}O_{22.2}$</td>
</tr>
<tr>
<td>FEP-CH$_2$O-CO-CCl$_3$</td>
<td>$C_{62.2}F_{13.5}O_{16.3}Cl_{1.8}$</td>
<td>$C_{68.0}F_{2.6}O_{17.1}Cl_{12.4}$</td>
</tr>
<tr>
<td>FEP-CH$_2$O-tresyl-GYIGSR</td>
<td>$C_{75.2}F_{3.3}O_{17.1}N_{3.4}$</td>
<td>$C_{72.9}F_{2.7}O_{18.6}N_{4.5}$</td>
</tr>
<tr>
<td>FEP-CH$_2$O-tresyl-GRGDS</td>
<td>$C_{73.2}F_{3.0}O_{20.0}N_{2.8}$</td>
<td>$C_{73.4}F_{4.1}O_{19.1}N_{2.5}$</td>
</tr>
<tr>
<td>FEP-CH$_2$O-tresyl-SIKVAV</td>
<td>$C_{76.2}F_{3.3}O_{17.7}N_{2.1}$</td>
<td>$C_{70.3}F_{3.0}O_{19.7}N_{6.1}$</td>
</tr>
<tr>
<td>Carboxylic acid functionalized films:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEP-COOH</td>
<td>$C_{33.3}F_{64.1}O_{2.6}$</td>
<td>$C_{31.5}F_{67.0}O_{1.5}$</td>
</tr>
<tr>
<td>FEP-COO-T1</td>
<td>$C_{35.3}F_{60.0}O_{3.8}Cl_{0.5}$</td>
<td>$C_{37.2}F_{56.5}O_{4.6}Tl_{1.7}$</td>
</tr>
<tr>
<td>FEP-COO-tresyl-GYIGSR</td>
<td>$C_{49.4}F_{40.3}O_{7.8}N_{2.2}$</td>
<td>$C_{53.3}F_{35.1}O_{8.6}N_{3.0}$</td>
</tr>
<tr>
<td>FEP-COO-tresyl-GRGDS</td>
<td>$C_{46.9}F_{42.1}O_{8.5}N_{2.3}$</td>
<td>$C_{45.0}F_{45.7}O_{7.9}N_{2.0}$</td>
</tr>
<tr>
<td>FEP-COO-tresyl-SIKVAV</td>
<td>$C_{37.8}F_{29.3}O_{10.2}N_{2.7}$</td>
<td>$C_{38.4}F_{28.3}O_{9.6}N_{3.6}$</td>
</tr>
</tbody>
</table>

FEP-C film samples were modified to produce hydroxyl or carboxylic acid functionalized surfaces by either hydroboration/oxidation or oxidation, respectively. The large decrease in both advancing and receding contact angles from FEP (120°/101°) to FEP-C (50°/19°) and then to FEP-CH$_2$OH (58°/25°) reflects the increased hydrophilic nature of the modified surfaces. FEP-CH$_2$OH is apparently less hydrophilic than FEP-C, likely as a result of the depleted surface fluorine concentration of FEP-C. Similarly, FEP-COOH (96°/44°) is more hydrophilic than FEP yet less than FEP-C owing to the oxidative removal of the reduced layer (Shoichet, 1990), producing carboxylic acid functionality at the fluoropolymer chain ends. The XPS data corroborates the contact angle data, demonstrating an increase in oxygen functionality in FEP-CH$_2$OH ($C_{59.7}F_{22.1}O_{18.2}$) and FEP-COOH ($C_{33.3}F_{64.1}O_{2.6}$) with respect to FEP ($C_{31.1}F_{68.9}$) and FEP-C ($C_{91.7}F_{4.4}O_{3.8}$). ATR FTIR spectra
confirm the presence of the hydroxyl functional group with a peak at 3660 cm⁻¹; the carboxylic acid functional group was not easily detected by ATR FTIR due to its low surface concentration and the inherent surface selectivity of this reaction.

To determine the percentage of oxygen functionality attributed to either hydroxyl or carboxylic acid functional groups, the hydroxyl-functionalized surfaces were labeled with trichloroacetyl chloride, while the carboxylic acid functionalized surfaces were labeled with thallous ethoxide. The XPS and contact angle data in Tables 2.1 and 2.2 reflect the presence of chlorine [on FEP-CHO-C(O)CCl₃] and thallium (on FEP-COO-Tl), indicating the presence of alcohol and carboxylic acid, respectively. ATR-FTIR data confirm that covalent bonds were formed with the disappearance of the hydroxyl stretch of FEP-CH₂OH at 3100-3400 cm⁻¹ and the appearance of a carbonyl stretch at 1740 cm⁻¹ of the newly formed ester.

2.5.2 Peptide Coupling to Functionalized FEP (FEP-X-peptide)

The hydroxyl and carboxylic acid functional groups on FEP film surfaces serve as reactive handles for further chemical modification. Using tresyl chloride as the coupling reagent, three peptides were coupled to the functionalized surfaces, the data of which are summarized in Tables 2.1 and 2.2. The advancing contact angles of the hydroxyl functionalized surfaces increased after peptide coupling, whereas the receding contact angles remained the same or showed a slight decrease. The increase in hysteresis reflects the chemical heterogeneity of the peptide-modified surfaces (Andrade, 1985). The contact angles of the peptide-modified surfaces were for the most part, indistinguishable from each other due to their similar composition. The XPS data confirmed that the change in contact angle data resulted from peptide modification. For all peptides, the presence of nitrogen, the
increase in carbon and the decrease in fluorine confirmed the peptide coupling reaction. While there are slight differences in the amount of nitrogen observed between the different peptides, these differences were not seen for carbon and fluorine, indicating that a similar amount of GYIGSR, GRGDS, and SIKVAV are coupled to the hydroxyl-functionalized surfaces.

Peptide modification of the carboxylic acid functionalized FEP surfaces was confirmed by decreased advancing and receding water contact angles with respect to FEP-COOH, and the presence of nitrogen, the relative increase in carbon, and relative decrease in fluorine. Unlike the hydroxyl-functionalized surfaces where the differences among peptides were insignificant, the XPS and contact angle data implied a greater concentration of SIKVAV than GYIGSR and GRGDS incorporated onto the FEP-COOH surface. By comparing the nitrogen concentration for peptide-modified FEP-X films that were reduced under the two conditions, -78°C and 25°C, a comparison of the relative amount of peptide introduced to the surface could be made. The XPS data indicated that a greater amount of GYIGSR and SIKVAV were introduced to films reduced at 25°C than those reduced at -78°C. The data for GRGDS showed an insignificant difference for films reduced under these two reaction conditions.

Three reagents, tresyl, TSU and SMCC, were compared quantitatively by scintillation counting and XPS in the coupling of GYIGSR to hydroxyl- and carboxylic acid-functionalized surfaces. (A cysteine terminated peptide, i.e. CGYIGSR, was used for SMCC coupling.) As summarized in Table 2.3, tresyl-activated FEP-CH₃OH (reduced at 3 h and 25°C) showed the highest concentration of GYIGSR with 150 ± 60 fmol/cm². Tresyl-activated FEP-COOH resulted in a lower (<8 fmol/cm²) coupling yield, likely as a result of
fewer surface carboxylic acids relative to surface hydroxyl groups. With SMCC coupling, the concentration of GYIGSR bound to the surface was between 5 and 30 fmol/cm² whereas with TSU it was between 7 and 80 fmol/cm².

Table 2.3 A comparison of the surface peptide concentration on peptide modified FEP-X films (FEP-X-peptide, where X is either hydroxyl CH₂O or carboxylic acid COO) as determined from the γ-radiation count of radiolabeled GYIGSR peptide and the surface nitrogen to fluorine atomic ratio as determined from XPS data. The GYIGSR peptides were coupled to FEP-X using tresyl chloride, SMCC or TSU. The mean ± standard error of the mean are tabulated (n = 3 samples per film type).

<table>
<thead>
<tr>
<th>Coupling Agent</th>
<th>Peptide Concentration (fmol/cm²)</th>
<th>Nitrogen/Fluorine XPS Atomic Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEP-C reduction at:</td>
<td></td>
</tr>
<tr>
<td>FEP-CH₃O-GYIGSR</td>
<td>-78°C, 20 min 25°C, 180 min</td>
<td>6.6 ± 1.8 150 ± 60 0.06 ± 0.02 0.80 ± 0.07</td>
</tr>
<tr>
<td>FEP-COO-GYIGSR</td>
<td></td>
<td>7.9 ± 1.5 7.8 ± 1.9 0.03 ± 0.00 0.03 ± 0.01</td>
</tr>
<tr>
<td>SMCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEP-CH₃O-GYIGSR</td>
<td>-78°C, 20 min 25°C, 180 min</td>
<td>15 ± 13 32 ± 6 0.20 ± 0.08 0.50 ± 0.20</td>
</tr>
<tr>
<td>FEP-COO-GYIGSR</td>
<td></td>
<td>4.8 ± 1.8 6.0 ± 2.3 0.03 ± 0.00 0.04 ± 0.01</td>
</tr>
<tr>
<td>TSU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEP-CH₃O-GYIGSR</td>
<td>-78°C, 20 min 25°C, 180 min</td>
<td>11 ± 8.3 81 ± 20 0.07 ± 0.04 0.20 ± 0.10</td>
</tr>
<tr>
<td>FEP-COO-GYIGSR</td>
<td></td>
<td>7.0 ± 2.0 22 ± 15 0.03 ± 0.01 0.02 ± 0.01</td>
</tr>
</tbody>
</table>

The XPS data summarized as the nitrogen to fluorine surface concentration ratio in Table 2.3 indicate that the greatest amount of peptide was introduced with tresyl chloride coupling to the hydroxyl-functionalized surfaces, thereby confirming the data obtained from radiolabeling experiments. Together, the data obtained from radiolabeling experiments and XPS analysis indicate that both the surface functional group (i.e., hydroxyl vs. carboxylic acid) and the coupling agent affect the amount of peptide introduced. Perhaps the greatest contribution to the amount of peptide incorporated is the surface functional group, with the FEP-CH₃OH surface (prepared from FEP-C at 25°C) repeatedly having the most peptide incorporated. Of the three coupling agents, tresyl chloride seemed to be most effective at
coupling the peptide to the functionalized surface, followed by TSU and SMCC.

2.5.3 Hippocampal Neuron-Fluoropolymer Interaction

Hippocampal neurons isolated from embryonic day 18 rats were plated from serum-free neurobasal medium onto the peptide-modified film samples. After 1 and 4 days, the number of neurites extending per cell of 50 cells were counted, with the mean and standard error of the mean summarized in Figures 2.3. The positive control of PLL/laminin-coated glass coverslips had 2.44 and 2.80 neurites/cell after 1 and 4 days, respectively, while the unmodified FEP control had only 0.96 and 0.06 neurites/cell at the same time points. Since hippocampal neurons are anchorage-dependent cells, they cannot survive on FEP. The hydroxyl- and carboxylic acid-functionalized surfaces served as additional controls for the peptide-modified surfaces. For films reduced at -78°C, the peptides GYIGSR and GRGDS on FEP-CH₃OH showed greater interaction with the hippocampal neurons than did SIKVAV and underivatized FEP-CH₃OH at 1 day. By 4 days, the peptide-modified surfaces were indistinguishable from each other and the positive control, but were statistically significantly different (p<0.05) from the FEP-CH₃OH control. For films reduced at 25°C, none of the FEP-CH₃O-peptide films were distinguishable at 1 day; however, at 4 days, the greatest neural cell interaction was observed on GYIGSR- and SIKVAV-modified surfaces.

Figure 2.3 The number of neurites per cell (averaged over 50 cells) were compared using embryonic day 18 hippocampal neurons cultured in serum-free medium on PLL/laminin-coated glass coverslips, FEP and FEP-X-peptide (X = COO or CH₃O; peptide = GYIGSR, GRGDS, SIKVAV) surfaces after (a) 1 day and (b) 4 days of incubation. Surfaces were prepared from FEP-C by reducing FEP for either 20 min, -78°C or 3 h, 25°C. The mean number of neurites per cell with the standard error of the mean are reported for 50 cells, repeated on three separate times. *Statistically significant differences in data (calculated using one-way ANOVA with p < 0.05) with respect to FEP-X controls of the same condition.
Mean number of neurites per cell

- PLL/Laminin
- FEP
- FEP-CH₃OH
- FEP-COOH
- FEP-CH₃O-GYIGSR
- FEP-COO-GYIGSR
- FEP-CH₃O-GRGDS
- FEP-COO-GRGDS
- FEP-CH₃O-SIKVAV
- FEP-COO-SIKVAV

Mean number of neurites per cell

- PLL/Laminin
- FEP
- FEP-CH₃OH
- FEP-COOH
- FEP-CH₃O-GYIGSR
- FEP-COO-GYIGSR
- FEP-CH₃O-GRGDS
- FEP-COO-GRGDS
- FEP-CH₃O-SIKVAV
- FEP-COO-SIKVAV

20 min, 78°C
3 h, 25°C
For films reduced at -78°C, the peptide-modified carboxylic acid-functionalized film surfaces showed either the same or slightly less hippocampal neuron interaction than similar FEP-CH₃OH peptide-modified film surfaces. The peptide-modified FEP-COOH film surfaces were indistinguishable from each other and the control FEP-COOH at 1 day; however, after 4 days, the peptide-modified film surfaces were statistically different from FEP-COOH, yet indistinguishable from the positive control (PLL/laminin). For films reduced at 25°C, GYIGSR and GRGDS modified film surfaces showed the greatest hippocampal neuron interaction at 1 day; however, by 4 days, GYIGSR and SIKVAV showed the greatest interaction and were distinguishable from GRGDS and FEP-COOH control film samples. Interestingly, the number of neurites decreased between 1 and 4 days on all control FEP films samples (i.e., FEP, FEP-CH₃OH and FEP-COOH), whereas the number of neurites increased on all peptide-modified surfaces, indicating these surfaces enhance prolonged hippocampal neuron interaction.

Figure 2.4 The percentage of cells with neurites longer than one cell body length were compared for 50 embryonic day 18 hippocampal neurons cultured in serum-free medium on PLL/laminin-coated glass coverslips, FEP and FEP-X-peptide (X = COO or CH₃O; peptide = GYIGSR, GRGDS, SIKVAV) surfaces after (a) 1 day and (b) 4 days of plating. Surfaces were prepared from FEP-C by reducing FEP for either 20 min. at -78°C or 3 h at 25°C.
Since neurite length is indicative of the extent of interaction of the cell with the substrate, the functionalized surfaces were compared with control surfaces in terms of the length of the neurites, as shown in Figure 2.4. Of the 50 random cells counted, the number of cells having at least one neurite longer than the cell body was determined. FEP, FEP-CH$_{2}$OH and FEP-COOH controls had a lower percentage of cells with longer neurites than either GYIGSR or SIKVAV functionalized surfaces after 4 d, thereby further demonstrating the enhanced interaction of hippocampal neurons with peptide-functionalized surfaces over control surfaces. Figure 2.5 includes representative optical fluorescent micrographs taken at 4 days of FEP-X-peptide, FEP and PLL/laminin control surfaces. The relative number of viable to non-viable cells and the lengths of their neurites confirm the enhanced interaction on peptide-modified film samples. However, due to the variability in plating concentrations, the actual viabilities of hippocampal neurons were qualitatively followed but not quantified.
Figure 2.5 Optical micrographs of fluorescently stained hippocampal neurons cultured in serum-free media on various surfaces, after 4 days of incubation, demonstrate differences in the number of live cells and the length of their neurites: (a) PLL / laminin-coated glass coverslips, (b) unmodified FEP, (c) FEP-CH₃O-GYIGSR, (d) FEP-CH₃O-GRGDS, (e) FEP-CH₃O-SIKVAV. All FEP-CH₃O-peptide surfaces were prepared from FEP reduced for 20 min at -78°C. (scale 100 μm = 22 mm)
2.6 Discussion

The reduction of FEP to FEP-C followed by hydroboration/oxidation to FEP-CH_3OH or oxidation to FEP-COOH was used as a means to introduce peptides to the normally chemically inert FEP surfaces. FEP-C samples readily oxidized when exposed to air, requiring that care be taken to limit its contact with air.

The reduction of FEP to FEP-C was conducted under two reaction conditions to determine whether subsequent surface modification would be affected. The number of hydroxyl groups per FEP repeat units were estimated from XPS data; the ratio of OH : CF_2CF_2 repeat units was 1 : 1 and 1 : 0.1, and similarly, the ratio of COOH : FEP repeat units were estimated at 1 : 18 and 1 : 30 for samples reduced -78°C and 25°C, respectively. The greater percentage of hydroxyl per FEP repeat unit versus carboxylic acid per FEP repeat unit reflects the surface depletion of fluorine groups in the top ~10 Å of the FEP-CH_3OH film surfaces. The FEP-CH_xOH surfaces were labeled with trichloroacetyl chloride, producing the trichloroacetate FEP-CH_xO-C(O)CCl_3 to determine the percentage of oxygen functionality attributable to hydroxyl. Similarly, the FEP-COOH surfaces were labeled with thallium ethoxide, producing the thallium carboxylate salt FEP-COO-TI, to determine the percentage of oxygen functionality attributable to carboxylic acid (Reilley, 1982). The ratios of chlorine to oxygen determined via XPS allowed us to conclude that 33% and 47% of elemental oxygen was present as hydroxyl functionality for reductions at -78°C and 25°C, respectively. Similarly, from the ratios of thallium to oxygen, 42% and 74% of elemental oxygen was present as carboxylic acid for reductions at -78°C and 25°C, respectively.

The yields for the peptide coupling reactions were calculated relative to the number of hydroxyl and carboxylic acid functional groups present on FEP-CH_3OH and FEP-COOH,
respectively, as shown in Equation (2).

\[
\% \text{ Yield} = \frac{\# \text{ peptides}}{\# \text{ functional groups}} \times 100 \quad (2)
\]

where \# peptides was calculated from the XPS nitrogen composition, taking into account the number of nitrogen atoms per peptide, and the \# functional groups was calculated from the XPS oxygen composition after subtracting the oxygen composition associated with the peptide.

While there was some variability in the data, the yields for peptide couplings were consistently higher on FEP-COOH modified surfaces than FEP-CH\textsubscript{2}OH surfaces. For film surfaces reduced at -78°C, FEP-COO-peptide had an average yield of 35% (between 22 and 52%), whereas FEP-CH\textsubscript{2}O-peptide had an average yield of 7% (between 5 and 12%). Similarly, for film surfaces reduced at 25°C, FEP-COO-peptide had an average yield of 26% (between 15 and 51%) whereas FEP-CHO-peptide had an average yield of 8% (between 2 and 25%). The yields for surfaces modified at -78°C were consistently higher than those modified at 25°C, indicating that the chemistry was more easily controlled at the lower time and temperature.

Although the yields are higher on FEP-COO-peptide coupled surfaces than on FEP-CH\textsubscript{2}O-peptide surfaces, the amount of peptide incorporated on FEP-CH\textsubscript{2}OH surfaces is greater as shown by the radiolabeled GYIGSR data (cf. Table 2.3). The amount of peptide covalently bound to FEP-functionalized surfaces falls within the range previously reported for peptide-surface modification of hydroxyl-functionalized poly(ethylene terephthalate) and hydroxyl-functionalized poly(tetrafluoroethylene) (Massia, 1991).

Peptide-modified surfaces were prepared in an attempt to mimic the interaction normally observed between neurons and laminin to determine whether the peptide-
functionalized surfaces could serve as replacement substrates in CNS injury repair strategies. Hippocampal neurons were plated on peptide-modified and control film samples to assess the impact of the peptides on CNS neuron interaction. By using E18 mice, a relatively homogeneous population of neurons were dissociated (Bottenstein, 1980, Goslin, 1991). Fluorodeoxyuridine/uridine was added to the medium to inhibit the proliferation of any mitotically active cells such as astrocytes that were plated with the neurons. In an attempt to determine an optimal surface for further investigation with CNS neurons, two reduced surfaces (i.e., -78°C and 25°C), two reactive handles (i.e., hydroxyl and carboxylic acid functionalities) and three peptides (i.e., GYIGSR, GRGDS and SIKVAV) were investigated in terms of the hippocampal neuron interaction. As was expected, unmodified FEP supported neither cell adhesion nor neurite outgrowth. Perhaps unexpectedly, FEP-CHxOH and FEP-COOH both supported cell adhesion and neurite outgrowth after 1 day; however, the number of neurites decreased after 4 days. Only FEP, FEP-CHxOH and FEP-COOH control film surfaces showed a decrease in the number of neurites over time, indicating the importance of the peptide presence on these modified surfaces.

For all peptide-modified surfaces, the hippocampal neuron interaction was either greater or equivalent on FEP-CHxO-peptide surfaces than the corresponding FEP-COO-peptide surfaces. This corresponds to the radiolabeled data that show more peptide on FEP-CHxOH than on FEP-COOH surfaces. FEP-CHxO-peptide surfaces that had been prepared from FEP-C at -78°C showed greater than or equivalent hippocampal neuron interaction to those prepared from FEP-C at 25°C. While it was difficult to differentiate between GYIGSR, GRGDS and SIKVAV-functionalized surfaces, GYIGSR and GRGDS showed greater interaction than SIKVAV after 1 day of plating which may indicate that SIKVAV is less
adhesive to neurons than the other peptides. While SIKVAV-modified surfaces were expected to provide the greatest neurite interaction (i.e. number and length of neurites), this effect was not observed until 4 days after plating. The representative optical micrographs included in Figure 2.5 demonstrate the qualitative differences observed between GYIGSR, GRGDS and SIKVAV surfaces, where GYIGSR had the greatest number of adherent cells and SIKVAV had the greatest proportion of longer neurites. The hippocampal neurons responded equivalently to PLL-laminin and all peptide-modified surfaces alike, indicating that the peptide-modified surfaces sufficiently mimic the interaction of laminin and hippocampal neurons.

2.7 Conclusions

We have demonstrated that chemical surface modification of fluoropolymers, specifically FEP, could be used to control the concentration of cell adhesive peptides introduced without affecting the surface topography. By isolating surface chemistry from surface morphology, the relative importance of surface functionality on the hippocampal neuron cellular response was assessed. Of all the surfaces analyzed, the greatest concentration of peptide was achieved with tresyl chloride activation of hydroxyl-functionalized FEP that had been reduced at 25°C for 3 h. After 4 days of incubation with hippocampal neurons, the peptide-functionalized surfaces were indistinguishable from each other and the positive PLL/laminin control yet distinguishable from the controls – FEP, FEP-CH₄OH and FEP-COOH – thereby underlying the importance of substrate adhesion molecules for an enhanced cellular response. Despite the differences in surface peptide composition and concentration, the hippocampal response was not easily differentiated.
While initially unexpected, this result agrees with recent findings by Condic and Letourneau (Condic, 1997) who demonstrated that for neurons, total integrin expression decreases with decreased ligand (i.e. laminin) availability, yet surface integrin expression increases. Thus, despite a lower concentration of available ligands, neuronal cell adhesion and neurite outgrowth increase. To better understand the differences between YIGSR-, IKVAV- and RGD-functionalized surfaces in terms of the hippocampal neuron response, future studies will probe cell adhesion and total integrin expression. To further enhance the cellular response, mixed peptide surfaces will be prepared and compared to monofunctionalized peptide and PLL/laminin surfaces in terms of cell adhesion, neurite outgrowth and total integrin expression.

The authors gratefully thank Professor Peter Pennefather for supplying the hippocampal neurons and for related discussions. The authors gratefully acknowledge the financial support provided by the Natural Sciences and Engineering Research Council of Canada and the University of Toronto.

2.8 References


Chapter 3. Defining the Surface Chemistry of Ammonia-modified Poly(tetrafluoroethylene-co-hexafluoropropylene) Films*

3.1 Overview

In Chapter 2, an initial study of hippocampal neurons cultured on FEP surfaces modified with cell-adhesive peptides proved that their interaction, specifically neurite outgrowth, was enhanced in a manner comparable to surfaces coated with PLL and laminin. Cell-adhesive peptides were coupled to oxygen functional groups created on FEP. This well-known technique of surface modifying FEP with sodium naphthalide is highly reactive and toxic. Thus, in this chapter, a novel method of functionalizing FEP with nitrogen functional groups was investigated. Using a simple one-step chemistry with only ammonia, a drop of mercury and ultra-violet (UV) irradiation, FEP was surface modified and the surface chemistry was defined using various techniques. Methods to increase the concentration of reactive functional groups such as amines and carboxylic acids were also investigated in this chapter.

3.2 Abstract

Poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) film samples were surface-modified by exposure to a UV-activated mercury/ammonia environment (called the "mercat reaction"), yielding a hydrophilic surface with a diversity of functional groups. We describe herein the functionality of mercat-modified FEP and ultimately hope to use these defined surfaces in biomedical applications, specifically to enhance the interaction of cells at the FEP interface. Following the mercat reaction, advancing and receding water contact angles decreased from 120°/101° for FEP to 71°/27° for mercat-modified FEP. By comparison to small molecule chemistry, we had expected the film samples to have imine and nitrile functional groups; however, XPS analysis of mercat-modified film samples indicated the presence of both nitrogen and oxygen functional groups. From a series of labeling reactions, the surface functional groups were found to include nitrile, imine, amine, carboxylic acid and hydroxyl and carbon-carbon double bonds. Hydrolysis of nitrile functional groups resulted in an increased carboxylic acid concentration from 10% to 38% and decreased water contact angles from 71°/27° to 61°/30°. Bromination/amination of carbon-carbon double bonds resulted in an increased amine concentration from 8% to 10% and decreased water contact angles from 71°/27° to 54°/24°. Future studies will correlate surface chemistry of these defined mercat-modified FEP film samples with neuronal cellular response.
3.3 Introduction

Surface chemistry and topography define a material’s interfacial properties, influencing the way in which a material interacts with others. Since cells are known to respond to both surface chemistry and morphology (Goodman, 1995, Massia, 1992) we are particularly interested in modifying surface chemistry without affecting surface roughness. By controlling surface chemistry alone, we aim to correlate a specific functionality with a cellular response.

Polymer surface modification has been studied for numerous years, and several techniques have been used to change the interfacial properties while maintaining those of the bulk (Bee, 1993). Fluoropolymers are particularly advantageous, allowing discrete interfaces to be prepared with specific functionality, such as carboxylic acid, hydroxyl, amine, etc. (McCarthy, 1995). Small molecule organic chemistry can be used to prepare well-defined fluoropolymer surface chemistries yet require highly reactive species to effect a reaction (Dias, 1984, Tong, 1998a). Defined plasma systems can also be used to modify surfaces, yet often result in the introduction of multiple functional groups (Garfinkle, 1984, Vargo, 1991). Recently, we demonstrated that fluoropolymer films could be modified in a UV-activated mercury-ammonia environment (called the mercat reaction) to introduce imine functionality (Tong, 1998b). The modified surface, while not as functionally uniform as those prepared by chemical modification, was relatively easy to prepare, hydrophilic and topographically smooth.
Figure 3.1 Proposed mechanism for the introduction of reactive functional groups to the surface of poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) by a mercury/ammonia UV-photosensitization reaction (Burdeniuc, 1995) to yield FEP-[N/O].

We describe herein the chemical functional groups of poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) film surfaces prepared by the mercat reaction which has been used for small molecules and poly(tetrafluoroethylene) (Burdeniuc, 1995). According to published literature for small molecules (Burdeniuc, 1995), and as shown in Figure 3.1 for FEP, we anticipated that nitrile and imine functional groups would be introduced to the FEP surface. We took advantage of the presence of these functional groups, oxidizing the nitrile to carboxylic acid and brominating/aminating the imine carbon-carbon double bonds to amine. These series of reactions allowed us to increase the number of reactive functional groups available for further modification with, for example, cell adhesive peptides. While the mechanism does not account for oxygen functionality, we found that mercat-modified FEP
film surfaces (FEP-[N]/[O]) were complicated, containing both nitrogen and oxygen functionalities. We gained insight into the types of functional groups present from a series of labeling reactions that we analyzed by X-ray photoelectron spectroscopy (XPS) and dynamic water contact angles. Figure 3.2 summarizes the series of reactions that were carried out on FEP-[N/O] film samples.
Figure 3.2 FEP-[N/O] film surfaces were labeled to determine the relative concentrations of reactive functional groups and then further modified to increase the surface concentration of carboxylic acid and amine functional groups.
3.4 Experimental

All chemicals were purchased from Aldrich (Milwaukee, WI) and used as received unless otherwise indicated. Tetrahydrofuran (THF, Fisher, Nepean, ON) was distilled from sodium benzophenone dianion and stored under nitrogen. FEP films (5 mil thickness, received from DuPont and cut into 4 cm x 2 cm samples) were Soxhlet-extracted in THF for 24 h. All water was deionized and distilled from Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) systems and used at 18 MΩ resistance. All reactions were carried out in a dry, inert argon atmosphere unless otherwise specified. All reagents were transferred by either cannula or syringe.

XPS data were collected on a Leybold LH Max 200 using a polychromatic MgKα X-ray source at 15 kV and 20 mA emission current. An aperture size of 13 x 7 μm was used to collect data at takeoff angles of 20° and 90° between sample and detector (n = 3 samples). Unless otherwise specified, all of the data presented were taken at a 20° takeoff angle. Advancing and receding water contact angles were obtained on a Ramé-Hart NRL telescopic goniometer using a Gilmont syringe with a 22-gauge flat-tipped needle. Values reported represent the average and standard deviation of five measurements per sample (n = 3 samples). Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were obtained on a Mattson Galaxy 5400 spectrometer using a germanium crystal (45°). Scanning electron microscopy (SEM) micrographs of samples coated with gold for 15 s were taken on a Hitachi S4500 field emission microscope at 5 kV acceleration voltage.
3.4.1 Introduction of Functional Groups to FEP (FEP-[N/O])

FEP-[N/O] film samples were prepared as previously described (Tong, 1998b, Burdeniuc, 1995). Briefly, a quartz Schlenk tube, containing FEP film samples and a drop of mercury, was evacuated (P < 0.01 mm Hg) and purged with argon (three times). After the fourth evacuation, the tube was re-filled with gaseous ammonia (99.99% purity; BOC, Etobicoke, ON) to 1 atm pressure. The tube was placed in a UV reactor (Rayonet, Branford, CT) and the films were irradiated at 254 nm with eight 15 W mercury lamps for 24 h.

3.4.2 Labeling the Amine Groups of FEP-[N/O] with 2,3,5-Trichlorobenzaldehyde (FEP-[O]/N-CH-C₆H₂Cl₃)

FEP-[N/O] film samples were immersed in 20 mL of a 0.012 M 2,3,5-trichlorobenzaldehyde solution in THF containing 1 mL of pyridine for 24 h at room temperature (RT). Film samples were then rinsed three times each in THF, methanol and then dichloromethane prior to drying under vacuum (P < 0.01 mm Hg). Unmodified FEP film samples were treated identically, thereby serving as controls.

3.4.3 Labeling the Amine and Hydroxyl Groups of FEP-[N/O] with Trichloroacetyl Chloride (FEP-NH-CO-CCl₃/O-CO-CCl₃)

FEP-[N/O] film samples were immersed in 20 mL of a 9 x 10⁻⁵ M trichloroacetyl chloride solution in THF containing 1 mL of pyridine for 24 h at RT. Film samples were rinsed three times each in THF, methanol and then dichloromethane prior to drying under vacuum (P < 0.01 mm Hg). Unmodified FEP film samples were treated identically, thereby serving as controls.
3.4.4 Labeling the Amine Group of FEP-[N/O] with Pentafluorobenzaldehyde and then the Hydroxyl Group of FEP-[N/O] with Trichloroacetyl Chloride (FEP-N-CH-C\textsubscript{6}F\textsubscript{5}/O-CO-CCl\textsubscript{3})

FEP-[N/O] film samples were immersed in 20 mL of a 5 x 10\textsuperscript{-5} M pentafluorobenzaldehyde solution in THF with 1 mL of pyridine for 24 h at RT. FEP-N-CH-C\textsubscript{6}F\textsubscript{5} film samples were then immersed in 20 mL of a 9 x 10\textsuperscript{-5} M trichloroacetyl chloride solution in THF with 1 mL of pyridine for 24 h at RT. The film samples were rinsed three times each in THF, methanol and then dichloromethane prior to drying under vacuum (P < 0.01 mm Hg). Unmodified FEP film samples were treated identically, thereby serving as controls.

3.4.5 Labeling the Carboxylic Acid Groups of FEP-[N/O] with Thallous Ethoxide (FEP-[N]/COOTI) (Shoichet, 1991)

FEP-[N/O] film samples were immersed in 10 mL of thallous ethoxide for 60 s at RT and then rinsed six times with ethanol prior to drying under vacuum (P < 0.01 mm Hg). Unmodified FEP film samples were treated identically, thereby serving as controls.

3.4.6 Hydrolysis of the Nitrile Groups of FEP-[N/O] (FEP-COOH) (Shoichet, 1994)

FEP-[N/O] film samples were immersed in 10 mL of concentrated HCl for 48 h at RT and then washed five times with water. Subsequently, the film samples were immersed in 10 mL of a 10 M NaOH solution for 48 h at RT and then rinsed five times each in water, 5\% of a 10 M oxalic acid solution, water, and then 95\% ethanol prior to drying under vacuum (P < 0.01 mm Hg). FEP-COOH film samples were labeled with thallous ethoxide (FEP-COOTI)
as described above for FEP-[N/O] film samples, with the latter serving as controls for the labeling of FEP-COOH.

3.4.7 Bromination of the Carbon-Carbon Double Bonds of FEP-[N/O] (FEP-[N/O]-Br) (Costello, 1987)

FEP-[N/O] film samples were immersed in 20 mL of a 0.2 M bromine solution in dichloromethane for 24 h at 0°C in the dark. The film samples were then sequentially rinsed five times each with dichloromethane, methanol and THF and dried under vacuum (P < 0.01 mm Hg).

3.4.8 Amination of Brominated FEP-[N/O]-Br (FEP-NH₂)

10 mL of ammonia was condensed onto sodium at -78°C and then distilled into a Schlenk tube containing FEP-[N/O]-Br film samples that were equilibrated at -196°C. The tube was allowed to warm to RT and the reaction proceeded for 24 h after which the film samples were sequentially washed three times each with methanol, dichloromethane and THF prior to drying under vacuum (P < 0.01 mm Hg). FEP-NH₂ film samples were labeled with 2,3,5-trichlorobenzaldehyde (FEP-N-CH-C₆H₂Cl₃) and trichloroacetyl chloride (FEP-NH-CO-CCl₃) as previously described for FEP-[N/O] film samples, with the latter serving as controls for the labeling of FEP-NH₂.
3.5 Results and Discussion

3.5.1 Introduction of Surface Functional Groups to FEP (FEP-[N/O])

FEP film samples were modified by the mercat reaction to introduce surface reactive functional groups. Since we previously showed that FEP films modified by the mercat reaction for 3, 24 and 72 h showed similar properties after 24 h (Tong, 1998b), all of the reactions described herein were based on 24 h-modified film surfaces. As was shown in Figure 3.1 (and as adapted from Burdeniuc et al.), the activated mercury-ammonia system led to the introduction of nitrile and imine functional groups. However, as shown in Table 3.1, the XPS data indicated the presence of both nitrogen and oxygen functional groups in the FEP-[N/O] film samples, thereby indicating a more complex surface chemistry than that accounted for by the proposed reaction scheme. The oxygen functional groups may have resulted from (1) air oxidation of nitrile or unsaturated carbon-carbon double bonds and/or (2) a non-specific reaction during (or after) the mercat reaction between the UV-activated surface and trace oxygen or water. However, control experiments where FEP film samples were irradiated similarly in the absence of ammonia or mercury did not show any modifications to the sample, with XPS composition (C$_{33.1}$F$_{66.9}$) similar to non-irradiated FEP.

After the 24 h mercat reaction, the surface was defluorinated while nitrogen, oxygen and carbon atomic concentrations increased (C$_{70.8}$F$_{5.6}$O$_{9.2}$N$_{14.3}$). Consequently, both the advancing and receding contact angles decreased from 120°/101° to 71°/27°, reflecting the increased hydrophilic nature of FEP-[N/O] film samples. SEM micrographs of FEP and FEP-[N/O] film samples were identical and smooth, having no topographical features. AFM images of FEP-[N/O] showed the surface roughness with root mean square value less than 20 nm. Thus the large hysteresis between advancing and receding contact angles likely reflected
the chemical heterogeneity of FEP-[N/O] film samples as opposed to surface roughness. The
ATR-FTIR spectrum of FEP had the characteristic fluoropolymer peaks with $\nu_{C-F}$ at 1210 cm$^{-1}$
and 1153 cm$^{-1}$. After the mercat reaction, additional small peaks were observed for $\nu_{N-H}$ at
3500 cm$^{-1}$, $\nu_{C=O}$ at 1780 cm$^{-1}$, and $\nu_{C=O}$ at 2420 cm$^{-1}$.

Table 3.1 Introduction and labeling of reactive functional groups of FEP-[N/O].

<table>
<thead>
<tr>
<th>Film Sample</th>
<th>C</th>
<th>F</th>
<th>O</th>
<th>N</th>
<th>Cl/TI</th>
<th>$\theta_A(\degree)$</th>
<th>$\theta_B(\degree)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEP</td>
<td>31.1</td>
<td>68.9</td>
<td>0</td>
<td></td>
<td></td>
<td>120±2</td>
<td>101±2</td>
</tr>
<tr>
<td>FEP-[N/O]</td>
<td>70.8</td>
<td>5.6</td>
<td>9.2</td>
<td>14.3</td>
<td></td>
<td>71±3</td>
<td>27±2</td>
</tr>
<tr>
<td>FEP-[O]-N-CH-C$_6$H$_2$Cl$_3$</td>
<td>68.4</td>
<td>10.7</td>
<td>13.0</td>
<td>6.9</td>
<td>1.0</td>
<td>64±2</td>
<td>24±1</td>
</tr>
<tr>
<td>FEP-NH-CO-CCl$_3$/O-CO-CCl$_3$</td>
<td>68.8</td>
<td>3.6</td>
<td>11.5</td>
<td>9.5</td>
<td>6.6</td>
<td>73±1</td>
<td>34±2</td>
</tr>
<tr>
<td>FEP-N-CH-C$_6$F$_3$/O-CO-CCl$_3$</td>
<td>57.6</td>
<td>12.1</td>
<td>18.9</td>
<td>6.6</td>
<td>4.8</td>
<td>69±2</td>
<td>30±3</td>
</tr>
<tr>
<td>FEP-[N]/COOTi</td>
<td>76.8</td>
<td>3.5</td>
<td>9.5</td>
<td>9.7</td>
<td>0.5</td>
<td>71±1</td>
<td>29±2</td>
</tr>
</tbody>
</table>

3.5.2 Identifying the Surface Functional Groups of FEP-[N/O]

In order to understand the complex surface chemistry resulting from the mercat
reaction, the available functional groups were labeled or further modified, as was shown in
Figure 3.2. The XPS and contact angle data for the labeling reactions are summarized in
Table 3.1. Amine functional groups present on the FEP-[N/O] film samples were labeled
with 2,3,5-trichlorobenzaldehyde to yield FEP-[O]/N-CH-C$_6$H$_2$Cl$_3$ ($C_{68.4}F_{10.7}O_{13.0}N_{6.9}Cl_{1.0}$).
The presence of chlorine, as detected by XPS, indicated the success of this reaction. While
the decrease in nitrogen atomic concentration may be explained by the presence of the bulky
phenyl ring, the increase in fluorine atomic concentration may suggest slight surface erosion
following modification.

Available amine and hydroxyl functional groups on FEP-[N/O] were labeled with
trichloroacetyl chloride, as evidenced by the presence of chlorine in the XPS data, to yield
FEP-NH-CO-CCl$_3$/O-CO-CCl$_3$ ($C_{68.8}F_{3.0}O_{11.5}N_{9.5}Cl_{6.6}$). The increase in oxygen and decrease
in nitrogen and fluorine concentrations relative to FEP-[N/O] further confirmed the success
of this reaction. As might be expected, the receding contact angle increased slightly, reflecting the relative hydrophobic nature of the ester and amide (73°/34°) of FEP-NH-CO-CCl₃/O-CO-CCl₃ to the hydroxyl and amine (71°/27°) of FEP-[N/O]. As a further gauge of the relative concentration of oxygen functionality present as hydroxyl, the amine groups were blocked by reaction with pentafluorobenzaldehyde, leaving only hydroxyl groups available for reaction with trichloroacetyl chloride. As expected, the resulting films, FEP-N-CH-C₆F₅/O-CO-CCl₃ (C₅₇.₆F₁₂.₁O₁₈.₉N₆.₆Cl₄.₈), had an XPS chlorine concentration less than FEP-N-CO-CCl₃/O-CO-CCl₃ film samples, indicating the success of these reactions. The increased fluorine and oxygen atomic concentrations in FEP-N-CH-C₆F₅/OCO-CCl₃ further confirmed this success.

Using the XPS data, the relative concentrations of surface functional groups were calculated assuming that (Batich, 1981, Everhart, 1981) (i) trichloroacetyl chloride reacted with all available hydroxyl and amine groups, (ii) trichlorobenzaldehyde and pentafluorobenzaldehyde reacted only and with all available amine groups, and (iii) that the labeling reactions were quantitative. For FEP-[N/O], 8 to 15% of the surface oxygen concentration was attributed to hydroxyl functional groups and 5 to 12% of the surface nitrogen concentration was attributed to amine functional groups.

To determine whether any of the remaining oxygen concentration could be attributed to carboxylic acid functional groups, FEP-[N/O] surfaces were labeled with thallous ethoxide. Assuming quantitative yields, we calculated that 10% of the surface oxygen existed as carboxylic acid groups on FEP-[N]/COOTl film surfaces. Since we are interested in using the FEP-[N/O] surfaces for further modification with peptides, we did not explore the range of additional possible oxygen functional groups present; however, we were interested in
determining whether the functional groups proposed in Figure 3.1 could be exploited to provide a surface with an increased number of functional groups for modification.

3.5.3 Increasing the Functionality of FEP-[N/O]: Hydrolysis and Bromination/Amination

As was described in the proposed mechanism for mercapt-modified FEP films (cf. Figure 3.1), FEP-[N/O] films contained both nitrile and imine functional groups. To both confirm the presence of these functional groups while at the same time increase the number of reactive handles available for further modification with peptides, nitrile groups were hydrolyzed to carboxylic acids and carbon-carbon double bonds were brominated and then aminated to amines (cf. Figure 3.2). The XPS and contact angle data are summarized in Tables 3.2 and 3.3.

**Table 3.2 Hydrolysis of nitrile groups of FEP-[N/O] and labeling of carboxylic acid groups with thallous ethoxide.**

<table>
<thead>
<tr>
<th>Film Sample</th>
<th>C</th>
<th>F</th>
<th>O</th>
<th>N</th>
<th>Tl</th>
<th>θA(°)</th>
<th>θR(°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEP-[N/O]</td>
<td>70.8</td>
<td>5.6</td>
<td>9.2</td>
<td>14.3</td>
<td></td>
<td>71±3</td>
<td>27±2</td>
</tr>
<tr>
<td>FEP-COOH</td>
<td>71.1</td>
<td>9.8</td>
<td>13.7</td>
<td>5.3</td>
<td></td>
<td>61±3</td>
<td>30±2</td>
</tr>
<tr>
<td>FEP-COOTl</td>
<td>63.6</td>
<td>12.3</td>
<td>17.2</td>
<td>3.4</td>
<td>3.5</td>
<td>71±1</td>
<td>40±2</td>
</tr>
<tr>
<td>FEP-[N/O] + EtOTl (control)</td>
<td>76.8</td>
<td>3.5</td>
<td>9.5</td>
<td>9.7</td>
<td>0.5</td>
<td>71±1</td>
<td>29±2</td>
</tr>
</tbody>
</table>

The nitrile groups of FEP-[N/O] film samples were hydrolyzed by strong acid and strong base to carboxylic acids, yielding FEP-COOH (C_{71.1}F_{9.8}O_{13.7}N_{5.3}). As shown in Table 3.2, relative to FEP-[N/O], the XPS atomic concentration of nitrogen decreased while oxygen increased, indicating the success of this reaction. In addition, the advancing contact angle decreased, reflecting the increased hydrophilicity of carboxylic acid-modified surfaces (61°/30°) over those with nitrile groups. To further confirm the conversion of nitriles to
carboxylic acids. FEP-COOH films were labeled with thallous ethoxide, yielding FEP-COOTl, and compared to FEP-[N/O] films labeled similarly (FEP-[N]-COOTl). As expected, the XPS concentration of thallium on FEP-COOTl \((C_{63.6}F_{123.0}O_{17.2}N_{3.4}Tl_{3.5})\) films was much greater than controls (FEP-[N]-COOTl). From the XPS data, and assuming that all nitrile groups were hydrolyzed to carboxylic acids, the relative percent of oxygen functionality attributed to carboxylic acid groups was calculated to increase from 10% for FEP-[N/O] to 38% for FEP-COOH. Based on these data, 70% of the surface nitrogen concentration was attributed to nitrile functional groups.

### Table 3.3 Bromination/amination of carbon-carbon double bonds of FEP-[N/O] and labeling of amine groups with either 2,3,5-trichlorobenzaldehyde or trichloroacetyl chloride.

<table>
<thead>
<tr>
<th>Film Sample</th>
<th>C</th>
<th>F</th>
<th>O</th>
<th>N</th>
<th>Cl/Br</th>
<th>(\theta_A(\degree))</th>
<th>(\theta_B(\degree))</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEP-[N/O]</td>
<td>70.8</td>
<td>5.6</td>
<td>9.2</td>
<td>14.3</td>
<td></td>
<td>71±3</td>
<td>27±2</td>
</tr>
<tr>
<td>FEP-[N/O]-Br</td>
<td>67.7</td>
<td>2.9</td>
<td>11.3</td>
<td>9.4</td>
<td>8.7</td>
<td>60±3</td>
<td>33±1</td>
</tr>
<tr>
<td>FEP-NH₂</td>
<td>69.4</td>
<td>2.9</td>
<td>16.0</td>
<td>11.7</td>
<td></td>
<td>54±3</td>
<td>24±2</td>
</tr>
<tr>
<td>FEP-N-CH-C₆H₂Cl₃</td>
<td>71.3</td>
<td>1.9</td>
<td>18.4</td>
<td>6.9</td>
<td>1.5</td>
<td>75±1</td>
<td>30±2</td>
</tr>
<tr>
<td>FEP-[N/O] + OHC-C₆H₂Cl₃ (control)</td>
<td>68.4</td>
<td>10.7</td>
<td>13.0</td>
<td>6.9</td>
<td>1.0</td>
<td>64±2</td>
<td>24±1</td>
</tr>
<tr>
<td>FEP-NH-COCCl₃</td>
<td>67.8</td>
<td>3.6</td>
<td>10.2</td>
<td>9.1</td>
<td>9.3</td>
<td>69±2</td>
<td>23±1</td>
</tr>
<tr>
<td>FEP-[N/O] + ClCOCCl₃ (control)</td>
<td>68.8</td>
<td>3.6</td>
<td>11.5</td>
<td>9.5</td>
<td>6.6</td>
<td>73±1</td>
<td>34±2</td>
</tr>
</tbody>
</table>

The carbon-carbon double bonds of FEP-[N/O] film samples were brominated by the reaction with bromine dissolved in dichloromethane to FEP-[N/O]-Br \((C_{67.7}F_{2.9}O_{11.3}N_{9.4}Br_{8.7})\) and then aminated by the reaction with ammonia to FEP-NH₂ \((C_{69.4}F_{2.9}O_{16.0}N_{11.7})\). Following the success of the bromination/amination reactions was facilitated by first the presence and then the absence of bromine in the XPS spectrum. Compared to brominated films, aminated films also had a higher concentration of nitrogen. On the basis of XPS data, the concentration of carbon present as carbon-carbon double bonds was estimated at 6%. As may be expected,
the advancing contact angle decreased following bromination (60°/33°) and then further decreased following amination (54°/24°).

To confirm that FEP-NH₂ film samples had more amine groups than FEP-[N/O] control film samples, both were labeled with either 2,3,5-trichlorobenzaldehyde or trichloroacetyl chloride. As shown in Table 3.3 for the reaction with 2,3,5-trichlorobenzaldehyde, FEP-N-CH-C₆H₂Cl₃ (C₇₁.₃F₁₈.₉O₁₈.₄N₆.₉Cl₁₃) had slightly more chlorine and carbon and less fluorine than control film samples. In addition, the contact angles of FEP-N-CH-C₆H₂Cl₃ increased (75°/30°) relative to FEP-NH₂, reflecting the increased hydrophobicity of benzaldehyde-labeled amines vs. unlabeled amines (54°/24°).

For the reaction with trichloroacetyl chloride, FEP-NH-CO-CCl₃ film samples (C₆₇.₂F₃.₆O₁₀.₂N₉.₁Cl₉.₃) had a greater concentration of chlorine than FEP-[N/O] control film samples labeled similarly, thereby further confirming the increase in amine functional groups of FEP-NH₂ vs. FEP-[N/O]. Relative to brominated/aminated FEP-NH₂ film samples, trichloroacetyl chloride-labeled FEP-NH-COCCl₃ film samples had a higher advancing contact angle (69°/23°), indicating a more hydrophobic surface, as may be expected.

Assuming that the concentration of hydroxyl groups was unaffected by the bromination/amination reactions, we calculated that the number of surface amine groups increased from ~8% for FEP-[N/O] to ~10% for FEP-NH₂ based on the XPS data.

3.6 Conclusions

FEP film surfaces were modified by the facile mercapt reaction introducing predominantly nitrile, imine, carboxylic acid and hydroxyl functional groups. We used a series of labeling reactions to determine the relative percentages of reactive functional groups.
and modified the existing functional groups to increase the surface chemical reactivity. These reactions indicated that of the nitrogen functional groups, 8% were attributed to amine and 70% to nitrile. On the basis of similar calculations of the oxygen functional groups, 8-15% were attributed to hydroxyl and 10% to carboxylic acid. Hydrolysis of the nitrile functional groups present on FEP-[N/O] surfaces resulted in an increased carboxylic acid concentration, accounting for 38% of the available oxygen. Bromination/amination of the imine carbon-carbon double bonds of FEP-[N/O], resulted in increased amine concentration, accounting for ~10% of the surface nitrogen functional groups. Relative to FEP-[N/O] film samples, both FEP-COOH and FEP-NH\textsubscript{2} film samples had higher concentrations of surface reactive functional groups available for further modification.

Future studies will take advantage of the increased reactivity of the FEP-COOH and FEP-NH\textsubscript{2} film surfaces by covalently bonding cell adhesive peptides derived from laminin. The peptide-modified films will be used in model systems of nerve regeneration to study the interaction of primary neurons of the central nervous system.

**Acknowledgment**

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**3.7 References**


Chapter 4. Enhancing the Interaction of Central Nervous System Neurons with Poly(tetrafluoroethylene-co-hexafluoropropylene) via a Novel Surface Amine-functionalization Reaction Followed by Peptide Modification

4.1 Overview

In Chapter 3, the chemistry of a novel and simple FEP surface modification method was studied. FEP surfaces were found to be functionalized with hydroxyl, carboxylic acid, amines, nitriles and carbon-carbon double bonds. In this chapter, these functional groups were used as reactive handles for the coupling of cell-adhesive peptides in a manner similar to that described in Chapter 2. The response of hippocampal neurons on these peptide-coupled surfaces was investigated and compared to surfaces coated with PLL and laminin to test the hypothesis that neuronal interaction can be controlled by the type of surface-coupled peptides.

4.2 Abstract

Poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) surfaces were modified with cell adhesive peptides, via a novel amination reaction, to enhance the neuron-substrate interaction. Amination of FEP surfaces was achieved by exposing FEP film samples to a UV-activated mercury/ammonia system for either 3 h or 24 h, yielding nitrogen compositions of 3.5% and 13.2%, respectively. By labeling the nitrogen functionality with trichlorobenzaldehyde, the surface amine compositions were calculated to be 14% and 4.3% for the 3 h and 24 h amination reactions, respectively. Three oligopeptide sequences derived from laminin (GYIGSR, GRGDS and SIKVAV) were coupled to the aminated FEP (FEP-[N/O]) surfaces and found to have almost identical surface concentrations as determined by XPS. Using radiolabeled GYIGSR, three coupling agents were compared and the concentration of peptide per surface area was calculated to be 3 and 6 fmoI/cm² for surfaces aminated for 3 h and 24 h, respectively, regardless of the coupling agent. The interaction of embryonic hippocampal neurons with the modified surfaces was compared to that with the positive poly(L-lysine)/laminin control in terms of number and length of extended neurites. After 1 day of incubation, neurite extension on the GYIGSR- and SIKVAV-coupled surfaces was similar to that on the positive control but significantly greater than that on FEP and FEP-[N/O] control surfaces. These peptide-coupled fluoropolymer surfaces enhance the neuron-fluoropolymer interaction, similar to that observed with PLL/laminin.
4.3 Introduction

Following injury, mature neurons of the central nervous system (CNS) will not regenerate spontaneously, but are capable of regenerating in an environment such as that provided by the peripheral nerve (Bunge, 1994, Cheng, 1996, David, 1981) or the embryonic CNS (Iwashita, 1994). The extracellular matrix (ECM), particularly laminin, mediates axonal elongation during development, providing contact adhesive cues and guidance to axonal growth cones. In an attempt to mimic the ECM found in the peripheral nerve (or during development), a series of laminin adhesive peptides were introduced to the surface of an amine-functionalized fluoropolymer and compared in terms of the response of primary CNS neurons.

Fluoropolymers, such as expanded poly(tetrafluoroethylene) ePTFE, enjoy widespread use as vascular grafts (Boyce, 1982) and dental implants (Ratner, 1993). Fluoropolymers are chemically inert and, consequently, few strategies exist to modify the surface and even fewer methods have been described to introduce nitrogen functionalities. For example, fluoropolymers have been modified by small molecule chemistry (Costello, 1984, Noh, 1997, Shoichet, 1991), plasma (Badey, 1994, Dekker, 1991, Vargo, 1991), or excimer laser processing (Ichinose, 1996) to introduce nitrogen functionalities, such as amine (Costello, 1984, Niino, 1996), hydrazide (Bening, 1990) and nitrile (Brennan, 1987). Recently, it was shown that tertiary fluorocarbons could be aminated using an activated mercury/ammonia system (Burdeniuc, 1995). We take advantage of this mercury/ammonia (“mercat”) reaction to introduce amine functionality to the surface of poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) which, unlike ePTFE, has the necessary tertiary carbon for successful amination. The surface amine groups on FEP serve
as reactive handles for coupling cell adhesive peptide sequences from laminin, thereby promoting cell interaction with the modified surfaces. The laminin amino acid sequences used to modify FEP-amine surfaces include Arg-Gly-Asp (RGD) (Grant, 1989, Pierschbacher, 1984), Tyr-Iso-Gly-Ser-Arg (YIGSR) (Graft, 1987) and Iso-Lys-Val-Ala-Val (IKVAV) (Jucker, 1991). It has been suggested that the amine functionality alone may act as a cell-adhesion mimic of glycosaminoglycan-binding domains (Hockberger, 1987, Massia, 1992). Previously, FEP that was peptide-modified via a radio frequency glow discharge (RFGD) oxygen functionalization technique (Vargo, 1995) required pre-treatment with albumin for an interaction with cell lines to be observed (Ranieri, 1995). Herein, the dual effect of amine- and peptide-functionalized surfaces is assessed in terms of the interaction of primary hippocampal neurons in serum-free conditions.

**Figure 4.1 Proposed mechanism for the introduction of amine functional groups to the surface of poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) by a mercury/ammonia UV-photosensitization reaction (Burdeniuc, 1995).**
FEP film samples were aminated by vapor phase mercury photosensitization with ammonia (Burdeniuc, 1995) for either 3 h or 24 h, as shown in Figure 4.1. The amine functionality was either quantified by an XPS labeling reaction, using 2,3,5-trichlorobenzaldehyde, or further modified with the laminin adhesive peptides, as shown in Figure 4.2. The surfaces were characterized in terms of relative hydrophilicity, atomic composition and chemical functionality. The hippocampal neuron-surface interaction was evaluated qualitatively, in terms of cell adhesion and viability, and quantitatively, in terms of number and length of extended neurites per cell body.

Figure 4.2 Amine functionalized poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP-[N/O]) film samples are either (a) coupled with cell adhesive peptides using either tresyl chloride, sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) or O-(N-succinimidyl)-N,N,N,N'-tetramethyluronium tetrafluoroborate (TSU); or (b) labeled with 2,3,5-trichlorobenzaldehyde.
4.4 Experimental

4.4.1 Materials

All chemicals were purchased from Aldrich (Milwaukee, WI) and used as received unless otherwise indicated. Tetrahydrofuran (THF, Fisher, Nepean, ON) was distilled from sodium benzophenone dianion and stored under nitrogen in Schlenk flasks. Hanks balanced salt solution (HBSS), neurobasal medium, B27 supplement and phosphate buffered saline (PBS) were sterile-filtered with 0.22 μm cellulose acetate filters (all from Gibco BRL, Burlington, ON). All oligopeptides were purchased from Vetrogen, ON and were used as received. FEP films (5 mil thickness, received from DuPont and cut into 2 cm x 2 cm samples) were Soxhlet-extracted in THF for 24 h. All water was deionized and distilled from Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) systems and used at 18 MΩ resistance.

X-ray photoelectron spectroscopy (XPS) data were collected of film samples within one week of modification on a Leybold LH Max 200 using a polychromatic MgKα X-ray source at 15 kV and 20 mA emission current. An aperture size of 13 x 7 μm was used to collect data at takeoff angles of 20° and 90° between sample and detector (n = 3 samples, 1 spot per sample). Unless otherwise specified, all the data presented were taken at a 20° takeoff angle. Advancing and receding water contact angles were obtained on a Ramé-Hart NRL telescopic goniometer. Values reported represent the average and standard deviation of 5 measurements per sample (n = 3 samples). Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of FEP film samples were obtained on a Mattson Galaxy 5400 spectrometer using a germanium crystal (45°). Scanning electron microscopy (SEM)
micrographs were taken on a Hitachi S4500 field emission microscope at 5 kV acceleration voltage. Iodine-125 radiolabeled tyrosine (Y) of GYIGSR was quantified using a scintillation counter (LKB Wallac 1282-802 Universal γ-Counter) with a 2 x 2 cm sodium iodide detector well of 80% efficiency. Embryonic mouse (CD1 Type, Charles River, ON) hippocampal neurons, that were cultured on all surfaces, were photographed under normal light or filters for fluorescently-stained cells at 20x magnification under an Axioverter 150 microscope.

4.4.2 Introduction of Nitrogen Functionality, FEP-[N/O]

FEP-NH₂ film samples were prepared using a technique similar to that described by Burdeniuc et al. (Burdeniuc, 1995). Briefly, a quartz Schlenk tube, containing FEP film samples and a drop of mercury, was evacuated ($P < 0.01 \text{ mm Hg}$) and purged with argon (three times). After the fourth evacuation, the tube was re-filled with gaseous ammonia (99.99% purity; BOC, Etobicoke, ON) to 1 atm pressure and the films were irradiated with eight 15 W mercury lamps (254 nm) in a UV photoreactor (Rayonet, Branford, CT) for either 3, 24 or 72 h.

4.4.3 Labeling FEP-[N/O], FEP-NH-CO-C₆H₂Cl₃

FEP-NH-CO-C₆H₂Cl₃ film samples were prepared by reacting FEP-[N/O] with 20 mL of a 0.012 M 2,3,5-trichlorobenzaldehyde solution in THF for 3 h and then rinsing three times each in THF, methanol and dichloromethane prior to drying under vacuum. Unmodified FEP film samples were treated identically, thereby serving as controls.
4.4.4 Peptide Coupling to Functionalized FEP, FEP-NH-peptide

Three peptides (GYIGSR, SIKVAV and GRGDS) were coupled to FEP-[N/O] film surfaces using trifluoroethanesulfonyl chloride (tresyl chloride). In addition to tresyl chloride, CGYIGSR was coupled to FEP-[N/O] film surfaces using O-(Nsuccinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TSU) or sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), as described below. Unmodified FEP film samples were treated identically and used as controls.

FEP-[N/O] film samples were immersed in a solution containing 200 μL of tresyl chloride in 1 mL pyridine/19 mL THF for 20 min at RT in a Schlenk flask under N₂. Film samples were then added to a beaker containing 10 mL of a 0.2 M pH 10 sodium carbonate-buffered solution and 0.1 μg/mL of peptide (i.e. GYIGSR, GRGDS or SIKVAV) for 24 h (Nilsson, 1981). The film samples were rinsed five times each with the buffer solution, dilute hydrochloric acid, water and THF prior to drying under vacuum.

FEP-[N/O] film samples were immersed in 20 mL of dimethyl formamide (DMF) containing 1.3 mM TSU and 1.3 mM diisopropylethylamine for 4 h at RT in a Schlenk flask under N₂. Film samples were then added to a beaker containing 10 mL of a 0.2 M pH 10 sodium carbonate-buffered solution containing 0.1 μg/mL of GYIGSR for 24 h (Drumheller, 1994). The film samples were rinsed five times each with the buffer solution, water and THF prior to drying under vacuum.

FEP-[N/O] were immersed in a beaker containing 30 mL of a 0.2 M sodium bicarbonate-buffered solution at pH 10 and containing 2 mg of SMCC. After 4 h, the activated film samples were immersed in a 0.2 M pH 10 buffered sodium bicarbonate solution containing 0.1 μg/mL of cysteine-terminated CGYIGSR for 24 h at RT (Mattson,
The film samples were rinsed five times each with the buffer solution, dilute hydrochloric acid, water and THF and then dried under vacuum.

4.4.5 Radioactive Labeling of Tyrosine

Tyrosine (Y) of GYIGSR was labeled as previously described (Drumheller, 1994) with radioactive iodine ($^{125}$I). Briefly, 2 mg of CGYIGSR was dissolved in 5 mL of a pH 11 buffer containing 20 mM sodium phosphate and 0.15 M sodium chloride and then reacted with 1 mCi of carrier-free Na$^{125}$I (ICN, Costa Mesa, CA) in the presence of Iodobeads (Pierce, Rockford, IL) for 15 min. Free iodide was removed by successive passes through columns packed with anion-exchange resin (Dowex 1-X8, Aldrich). The labeled peptides were coupled to FEP-[N/O] surfaces as described above. The FEP-NH-peptide films were also rinsed with 10 mM sodium iodide to desorb any trace I$^{-}$.125 before counting by scintillation.

4.4.6 Hippocampal Neuron-Fluoropolymer Interaction

FEP-NH-peptide films and controls (FEP and FEP-[N/O]) were immersed in 70% ethanol for 1 h and then rinsed four times with sterile distilled water before air-drying. Positive control surfaces were prepared by coating glass coverslips with 1 mL of an aqueous 1 mg/mL solution of poly(L-lysine) (PLL, Sigma, MW = 37,000 g/mol) for 24 h at 37°C and then with 10 μL of a 1 mg/mL aqueous solution of laminin (Gibco) for 2 h at 37°C. PLL/laminin-coated glass coverslips were rinsed with sterile, distilled water and then air-dried. Embryonic day 18 (E18) mouse hippocampal neurons were isolated, as previously described (Brewer, 1993), by dissociation with papain (Worthington Biochemical
Corporation) and DNase (Sigma) for 30 min and mechanical trituration in calcium-free HBSS. 1 mL of a hippocampal neuron suspension was then plated at 1x10^6 cells/mL (or 2.5x10^5 cells/cm²) in serum-free medium (SFM) on each film sample. The SFM consisted of 2 mL B27 supplement, 100 mg chicken egg albumin (Sigma), 10 mg pyruvic acid (Sigma), 1 mL glutamine (Sigma) and 1 mL penicillin/streptomycin (Gibco, 10,000 unit/mL and 10,000 μg/mL, respectively) in 100 mL of neurobasal medium. The anti-mitotic agent, fluorodeoxyuridine/uridine (Sigma), was added after 24 h. The cells were incubated at 37°C in 5% CO₂ for 4 days. The cell-material interaction was assessed after 1 and 4 days in terms of the number and relative length of extended neurites per cell body (n = 50 cells). Phase contrast micrographs were taken after 1 and 4 days. To assess cell viability at 4 days, samples were incubated for 30 min at 37°C with 150 μL of stock viability assay solution (20 μL ethidium homodimer and 5 μL calcein AM in 10 mL PBS; Molecular Probes, Eugene, OR) and examined under fluorescent filters of the optical microscope.

4.4.7 Statistics

Triplicate experimental data were subjected to statistical analysis using SAS (SAS Institute Inc., Cary, NC) on a SGI Challenge L Unix-based computer system. One-way ANOVA analysis assuming a constant variance and a 95% confidence interval was used to determine statistical differences of various data sets. Results are reported as the mean ± the standard error of the mean and statistically significantly different data sets are labeled with different letters.
4.5 Results and Discussion

4.5.1 Introduction of Nitrogen Functionality, FEP-[N/O]

The exposure of FEP film samples to mercury-activated ammonia (i.e. mercat reaction) resulted in the introduction of amine functional groups (FEP-[N/O]) as determined by XPS and contact angle. The surface composition of the top 10-40 Å was measured at a takeoff angle of 20° while that of the top 40-100 Å was measured at a takeoff angle of 90°. In Table 4.1, the 20° takeoff angle data is summarized. After a 3 h exposure, amination was evidenced by a decreased contact angle (84°/31°) relative to FEP (120°/101°) and by the presence of nitrogen in the XPS spectrum (C_{45.2}F_{46.4}O_{5.9}N_{3.5}). The increased hydrophilicity of FEP-[N/O] relative to FEP is reflected by the lower contact angles and the XPS composition having a higher nitrogen and oxygen and lower fluorine concentrations. After a 24 h exposure, the contact angle was further decreased (27°/22°) and the nitrogen concentration further increased (C_{72.5}F_{3.7}O_{10.6}N_{13.2}), relative to the 3 h exposure, indicating that the extent of amination can be controlled by exposure time. The reaction was self-limiting as amination did not increase at exposure times greater than 24 h. For example, after 72 h of exposure to the mercat reaction, aminated FEP film samples had similar properties to those aminated for only 24 h. After the 72 h exposure, the water contact angles were 31°/20° and the atomic composition at a 20° takeoff angle was C_{71.4}F_{4.5}O_{11.1}N_{13.0}. Scanning electron micrographs of FEP, FEP-[N/O]-24h and FEP-[N/O]-72h were identical and smooth, having no topographical features and indicating that surface chemistry was modified independently from surface morphology. Due to the similarities observed between FEP-[N/O]-24h and FEP-[N/O]-72h, further studies were conducted on FEP aminated for 3 h and 24 h only.
Table 4.1. Average contact angle and XPS data of surface modified FEP film samples (n = 3 samples). XPS data were taken at a takeoff angle of 20° between the sample and detector.

<table>
<thead>
<tr>
<th>Film Type</th>
<th>Contact Angle ($^\circ_{\theta_A}$ / $^\circ_{\theta_B}$)</th>
<th>XPS Atomic Composition (%)</th>
<th>Contact Angle ($^\circ_{\theta_A}$ / $^\circ_{\theta_B}$)</th>
<th>XPS Atomic Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clean FEP</td>
<td>120±2 / 101±2</td>
<td>C_{31.1}F_{68.3}</td>
<td>120±2 / 101±2</td>
<td>C_{31.1}F_{68.3}</td>
</tr>
<tr>
<td>FEP-[N/O]</td>
<td>84±2 / 31±2</td>
<td>C_{46.2}F_{64.4}O_{3.9}N_{3.5}</td>
<td>27±2 / 22±1</td>
<td>C_{72.5}F_{7.5}O_{10.6}N_{13.2}</td>
</tr>
<tr>
<td>FEP-NH-CO-C_{6}H_{2}Cl_{3}</td>
<td>86±2 / 46±2</td>
<td>C_{42.7}F_{49.0}O_{5.2}N_{2.5}Cl_{0.9}</td>
<td>66±3 / 20±1</td>
<td>C_{82.8}F_{13.2}O_{12.8}N_{10.6}Cl_{1.3}</td>
</tr>
<tr>
<td>FEP + C_{6}H_{12}Cl_{5}-CHO (control for labeling)</td>
<td>119±2 / 101±2</td>
<td>C_{12.7}F_{68}</td>
<td>119±2 / 101±2</td>
<td>C_{32.7}F_{68}</td>
</tr>
<tr>
<td>FEP-NH-GYIGSR</td>
<td>74±4 / 21±1</td>
<td>C_{51.8}F_{36.6}O_{7.7}N_{1.9}</td>
<td>56±2 / 20±3</td>
<td>C_{70.0}F_{6.5}O_{14.6}N_{9.5}</td>
</tr>
<tr>
<td>FEP-NH-GRGDS</td>
<td>77±3 / 22±2</td>
<td>C_{33.3}F_{35.8}O_{7.2}N_{1.7}</td>
<td>59±2 / 14±1</td>
<td>C_{71.8}F_{43.0}O_{14.7}N_{9.3}</td>
</tr>
<tr>
<td>FEP-NH-SIKVAV</td>
<td>71±1 / 18±2</td>
<td>C_{22.3}F_{36.8}O_{7.0}N_{3.9}</td>
<td>67±3 / 13±1</td>
<td>C_{60.7}F_{6.2}O_{12.2}N_{8.6}</td>
</tr>
</tbody>
</table>

In order to determine the surface selectivity of the amination reaction, angle resolved XPS was performed by comparing the 20° and 90° takeoff angle data. For a 3 h exposure, FEP-[N/O]-3h film samples had more nitrogen and less carbon and fluorine at a 20° takeoff angle ($C_{46.2}F_{64.4}O_{3.9}N_{3.5}$) than that at a 90° takeoff angle ($C_{41.4}F_{53.3}O_{3.1}N_{2.3}$), indicating the surface selectivity of this reaction. For a 24 h exposure, the FEP-[N/O]-24h film samples had an equivalent amount of fluorine and less nitrogen at the 20° takeoff angle ($C_{72.5}F_{7.5}O_{10.6}N_{13.2}$) than that at the 90° takeoff angle ($C_{65.2}F_{3.7}O_{10.8}N_{20.4}$), indicating that reactive ammonia penetrated below the FEP surface. Up to 24 h, the longer the exposure time, the deeper the modification. The increased nitrogen concentration at greater depths for FEP-[N/O]-24h reflects the solubility of ammonia in FEP. By comparing the 20° and 90° takeoff angle XPS data, it is clear that the surface of FEP-[N/O]-24h is enriched with carbon and depleted in nitrogen which may result from surface rearrangement of the functional groups to minimize surface free energy. While FEP-[N/O]-24h films were modified to a greater depth of modification than FEP-[N/O]-3h films, no peaks, other than those ascribed to unmodified FEP, were evident in the ATR-FTIR spectra of all film samples. This indicates
that the mercat reaction was limited to depths of modification significantly less than 1 μm (the sampling depth of ATR).

The presence of surface oxygen functionality, although not accounted for by the mechanism in Figure 4.1, likely results from air-oxidation of either or both the carbon-carbon double bonds or the nitrile group.

4.5.2 Labeling FEP-[N/O], FEP-NH-CO-C₆H₅Cl₃

The labeling reaction of FEP-[N/O] with trichlorobenzaldehyde (FEP-NH-COC₆H₅Cl₃) was used to estimate the concentration of surface primary and secondary amines that would be available for peptide modification. As shown in Table 4.1, relative to FEP-[N/O] (3 h or 24 h), FEP-NH-CO-C₆H₅Cl₃ surfaces were less hydrophilic, as expected, due to the phenyl ring. FEP-NH-3h-CO-C₆H₅Cl₃ surfaces had an increased receding contact angle whereas FEP-NH-24h-CO-C₆H₅Cl₃ surfaces had an increased advancing contact angle relative to FEP-[N/O]-3h or FEP-[N/O]-24h, respectively. The increased hysteresis between advancing and receding contact angles of FEP-NH-24h-CO-C₆H₅Cl₃ film surfaces reflects their chemical heterogeneity relative to that of FEP-NH-3h-CO-C₆H₅Cl₃ films. Since the control reaction of FEP with trichlorobenzaldehyde resulted in FEP (cf. Table 4.1 for XPS and contact angle data), we can assume that all of the chlorine results from the reaction of trichlorobenzaldehyde and primary and secondary amine groups of FEP-[N/O]. Assuming 100% yield for the amine labeling reaction with trichlorobenzaldehyde, the XPS atomic chlorine to nitrogen ratios can be used to calculate the minimum percentage of nitrogen present as primary and secondary amines. For example, for FEP-NH-3h-CO-C₆H₅Cl₃ films, the surface chlorine concentration was 0.9 at.% indicating that 0.3 at.% of the total 2.3 at.%
surface nitrogen concentration exists as primary or secondary amines. Thus 14% of the nitrogen functionality of FEP-[N/O]-3h is available for further modification with peptides. By a similar analysis, FEP-NH-24h-CO-C₆H₂Cl₃ films had 1.3 at.% chlorine and thus 0.43 at.% of the total 10.0 at.% (or 4.3%) surface nitrogen was available for further modification with laminin adhesive peptide sequences. The greater hysteresis in the contact angle data for FEP-NH-24h-CO-C₆H₂Cl₃ is consistent with the greater chemical heterogeneity observed. The relative number of surface amine groups to FEP repeat units was determined from both the 20° XPS fluorine to nitrogen data and the calculated amine concentrations. For FEP-[N/O]-3h, there was 1 amine group per 23 FEP repeat units and for FEP-[N/O]-24h, there was 1 amine group per 1.6 FEP repeat units.

Since a low percentage of nitrogen functionality was available for further chemical modification with peptides, methods will be developed to increase both their reactivity and the percent of surface amine groups. For example, based on the mechanism in Figure 4.1, the amine groups will be more reactive after reducing the carbon-carbon double bonds to single bonds. To further increase the number of amine groups, the nitrile group will be reduced to primary amine.

4.5.3 Peptide Coupling to Functionalized FEP, FEP-NH-peptide

FEP-[N/O] surfaces were modified with three cell adhesive peptides (GYIGSR, GRGDS, SIKVAV) using tresyl activation. For the FEP-[N/O]-3h films, the contact angles decreased after peptide modification reflecting the increased hydrophilic nature of the peptide group on FEP-NH-peptide relative to FEP-[N/O]-3h (cf. Table 4.1). The XPS data confirm the contact angle data: FEP-NH-peptide surfaces have depleted fluorine and
increased carbon, oxygen and nitrogen. For FEP-[N/O]-24h, the advancing contact angles increased while the receding contact angles decreased after peptide modification. The XPS data indicate that similar amounts of the three peptides GYIGSR, GRGDS and SIKVAV were introduced to the FEP-[N/O] film surfaces for each amination time (i.e. 3 h and 24 h); however, a greater concentration of peptides were introduced to FEP-NH2-24h than to FEP-[N/O]-3h. The increased hysteresis observed for FEP-NH-24h-peptide indicates a chemically heterogeneous surface and likely less peptide per nitrogen than that observed for FEP-NH-3h-peptide as confirmed by the labeling reaction. The XPS data confirm this observation with a surface enrichment of oxygen and fluorine after peptide modification for FEP-[N/O]-24h.

The increased fluorine concentration may reflect surface rearrangement, facilitated by the use of THF that slightly swells FEP.

Figure 4.3 The surface peptide concentrations of FEP-NH-GYIGSR films were determined by counting the γ-radiation of 1-125 radiolabeled tyrosine (Y) of GYIGSR. The radiolabeled GYIGSR peptide was coupled to FEP-[N/O] using tresyl chloride, SMCC or TSU. The mean with the respective standard error of the mean are plotted for films aminated for 3 h (■) and 24 h (□).
To determine which of the three coupling agents, tresyl chloride, SMCC and TSU, could yield the greatest amount of peptide per surface area, $^{125}$I radiolabeled tyrosine (Y) of CGYIGSR was coupled to FEP-[N/O] surfaces. As shown in Figure 4.3, the amount of peptide that was introduced to FEP-[N/O]-24h was greater than that introduced to FEP-[N/O]-3h for all coupling reagents; however, the differences between the three reagents were statistically indistinguishable within 95% confidence. Given the low amount of peptide introduced, the lack of differentiation among the coupling reagents may reflect the low surface concentration of reactive amine groups.

4.5.4 Hippocampal Neuron-Fluoropolymer Interaction

The peptide-functionalized surfaces were compared, in terms of the response of hippocampal neurons, to FEP-[N/O], FEP and a positive control, i.e. glass modified with PLL/laminin. The number of neurites extending per cell body (averaged over 50 cells) is summarized in Figure 4.4 after 1 day and 4 days of plating. In Figure 4.4, statistical differences in the data are designated with different letters. The biggest differences between the surfaces were observed after 1 day of plating. The response observed, from greatest to least, on the FEP-[N/O]-3h surfaces was FEP-NH-3h-GYIGSR ~ PLL/laminin ~ FEP-NH-3h-SIKVAV > FEP-NH-3h-GRGDS > FEP-[N/O]-3h ~ FEP. For FEP-[N/O]-24h, the order was FEP-NH-24h-GYIGSR ~ PLL/laminin > FEP-NH-24h-SIKVAV ~ FEP-NH-24h-GRGDS ~ FEP-[N/O]-24h > FEP. After 4 days of plating, the differences among the peptide-functionalized surfaces, FEP-NH$_2$ and PLL/laminin were difficult to elucidate; however, no cells were supported on FEP. While there was no statistical difference between PLL/laminin controls and GYIGSR- and SIKVAV-functionalized surfaces, PLL/laminin
supported statistically more axonal interaction than both the GRGDS- and [N/O]-
functionalized FEP surfaces.

Figure 4.4 The number of neurites extending per cell (averaged over 50 cells) of embryonic day 18 (E18) hippocampal neurons cultured in serum-free medium on PLL/laminin-coated glass coverslips, FEP and FEP-NH-peptide (peptide = GYIGSR, GRGDS, SIKVAV) surfaces after (a) 1 day and (b) 4 days of plating. Surfaces were prepared from FEP-[N/O] for 3 h vs. 24 h. The mean numbers of neurites per cell body with the standard error of the mean are reported for n = 50 cells, repeated twice. Data sets with a similar letter are statistically the same whereas those with different letters are statistically different from FEP-[N/O] controls of the same conditions. (The data were calculated using one-way ANOVA with $p < 0.05$)
In order to further gauge the cell-material interaction, the relative length of neurites on the modified surfaces was compared by calculating the percentage of cells having one or more neurites greater than the cell body length, as shown in Figure 4.5. After 1 day of plating, the response was greatest on FEP-NH-GYIGSR and least on FEP, with the other surfaces showing similar percentages of cells having long neurites between these two extremes. After 4 days of plating, all peptide-modified surfaces had similar percentages, with FEP-NH-GRGDS having the lowest response of the peptide surfaces. By using both number and length of neurites as indicators of the cellular response to the modified surfaces, it is
clear that of the three peptides studied, the GYIGSR-functionalized surfaces are the most conducive to cellular interaction while the GRGDS-functionalized surfaces are the least conducive. These results are consistent with other results we have obtained using different surface chemistry to achieve peptide immobilization (Tong, 1998). The phase contrast micrographs, included in Figure 4.6, represent the range of responses observed between the hippocampal neurons and the FEP surfaces. FEP supported neither cell adhesion nor neurite outgrowth (cf. Figure 4.6a). FEP-[N/O] supported both cell adhesion and neurite outgrowth. yet the large cell clusters indicate that the neurons prefer to grow on each other than on the surface (cf. Figure 4.6b). FEP-NH-GYIGSR supported both cell adhesion and neurite outgrowth; the enhanced interaction of neurons with this surface is represented by the distribution of cells (cf. Figure 4.6c).

Figure 4.5 The percentage of cells (n = 50 cells, for 2 film samples) with neurites longer than one cell body length, of embryonic day 18 (E18) hippocampal neurons cultured in serum-free medium, were compared on PLL/laminin-coated glass coverslips, FEP and FEP-NH-peptide (peptide = GYIGSR, GRGDS, SIKVAV) surfaces after (a) 1 day and (b) 4 days of plating. Films were aminated for either 3 h or 24 h.
4.6 Conclusions

FEP films were surface-modified with amine functional groups after exposure to the mercapt reaction for 3 h and 24 h, resulting in 14% and 4.3% of reactive amines, respectively. Despite the lower percentage of nitrogen present as amine groups for the modification at 24 h, more nitrogen was present on FEP-NH$_2$-24h than on FEP-NH$_2$-3h. Coupling the oligopeptide CGYIGSR with FEP-NH$_2$-3h and -24h surfaces resulted in surface peptide concentrations of 3 and 6 fmol/cm$^2$, respectively, using tresyl chloride, SMCC or TSU. The hippocampal neuron-material interaction was compared on the following surfaces: nitrogen-functionalized FEP, peptide-coupled films, including GYIGSR-, SIKVAV- and GRGDS-functionalized FEP, unmodified FEP (control) and PLL/laminin-coated glass coverslips (positive control). From the neurite extension and neurite length results, we conclude that the peptide-modified surfaces, and in particular FEP-NH-GYIGSR, enhanced the hippocampal neuron interaction and best mimicked the effects of PLL/laminin surfaces in vitro. These surface modification methods may impact strategies used to enhance nerve regeneration.
Figure 4.6 Phase contrast micrographs of hippocampal neurons plated on different functionalized surfaces after 1 day of incubation demonstrate the enhanced interaction after peptide modification: (a) PLL/laminin control, (b) FEP-[N/O]-24h, (c) FEP-NHG-YIGSR, (d) FEP-NH-SIKVAV, and (e) FEP-NH-GRGDS. (scale: 100 μm = 22 mm)

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4.7 References


Dekker, A., Reitsma, K., Beugeling, T., Bantjes, A., Feijen, J., and Van Aken, W.G.


Massia, S.P. and Hubbell, J.A. “Immobilized Amines and Basic Amino Acids as Mimetic Heparin-binding Domains for Cell Surface Proteoglycan-mediated Adhesion”, *J.*


Nilsson, K. and Mosbach, K. “Immobilization of Enzymes and Affinity Ligands to Various Hydroxyl Group Carrying Supports Using Highly Reactive Sulfonyl Chlorides”.


Tong, Y.W. and Shoichet, M.S. “Peptide surface modification of poly(tetrafluoroethylene-co-hexafluoropropylene) enhances its interaction with central


Chapter 5. Enhanced Neuronal Interaction on Surface Modified Fluoropolymers

5.1 Overview

In the previous chapters, FEP surfaces were shown to be modified with functional groups and subsequently coupled with cell-adhesive peptides in an attempt to enhance the response of neurons. Preliminary tests of cultured hippocampal cells on the peptide-modified surfaces have shown that neurite outgrowth was increased in a comparable manner as the positive control of surfaces coated with PLL and laminin. In this chapter, an in-depth study of the neuronal response on several different peptide-modified surfaces is presented. The specificity of the surface-bound peptides was tested using a competitive assay of peptides dissolved in the culture media. New methods of quantifying neuronal response were also developed as previous methods were found to inadequately distinguish between different surfaces.

5.2 Abstract

Embryonic hippocampal neurons cultured on surface modified fluoropolymers showed enhanced interaction and neurite extension. Poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) film surfaces were aminated by reaction with a UV-activated mercury ammonia system yielding FEP-[N/O]. Laminin-derived cell-adhesive peptides (YIGSR and IKVAV) were coupled to FEP surface functional groups using tresyl chloride activation. Embryonic (E18) hippocampal neurons cultured in serum-free media for up to a week on FEP film surfaces modified with one or both of GYIGSR and SIKVAV were compared to controls of unmodified FEP, FEP-[N/O] and poly(l-lysine)/laminin coated tissue culture dishes. Neuron-surface interactions were analyzed in terms of neurite outgrowth (number and length of neurites) and cell adhesion over time. Neurite outgrowth and adhesion was significantly better on peptide-modified surfaces than either FEP and FEP-[N/O]. Cells on the mixed peptide surface demonstrated similar behavior to those on the positive PLL/laminin control. The specificity of the cell-peptide interaction was studied with a competitive assay where dissociated neurons were incubated in media containing 0.1 mg/mL of GYIGSR and SIKVAV for 30 minutes prior to plating. Cell adhesion and neurite outgrowth diminished on all surfaces where hippocampal neurons were preincubated with dissolved peptides prior to plating.

5.3 Introduction

Central nervous system (CNS) neurons, unlike those of peripheral nervous system, do not regenerate spontaneously following injury; however, CNS neurons can regenerate in a peripheral nerve graft (David, 1981; Richardson, 1980) or in an environment that mimics that
of the peripheral nerve (Bunge, 1994). In an attempt to mimic the contact-mediated attractive cues to which axons respond, we have prepared surfaces with well-defined chemistry and topography using poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP) (Tong, 1998a, 1998b, 1999). Because FEP is chemically inert, functional groups can be introduced to defined depths with minimal or no effect on surface topography (Shoichet, 1991). We previously demonstrated that FEP surfaces modified with a UV-activated mercury ammonia system (the mercat reaction) resulted in heterogeneous functionalized surfaces (Tong, 1999) having amine, hydroxyl and carboxylic acid groups available for peptide coupling. We chose to modify these surfaces with two laminin-derived peptides that are known to enhance cell adhesion (YIGSR) (Yamada, 1992) and neurite outgrowth (IKVAV) (Jucker, 1991).

In previous studies, we demonstrated that hippocampal neurons responded to the peptide-modified surfaces in a similar manner to glass-coverslips coated with poly(L-lysine) (PLL) and laminin. We hypothesized that surfaces modified with both YIGSR and IKVAV would yield an even better cellular response. We also examined the effects of peptide mobility by introducing a spacer group between the peptide and the surface. In this paper, the spacer group used was a pentaglycine peptide, thus neuronal response of GYIGSR was compared to that of GGGGGGYIGSR. To this end, we focused on the cell-material interaction, going into greater depth of analysis on cell adhesion, viability, neurite number and length, and specificity of interaction.

5.4 Materials and Methods

All chemicals were purchased from Aldrich (Milwaukee, WI) and used as received unless otherwise indicated. Tetrahydrofuran (THF, Fisher, Nepean, ON) was distilled from
sodium benzophenone dianion and stored under nitrogen in Schlenk flasks. Neurobasal medium, B27 supplement and phosphate buffered saline (PBS) were sterile-filtered with 0.22 μm cellulose acetate filters (all from Gibco BRL, Burlington, ON). All peptides were purchased from Vetrogen (London, ON) and used as received. FEP films (5 mil thickness, received from DuPont and cut into 2 cm x 2 cm samples) were Soxhlet-extracted in THF for 24 h prior to use. All reactions were done under inert nitrogen atmosphere unless otherwise indicated. Deionized distilled water was obtained from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) and used at 18 MΩ resistance.

Embryonic rat hippocampal neurons (BrainBits, Springfield, IL) were obtained as undissociated brain tissue. Cultures on all surfaces were photographed under normal light or filters for fluorescently-stained cells at 20X magnification under an Axiovert S-100 inverted microscope (Zeiss, Germany) using a digital camera DC-130 (Sony, Japan) connected to a computer. Digital images were then analyzed using the Empix Imaging Northern Eclipse software (version 5.0, Toronto, ON) for neurite length, number and cell count.

5.4.1 Introduction of Nitrogen Functionality by the Mercat Method, FEP-[N/O]

FEP-[N/O] film samples were prepared as previously described (Tong, 1998b, 1999). Briefly, a quartz Schlenk tube, containing FEP film samples and a drop of mercury, was evacuated (P < 0.01 mm Hg) and purged with argon (3 times). After the fourth evacuation, the tube was re-filled with gaseous ammonia (99.99% purity, BOC, Toronto, ON) to 1 atm pressure and the films were irradiated with eight 15 W mercury lamps (254 nm) in a UV photoreactor (Rayonet, Branford, CT) for 24 h. The reacted samples were then washed three times each with THF, methanol and dichloromethane.
5.4.2 Peptide Coupling to Functionalized FEP, FEP-[N/O]-peptide

The peptides (GYIGSR, GGGGGGYIGSR and SIKVAV) were coupled to FEP-[N/O] film surfaces using the trifluoroethanesulfonyl chloride (tresyl chloride) coupling agent as previously described (Nilsson, 1981, Tong, 1998a). Briefly, FEP-[N/O] film samples were immersed in a solution containing 200 μL of tresyl chloride, 1 mL pyridine and 19 mL THF for 20 min at RT. Activated films were then transferred to a beaker containing 10 mL of a 0.2 M pH 10 sodium carbonate-buffered solution and 0.1 μg/mL of peptide for 24 h: either 100% GYIGSR (FEP-[N/O]-GYIGSR), 100% GGGGGGYIGSR (FEP-[N/O]-G3GYIGSR), 100% SIKVAV (FEP-[N/O]-SIKVAV), or 50% GYIGSR and 50% SIKVAV (FEP-[N/O]-Mix). The films samples were sequentially rinsed five times each with the pH 10 buffer solution, dilute hydrochloric acid, water and THF prior to drying under vacuum.

5.4.3 Hippocampal Neuron-Fluoropolymer Interaction

FEP-[N/O]-peptide films and controls (FEP and FEP-[N/O]) were immersed in 70% ethanol for 1 h and then rinsed four times with sterile water before air-drying. Positive control surfaces were prepared by coating 24-well tissue culture plates with 1 mL of an aqueous 1 mg/mL solution of poly(L-lysine) (PLL, Sigma, MW = 37,000 g/mol) for 24 h at 37°C and then with 10 μL of a 1 mg/mL aqueous solution of laminin (Gibco) for 2 h at 37°C. The PLL/laminin-coated wells were rinsed with sterile water and air-dried prior to plating the hippocampal neurons. Embryonic day 18 (E18) mouse hippocampal neurons were dissociated by incubating with papain (Sigma) for 30 min and mechanically triturated in serum-free media (SFM). 1 mL of the dissociated hippocampal neurons was then plated at 10^5 cells/mL in SFM on each film sample. After 3 h, the media was replaced with fresh SFM.
The SFM was made with 2 mL B27 supplement, 100 mg chicken egg albumin (Sigma), 10 mg pyruvic acid (Sigma), 1 mL glutamine (Sigma) and 1 mL penicillin/streptomycin (Gibco, 10,000 unit/mL and 10,000 µg/mL, respectively) in 100 mL neurobasal medium. The anti-mitotic agent, fluorodeoxyuridine/uridine (Sigma), was added after 24 h. The cells were incubated at 37°C in 5% CO₂ for up to a week.

To assess cell viability, samples were incubated for 30 min at 37°C with 150 µL of stock viability assay solution (20 µL ethidium homodimer and 5 µL calcein AM in 10 mL PBS, Molecular Probes, Eugene, OR) in fresh SFM. The cell-material interaction was assessed each day from day 1 to 4 in terms of the number and length of extended neurites, and the number of cells. At each time point, 50 cells were chosen at random and the number of neurites per cell longer than the cell body length, and the length of the neurites were quantified. The number of live neurons per field was also counted for 10 fields per sample (n=3).

5.4.4 Competitive Assay

To assess for the specificity of the cell-peptide interaction, dissociated neurons were incubated with 0.1 mg/mL of dissolved GYIGSR and SIKVAV in SFM for 30 min prior to plating on the surfaces. After 3 h, the media was replaced with fresh SFM (without peptides) and the cultures analyzed as described above.

5.4.5 Statistics

For every surface, on which 50 cells were evaluated, triplicate experimental data sets were subjected to statistical analysis using SigmaStat (Chicago, IL). One-way ANOVA
analysis assuming a constant variance and a 95% confidence interval was used to determine statistical differences of various data sets. Results are reported as the mean ± the standard error of the mean and statistically significantly differences are labeled with an asterix (*).

5.5 Results

FEP film samples were surface modified by the mercat reaction to yield a hydrophilic surfaces, composed of nitrogen and oxygen functional groups (Tong, 1999). These functional groups served as reactive handles to couple GYIGSR and SIKVAV peptides individually or together (1:1, w/w) yielding 3-6 fmol/cm² of peptide, as previously determined by radioactive labeling (Tong, 1999). The neuronal response to the modified surfaces was investigated by the number and length of neurites, and the number of adherent neurons on a given surface.

5.5.1 Length of Neurites

The average length of the longest neurite for 50 randomly chosen cells was measured by tracing the distance along the neurite using a calibrated image analysis program. As shown in Figure 5.1, neurite length increased steadily over time for all surfaces. While single peptide-modified surfaces of YIGSR or IKVAV alone had similar neurite lengths - from 40 μm to 87 μm, mixed peptide surfaces, containing both YIGSR and IKVAV, had significantly longer neurites - from 35 μm to 124 μm. The shortest neurites were observed on FEP-[N/O] control surfaces and the longest on PLL/laminin positive controls, where neurites increased from 61 μm to 190 μm. Interestingly, rate of neurite extension on the mixed peptide surface was the greatest, increasing over the 3 day period by 3.5 times, as compared to that on
PLL/laminin of 3.1 times. The effect of using a spacer group between the peptide and the surface can be observed in Figure 5.2, where GGGGGGYIGSR (a pentaglycine spacer group) showed significantly longer neurites than GYIGSR (no spacer group). It should be noted that for all time points, neurite length on the spacer group surfaces with a single peptide were comparable to the non-spacer group surface with mixed peptides.

Figure 5.1. The length of the longest neurites extended per cell averaged over 50 cells per sample (n=3) from day 0 to day 3 of E18 hippocampal cell culture in serum free media.
Figure 5.2. The length of the longest neurites extended per cell averaged over 50 cells per sample (n=3) from day 0 to day 3 of E18 hippocampal cell culture in serum free media, comparing GYIGSR coupled directly to the surface, or coupled through a spacer group to the surface.

To test the specificity of peptide influence on neurite length, a competitive assay was done where cells were pre-incubated with peptides for all surfaces prior to plating. As shown in Figure 5.3, neurite length decreased after pre-incubation on all surfaces except on control FEP-[N/O] film samples, where the cell-surface interactions are non-specific. Neurite length decreased most significantly on IKVAV surfaces, as was expected given that IKVAV is known to promote neurite outgrowth (Jucker, 1991). It is worth noting that the cells recovered over time and continued to extend long neurites after day 2 (data not shown).
Figure 5.3. The length of the longest neurites extended per cell averaged over 50 cells per sample (n=3) from day 0 to day 3 of E18 hippocampal cell culture in serum free media after preincubating the cells with 0.1 mg/mL of peptides for 30 minutes.

5.5.2 Number of Neurites

The number of neurites per cell were quantified for each surface, the averages of which are summarized in Figure 5.4. As shown, all peptide-modified surfaces supported a greater number of neurites per cell than the aminated FEP (FEP-[N/O]) controls, yet fewer neurites per cell than PLL/laminin positive control surfaces. The differences between YIGSR and IKVAV surfaces were insignificant; however, FEP-[N/O]-Mix surfaces had significantly more neurites per cell than single-peptide-modified films on day 3. Figure 5.5 shows that the effects of a pentaglycine spacer group on the number of neurites extended were only distinguishable at day 3, where GGGGGGYIGSR surfaces had significantly greater number of neurites than GYIGSR surfaces.
Figure 5.4. The number of neurites extended per cell averaged over 50 cells per sample (n=3) from day 0 to day 3 of E18 hippocampal cell culture in serum free media.
Figure 5.5. The number of the neurites extended per cell averaged over 50 cells per sample (n=3) from day 0 to day 3 of E18 hippocampal cell culture in serum free media, comparing GYIGSR coupled directly to the surface, or coupled through a spacer group to the surface.

In the competitive assay study of hippocampal neurons incubated with dissolved GYIGSR and SIKVAV peptides for 30 min prior to plating, the number of neurites per cell decreased on all surfaces, as shown in Figure 5.6. The greatest percent decrease was observed on the IKVAV surfaces, as was expected, given that IKVAV promotes neurite outgrowth (Jucker, 1991). A small decrease in the number of neurites per cell was observed for PLL/laminin and FEP-[N/O] control surfaces, indicating that merely saturating cellular receptors influenced outgrowth. Interestingly, the neurons again recovered over time, extending more neurites per cell from day 2 onwards.
Figure 5.6. The number of neurites extended per cell averaged over 50 cells per sample (n=3) from day 0 to day 3 of E18 hippocampal cell culture in serum free media after preincubating the cells with 0.1 mg/mL of peptides for 30 minutes.

5.5.3 Number of Adherent Cells

The number of adherent cells on each surface was estimated by averaging the number of live cells over 10 fields of images (field size of $2.5 \times 10^5 \mu m^2$), using samples stained with calcein AM for live cells and ethidium homodimer for dead cells. As shown in Figure 5.7, there are statistically significant differences between the surfaces over time. While there were fewer adherent cells on FEP-[N/O] controls than on other surfaces, the variability in the cell adhesion data made it difficult to differentiate among our surfaces. Studying the strength of adhesion may be a better tool to assess cell-surface interactions.
Figure 5.7 The number of cells per field (field size = $2.5 \times 10^5 \mu$m$^2$) averaged over 10 fields per sample ($n=3$) from day 0 to day 3 of E18 hippocampal cell culture in serum free media.

5.6 Discussion

The goal of peptide modification is to create analogs of protein modified surfaces to obviate the use of full proteins. This allows control of concentration and orientation of the peptides on the surfaces and overcomes the use of natural proteins derived from animal tissues. YIGSR and IKVAV were studied because they promote adhesion (Yamada, 1992) and outgrowth (Jucker, 1991) respectively, and are derived from laminin, which is an important extracellular matrix protein for neurons (Ruoslhtaht, 1988).
Previous research in our (Tong, 1998a, 1998b, 1999) and other laboratories (Massia, 1993) have demonstrated the utility of single peptide-modified surfaces, yet few, if any, have studied the additional, synergistic effect of mixed peptide surfaces. Our results demonstrate that the mixed peptide surface is a better analog for laminin than single peptide surfaces, as shown by both the neurite length and number results. While the longest neurite was not as long as that observed on PLL/laminin positive control surfaces, the percent increase on the mixed peptide surface was the greatest of all surfaces studied. Our results of using a spacer group extending the peptide from the surface demonstrate that peptide mobility is an important factor in mimicking the natural conformation of laminin. By adding a pentaglycine spacer, the neuronal interactions were significantly enhanced to a comparable manner as the mixed peptide surface without spacer groups.

It is also important to note that positive controls were prepared on tissue culture polystyrene dishes whereas peptide-modified films were prepared on FEP. The underlying surface chemistry/hydrophilicity may have impacted our results. Notwithstanding these differences, the data indicate that neurite extension requires stimulation of multiple receptor types. Therefore, in order to fully mimic the neuron-laminin response, other peptide sequences of laminin such as RGD (Grant, 1989) or RKRLQVQLSIRT (Richards, 1996), and extension of the peptides from the surface using spacer groups, may be required.

The differences observed between laminin and mixed peptide surfaces were most obvious in the number of neurites per cell. In order to match positive control results, two additional studies may be required: (i) investigating several relative concentrations of YIGSR and IKVAV (herein we investigated only 1:1 w/w ratios), or (ii) using longer amino acid sequences to accommodate the three-dimensional conformation observed in laminin.
5.7 Conclusions

Peptide-modified FEP film surfaces enhanced cellular interaction of hippocampal neurons. FEP surfaces containing a 1:1 mixture of YIGSR and IKVAV peptides promoted a greater number of neurites per cell and longer neurites than surfaces modified with one peptide type. The interaction of the neurites with the surface was blocked by competitive binding of soluble peptides in the media, indicating that cell-surface interactions are peptide-receptor specific. The mixed peptide-coupled FEP surface served as the best analog for poly(L-lysine)/laminin coated tissue culture polystyrene surfaces. To enhance the cell-surface interactions further, future studies will investigate multiple synergy site peptides having the conformation similar to that observed in laminin.

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5.8 References


Chapter 6. Conclusions and Future Work

6.1 Conclusions

From the observations and results obtained, it can be concluded that:

(a) FEP surfaces were successfully functionalized with hydroxyl, carboxylic acid and amine functional groups to different surface concentrations using the established sodium naphthalide method and the novel mercat method;

(b) The surface chemistry and morphology of FEP films modified with the novel mercat method was fully defined, and was found to be heterogeneously composed of nitrogen groups (of which 8% are amines and 70% are nitriles), oxygen groups (of which 8-15% are hydroxyls and 10% are carboxylic acids), and carbon-carbon double bonds (6% of total carbon);

(c) Covalent coupling of three different cell adhesive peptides onto the functionalized FEP surfaces were achieved using three different coupling reagents at concentrations ranging from 3 to 150 fmol/cm²;

(d) Three different cell adhesive peptides, YIGSR, IKVAV and RGD, were introduced onto modified FEP surfaces either individually, or in a 1:1 w/w mixture of YIGSR and IKVAV;

(e) YIGSR was successfully coupled onto the mercat modified FEP films either directly as GYIGSR, or via a peptide spacer group as GGGGGGYIGSR;

(f) New methods of quantifying neuron-surface interactions were developed by measuring neurite number and length, and cell number/viability at different time points;
The interactions of hippocampal neurons with peptide modified FEP were enhanced compared to unmodified FEP and functionalized FEP, and were similar to PLL/Laminin coated surfaces; FEP surfaces with a 1:1 mixture of YIGSR and IKVAV promoted greater neurite outgrowth than FEP surfaces with single peptides of YIGSR and IKVAV; Neuronal interactions were not found to be affected by the range of peptide concentration used; GYIGSR peptide coupled to FEP surfaces through a peptide spacer group promoted greater neurite outgrowth than without the spacer group.

6.2 Future Work

In this thesis, a model system for mimicking the permissive environment of the PNS was developed with the eventual goal for curing spinal cord injuries. Fluoropolymer surfaces that were successfully modified with cell-adhesive peptides were proven to support the outgrowth of neurites and the viability of neurons equal to the best known surface for culturing cells, that is, surfaces coated with PLL and laminin. These conclusions lead to other possible research along similar lines.

One possible avenue of research is to further enhance neuronal interactions to be better than the PLL/laminin surfaces. As a mixture of YIGSR and IKVAV on a surface has shown to synergistically enhance neurite outgrowth, other laminin-derived peptide sequences such as LIGRKK and KNRLTIELEVRT could be added to the mixture for ever greater synergism. The use of spacer groups between the peptide and surface was also shown to be more effective in neurite outgrowth than peptides attach directly to a surface. Thus, peptide
conformation could be investigated further using longer spacer groups, or different types of spacers.

Other possible research avenues include application of peptide surface modification to other polymers that could be used eventually *in vivo*. As fluoropolymers are non-biodegradable and does not possess similar mechanical properties to the spinal cord, other polymers that does not have these disadvantages should be used. Preliminary *in vivo* studies of peptide-modified fluoropolymers as peripheral nerve grafts could also be done to prove the viability of such a model.
Appendix A

The chemistry of coupling peptides to oxygen or nitrogen functionalized surfaces:

(i) Tresyl coupling chemistry:

(ii) SMCC coupling chemistry:

A-1
(iii) TSU coupling chemistry:

\[
\begin{array}{c}
\text{COOH} \\
\text{CH}_2\text{OH} \\
\text{NH}_2
\end{array}
\begin{array}{c}
\text{TSU} \\
\text{TSU} \\
\text{TSU}
\end{array}
\begin{array}{c}
\text{BF}_4^- \\
\text{BF}_4^- \\
\text{BF}_4^-
\end{array}
\begin{array}{c}
\xrightarrow{\text{FEP}} \\
\xrightarrow{\text{FEP}} \\
\xrightarrow{\text{FEP}}
\end{array}
\begin{array}{c}
\text{O} \\
\text{R} \\
\text{NH}_2
\end{array}
\begin{array}{c}
\text{O} \\
\text{C} \\
\text{CH} \\
\text{NH}_2
\end{array}
\begin{array}{c}
\text{FEP-X} \\
\text{FEP-X} \\
\text{FEP-X-peptide}
\end{array}
\]