INFLUENCE OF POLYMER SURFACE CHEMISTRY ON THE RECRUITMENT OF STEM CELLS IN MICE

by

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ABSTRACT

INFLUENCE OF POLYMER SURFACE CHEMISTRY

ON THE RECRUITMENT OF STEM CELLS IN MICE

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The most troublesome biological response to the implantation of blood-contacting biomaterials is inflammation, which thereby-sets in motion a cascade of adverse host responses. If devices for therapy and drug delivery could be developed that prevent such responses from occurring altogether, a new generation of "stealth" biomaterials would be born. Such was the motivation of this project, which sought to investigate using mouse subcutaneous implantation model, the implant mediated host tissue responses to surfaces differing in their functionalities. During the course of a previous study in our laboratory, we accidentally discovered the presence of stem cells and their accumulation at the capsule around microparticle implants.[31] In our present study, as the recruitment of stem cell coincided with that of the inflammatory cells, we believe that stem cells get actively recruited by the inflammatory response induced by

the biomaterial implantation. As the first stage of our investigation, to test this hypothesis, we evaluated the host tissue responses to polypropylene particles with surfaces, modified using radio frequency glow discharge plasma polymerization to have high concentrations of -OH, $-NH_2$, $-CF_3$ and -COOH groups. The extent of inflammatory responses mediated by the biomaterial implantation and corresponding stem cell recruitment were assessed following implantation, using immunohistological analyses.

Our results indicate that surface functionalities significantly affect both capsule formed around the implant and the inflammatory cells, with leukocyte marker CD11b, recruited to the implant. In addition, we have also uncovered many cells that stain positive with stem cell markers SCF, Nanog and SH2B. Our results reveal that chemical characteristics of material surfaces play important roles in biomaterial mediated tissue responses. Surfaces with $-NH_2$ and -OH groups showed the highest number of inflammatory cells at the capsule along with the thickest capsule measured in microns. Interestingly, we observed that all five surfaces provoked different extents of recruitment of these stem cells at the capsule. The -COOH group showed the maximum number of positive cells for all three stem cell markers. Unexpectedly, an inverse relation between recruited inflammatory and stem cells was found for most surfaces , suggesting that stem cells are different and distinct from the inflammatory cells, though both were influenced by the implant mediated foreign body response. The underlying mechanism is yet to be determined. Overall, our results indicate that the surface

functional groups influence not only the inflammatory responses but also the biomaterial mediated stem cell recruitment, inside the host.

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LIST OF ABBREVIATIONS

ESC.. …….	Embryonic Stem Cell
ASC ……..	Adult Stem Cell
HSC ……..	Hematopoietic Stem Cell
MSC …….	Mesenchymal Stem Cell
PP …….	Polypropylene
EDA ……..	Ethylene Diamine
EO2V..……...	Di (ethylene glycol) vinylacetate ester
C6F14 ……	Perflurohexane
VAA ……..	Vinyl Acetic Acid
SCF …….....	Stem Cell Factor
SH2B ……..	Src Homology 2 B
CD11b/Mac-1 …….	Alpha M Integrin Chain

CHAPTER 1

GENERAL INTRODUCTION

1.1 Particles in Drug Delivery : A Brief Introduction and ChallengeF aced

Systemic drug delivery has traditionally been employed to treat localized disease conditions pertaining to specific anatomical sites. With pharmaceutical treatments, there are a variety of disease states where systemic drug delivery has poor efficacy or significant side effects. This problem is especially pronounced when the accessibility of drug to a specific anatomical site proves to be problematic. Targeted local drug delivery has been considered the preferred method of treatment in such. However, there still persist limitations in the functional life of the targeted drug delivery system because of the inherent foreign body responses within the host. Biological response such as infection, inflammation, immune system targeting, and thrombogenesis, each are known to inhibit the functioning of such systems. Pharmaceuticals or antimicrobial agents are often given to a patient to enable the performance of the implanted drug delivery carriers. In some cases, this approach has been insufficient or entirely ineffective.

Thus the strive to design a drug delivery carrier with properties to elicit minimal or no host responses and enhance biocompatibility has been relentless. And this has, unconditionally, been the impetus for this study.

1.1.1 Biomaterials in Drug Delivery Systems

Biomaterials are considered to nonviable materials that become a part of the body either temporarily or permanently to restore, augment, or replace the natural functions of the living tissues or organs in the body. A biomaterial may be defined as: "a synthetic material used to replace part of a living system or to function in intimate contact with living tissue.[1]A biomaterial may be further identified as belonging to one of the three classes[2]:

- Class1: Devices intended for percutaneous, temporary use, such as Band-Aids™;
- Class 2: Devices used for internal applications, such as Drug delivery systems;
- Class 3: Devices that are permanently implanted in a body and intended to integrate and function with the living body, such as vascular grafts.

A number of biomaterials have been used for medical applications including controlled drug delivery.[3,4] Controlled drug delivery technology represents one of the frontier areas of science, which involves multidisciplinary scientific approach, contributing to human health care.[5] These delivery systems offer numerous advantages compared to conventional dosage forms, which include improved efficacy, reduced toxicity, and improved patient compliance and convenience. Such systems often use macro molecules as carriers for drugs. By doing so, treatments that would not otherwise be possible are now in conventional use. A variety of synthetic or natural polymers have been employed as the controlled-release drug delivery system in humans.[6] Among them, biodegradable polymers have become increasingly important in the development

of implantable biomaterials and drug delivery devices.[7] A major advantage of these systems is that an invasive technique such as surgery is not required for their removal.[7]

Nanoparticles and microparticles have attained much importance and occupy a unique position in drug delivery technology.[8] Nanoparticles are materials in the nanometer range with many potential applications in clinical medicine and research.[9] Devised initially as carriers for vaccines and anti-cancer drugs, nanoparticles have been found of use in a wide variety of drug delivery applications.[10] These materials being at the nanometer scale exploit novel physical, chemical and biological properties. They are also useful as drug carriers for the effective transport of poorly soluble therapeutics. When a drug is suitably encapsulated, in nanoparticulate form, it can be delivered to the appropriate site, released in a controlled way and protected from undergoing premature degradation.[11, 12] Polymer-based nanotechnologies are now proposed as an alternative to classical formulations for drug administration, delivery and targeting.

Microparticles are defined, as spherical particles with the size varying in between 50nm to 2mm containing a core substance. Microspheres are in strict sense, spherically empty structures.[12,13] Polymeric microspheres have been widely investigated as controlled release system for proteins and peptides.[14,15]

1.1.2 Biocompatibility: Challenge for Particles

The aim of targeted drug delivery and a controlled release is to better manage drug pharmacokinetics, pharmacodynamics, non-specific toxicity, immunogenicity and biorecognition of systems in the quest for improved efficacy. The advantage of using biomaterials in drug delivery is the ability to deliver drugs locally ,at a desired

degradation rate ,for therapy.[2,5,8,11,16] However, the design and development of biodegradable microparticles and nanoparticles containing bioactive agents for therapeutic application requires a fundamental understanding of the in vivo biodegradation phenomena as well as the cellular and tissue responses which determine the biocompatibility of the particles.[2,16] These biomaterial components, must sustain long-term structural and functional properties in order to endure the harsh physiological environment. Along with this harsh physiological environment is the potential immune response towards them *in vivo* due to the cellular component.[11,16]

The cellular responses to delivery systems that utilize biodegradable, biocompatible polymer particles are drawing considerable interest. Inflammation, wound healing and foreign body responses are generally considered as components of the tissue or cellular host responses to injury.[16,17,18] The response to injury is initiated by the implantation procedure, which for the particles involves injection of the formulation within a solvent vehicle. The particles are said to encounter macrophages as the primary host defense. Since inflammatory responses to polymeric materials in the body occur frequently, it is important to investigate the compatibility of polymeric materials used in drug delivery systems.[17,18] The properties of polymeric microparticles that affect phagocytosis have been studies in detail by Tabat and Ikada.[10] The main factors found to influence phagocytosis were particle size, surface charge and surface coating.[2,3,5,8,11,16,19]

1.2 Concept of this Thesis

The research described in this Thesis deals with the influence of surface modification of particles on foreign body responses. The major part of the work presented is directed towards the study of the modulation of inflammatory and fibrotic responses by functional groups, coated using plasma polymerization of polypropylene containing monomers.

Chapter 2 gives an overview of the most commonly used techniques for surface modification of polymers. This chapter also serves as a general introduction to plasma polymerization. A general description of the plasma reactor is also given. The particles used for the purpose of this study and monomers used for providing the required functional groups are also discussed

The influence of the various surface functional groups on foreign body reactions, studies using a subcutaneous mouse implantation model are discussed in Chapter 3 A major part of this section focuses on the extent of inflammatory and fibrotic responses, analyzed using histological techniques, like Hematoxylin & Eosin (H&E) staining . The extent of recruitment of inflammatory cells, to the implantation site, is also explained using Immunohistochemistry with the inflammatory marker, CD11b. The presence of non-inflammatory cells is also observed at the implantation site.

Chapter 4 is an introduction to stem cells. The influence of surface functionality on the recruitment of stem cells to the implantation site is presented. The study comprises of immunohistochemistry with three different stem cell markers, namely ,

Stem cell Factor (SCF), Nanog and SH2-B (Src Homology) to study the extent of stem cell recruitment due to each surface functionality.

In Chapter 5, relation between foreign body response and stem cell recruitment is discussed. The co-existence of inflammatory and stem cells , at the site of implantation was analyzed using overlapped images from immunohistochemistry , with CD11b and stem cell markers SH2-B, respectively. The relation between recruitment of both stem cells and inflammatory cells is discussed.

CHAPTER 2

SURFACE MODIFICATION AND ITS INFLUENCE ON BIOMATERIAL MEDIATED FIBROTIC RESPONSE

2.1 Introduction

Surface properties of biomaterials, including chemistry, wettability, domain composition, and morphology, have been seen to influence protein adsorption and subsequent cellular responses to biomaterial implants.[19,20] Surface modifications of biomaterials, represents an exceedingly active research area. Despite substantial work in this area, it is still unclear which surface properties may be critical to the host responses.[21] A diverse and imaginative ranges of experimental techniques have been developed to achieve surface modifications.[21] Some of these surface modification techniques will be discussed briefly here below. Firstly, some surface treatment methods will be presented, that enable the alteration of chemical and physical properties of polymer surfaces without affecting their bulk properties. Secondly, surface modification by attachment of a monolayer or thin polymer film to the surface will be discussed.

2.1.1 Surface Treatment of Polymers

Flame Treatment

Flame treatments have been used commonly in the polymer industry to improve adhesive characteristics of surfaces, or more particularly to enhance ink permanence on

polymer surfaces. The high flame temperature (1000-2000 °C) and reaction with excited species in the flame, basically leads to an increased oxygen concentration at the treated surface.[22,23]

Corona Discharge

A corona discharge (atmospheric pressure plasma) is produced when air is ionized by a high electric field. Often a corona discharge system is used for continuous treatment of films, installed downstream of an extruder. Similar to a flame treatment, a corona treatment causes surface oxidation of polymers. Electrons, ions, excited species and photons that are present in a discharge react with the polymer surface to form radicals. These radicals react rapidly with atmospheric oxygen.[22,24]

Advantages of corona and flame treatments are that these processes can be used in continuous operation, and that the required equipment is very simple and cost effective. The disadvantages arise from the fact that both treatments are carried out in open air, which often makes it difficult to control the uniformity or chemical nature of the modification, due to variations in ambient conditions such as temperature and humidity or contaminations.[22,23,24]

2.1.2 Surface Modification by Attachment of Monolayers

Langmuir-Blodgett-Kuhn Technique

In the first step, amphiphilic molecules are spread on an aqueous subphase in a trough. In the second step, the surface area of the molecules is slowly reduced by two barriers, resulting in orientation of the molecules. The hydrophilic headgroups are dissolved in the subphase, while the compressed hydrophobic chains stand out of the

solution. In the third step, a substrate is dipped in and out of the solution, while the surface pressure is kept constant by the barriers. At every dip a well defined monolayer is transferred on to the substrate, and highly ordered multilayers can be deposited.[22,25]

Polyelectrolyte Multilayer Deposition

By dipping a charged substrate alternating in two polyelectrolyte solutions with oppositely charged polymers, it is possible to transfer over 100 monolayers of constant thickness to the substrate. By functionalization of polyelectrolytes with various chemical groups, stable thin films with different properties can be prepared.[22,26]

Block Copolymer Thin Films

Block copolymers are macromolecular architectures, consisting of two or more chemically different, covalently linked polymer chains. In general, a substrate is subjected to the block copolymer solution. In principle, the polymer chains in solution are highly mobile, and the thermodynamic driving force minimizes the total free energy of the system by segregation of the component with the lowest surface free energy at the surface.[22, 27]

2.1.3 Surface modification using Pulsed Plasma

Plasma polymerization is accepted as an important process for the formation of entirely new materials, and as a valuable technique to modify the surfaces of polymers or of other materials.[21,28] It can be defined as the formation of polymeric materials under the influence of plasma.[29] Functional groups are introduced at the surface of the polymer by reaction of gas-phase species and surface species.[21,28,29] Additionally, a wide range of compounds can be chosen as a monomer for plasma polymerization, even

saturated hydrocarbons, providing a great diversity of possible surface modifications Pulsed plasma technique is used to improve the adhesion of plasma-deposited films to underlying solid substrates. The pulsed plasma is initiated for a brief period at a relatively high duty cycle which is then progressively reduced to a final value to provide a desired surface density of functional groups.[21,22,28,29] It is clear that this process provides an excellent starting point for molecular surface tailoring via subsequent chemical derivatization of controlled surface density of reactive functional groups introduced during the pulsed plasma deposition.[21,28,29]

Plasma Reactor

Figure 2.1 provides a schematic diagram of a plasma reactor employed for all plasma depositions described in this work. The reaction chamber consists of a Pyrex glass cylindrical tube, 30 cm in length and 10 cm in width. Gases fed through the system pass two cold traps, cooled with liquid nitrogen, for collection of excess reactant before reaching the pump. Side arms at the reactor inlet allow the introduction of non-polymerizable gases such as oxygen, nitrogen and argon, and the monomer vapors. The flow rate of the O2, N2 and Ar is controlled by MKS gas flow meters, while that of the monomer vapor is controlled by a Kobold floating ball flowmeter. A MKS baratron (type 122) is also connected to one of the inlets, to monitor the reactor pressure. A butterfly valve at the other reactor end is used in conjunction with the pressure transducer, to allow accurate control over the process pressure. The flow meters, the baratron and the butterfly valve are controlled by a central multigas controller. A Tektronix PG 501 pulse generator controls the pulsing of the radio frequency signal,

obtained from a 20 MHz function generator. The signal is amplified by an ENI 300 W amplifier, and passed via a bidirectional coupler, an analogue wattmeter and a matching network to the electrodes, consisting of two external concentric metal rings with a spacing of 10 cm. An oscilloscope, connected to the bi-directional coupler, was used to tune the rf circuit, by minimizing the reflected power. [22,28,29]

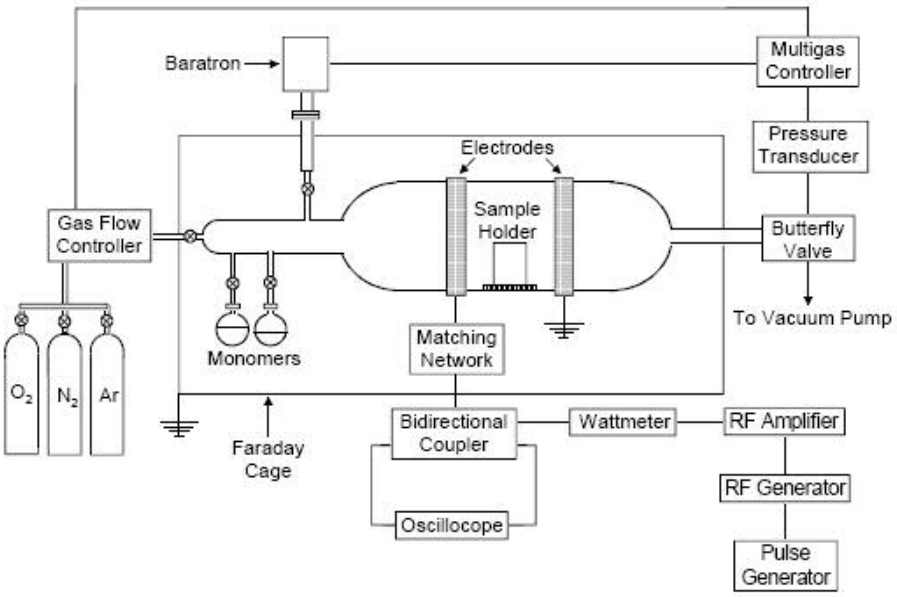

Figure 2.1 Schematic diagram of a plasma reactor and the electrical components.[22,28,29]

Advantages of plasma polymerization include the fact that pinhole free, conformal thin films can be deposited on most substrates, using a relatively simple one-step coating procedure.[22,28,29]

Though it is believed that the extent and duration of the host tissue response will depend on the implant procedure as well as the physical and chemical properties of the biomaterial implanted and that plasma surface tailoring will help improve

biocompatibility, it is still unclear whether surface functionalities would have any effect on subsequent fibrotic responses and biocompatibility.[17-20]

2.1.4 Inflammatory Response to Particles

The response to injury is initiated on implantation. Humoral and cellular mechanisms are activated to produce inflammation and healing.[16-19,30,31] The degree to which these mechanisms are perturbed and the extent of the pathophysiological responses and their resolution are a measure of the host reaction to drug delivery system.[16-19] The sequence of events following implantation of particle drug delivery system is: injury, acute inflammation, chronic inflammation, granulation tissue, foreign body reaction and fibrosis. The size, shape, chemical and physical properties of the biomaterial, and the physical dimensions and properties of the material may be responsible for variations in intensity and time duration of inflammatory and wound healing processes.[16,18,30,31] In addition the high surface area/low volume characteristics of implanted particles are also seen to lead to the tissue response. The volume of the particles is also seen to elicit responses which are seen early as a granulation tissue response which leads to fibrous encapsulation of the entire microspheres implant.[31] Presence of the inflammatory cells can be determined using immunohistochemistry, employing suitable inflammatory markers, like CD11b.[31]

CD11b

The adhesion molecule CD11b, a member of the *b*2-integrin family, strengthens the initial contact and is involved in the migration into the inflamed tissue. It is a cell surface antigen, and is also known as MAC-1 (CD11b/CD18/alpha M integrin chain).

CD11b is mobilized from intracellular pools upon activation and plays an important role in the innate immunity towards invading microbes. It is upregulated during neutrophil activation[31,43,44] It is stored in specific granules which are shuttled to the granulocyte surface. It exists as a chemoattractant activation-dependent molecule that undergoes a conformational change upon stimulation. Expression of new epitopes on Mac-1 can be detected after activation by specific reporter monoclonal antibodies. Until stimulation occurs, Mac-1 remains in a resting, non-adhesive state. Activation of Mac-1 may play a role during neutrophil recruitment to the inflamed site.[31,43,44]

2.1.5 Previous Observation

The implantation of a biomaterial leads to a response to injury that activates mechanisms of healing of the damaged tissue. A sequence of events is initiated starting with an acute inflammatory response and involving granulation tissue development, a foreign body reaction and fibrous capsule development. The size, shape and chemical and physical properties of the biomaterial may be responsible for variations in the intensity and time duration of the inflammatory and wound healing process.[2,5,16,18,30,31]

As biomaterial –mediated inflammatory responses have been related to subsequent fibrotic responses and nature of biomaterial used, we had reasons to believe that surface functionalities might influence the extent of biomaterial mediated inflammatory and fibrotic responses.

2.2 First Hypothesis

Surface chemistry of the biomaterial influences the extent of host tissue response at the site of implantation.

2.3 Experimental Section

2.3.1 Materials and Methods

Ethylene diamine, Di (ethylene glycol) vinyl Acetate and Vinyl Acetic acid were obtained from Sigma Chemical Company (St.Louis,MO) and Perflurohexanes from PCR Chemical (Gainesville,FL). This was done in collaboration with Dr Richard B Timmons Laboratory at the University of Texas at Arlington

Primary antibodies against CD11b was purchased from Santa Cruz Biotechnology Inc. Texas Red was from Jackson ImmunoResearch laboratories, Inc. (West Grove, PA). DAPI (1:200) for nuclear staining was obtained from Cambio Ltd., UK. Prolong gold antifade reagent (Molecular Probes, OR) was mounted on the samples after the experiment to preserve the fluorescence. Microscopy analysis were done using the Leica Standard Microscope (Leica Microsystems, Wetzlar, GmbH) equipped with a Nikon E500 Camera(8.4V,0.9A, Nikon Corp., Japan)

Modification of Polymeric Surfaces

Surfaces with different functional groups with hydrophobicity were produced using a flowing gas system in which ionized gas plasma is repeatedly generated (with on and off cycles) to provide , via polymerization of appropriate monomer, thin film coatings with different functional groups. The functional group density at the surface was controlled by using different monomers and by variation of the input power during the plasma deposition. In the present work, pulsed plasma was used to coat polypropylene (PP) particles with different surface functionalities, including –OH (from

Di (ethylene glycol) vinyl Acetate), -NH2 (from Ethylenediamine), -CF3 (from Perflurohexane), -COOH (from Vinyl Acetic Acid).

Polypropylene

Polypropylene or polypropene (PP) is a thermoplastic polymer, used in a wide variety of applications. An addition polymer made from the monomer propylene, it is unusually resistant to many chemical solvents, bases and acids. Polypropylene has very good resistance to fatigue. Medical utilization includes yarns for suture; films for sterile condition bags; thermoformed external prostheses; cast bodies for syringes, rigid nozzles and sterilizable vessels.[42]

Table 2.1 List of Monomers used in this study

Monomer Name	Representation	Plasma Condition	Water Contact Angle	Surface Functionality
Ethylene diamine	EDA	15/35, 200W, 78-80 mTorr, 15min	12-14 degree	$-NH_2$
Vinyl Acetic Acid	VAA	0.75/20, 200W, 80 mTorr, 15 min	29-30 degree	-COOH
Di (ethylene glycol) vinyl Acetate	EO2V	20/300,33.7W,60 mTorr,60 min	45 degree	-OH
Perfluro hexane	C6F14	25/200, 200W, 200 mTorr, 30 min	135 degree	$-CF_3$

2.3.2 Animal Implantation Model

In order to test this hypothesis we employed animal models in which test specimens of Polypropylene particles coated with ethylene diamine (EDA), di(ethylene

glycol)-vinylether (EO2V), perflurohexane(C6F14) and vinyl acetic acid (VAA), using Plasma Polymerization, as mentioned in section 2.2, were implanted subcutaneously in Balb/C mice (male, female) from Taconic Farms (Germantown, NY, USA).. Polypropylene particles without any surface modification, were employed as Control specimen, for the study. After implantation for 2 weeks, implant-bearing mice were sacrificed and the implants and the surrounding tissues were then recovered for histological and immunohistochemical analyses. To assess the biomaterial-implant mediated cell recruitment and tissue response, in each case, the explants were fixed and frozen sectioned.

2.3.3 Histological & Immunohistochemistry Analyses

Histological Evaluation

Skin tissue samples obtained from the animal were directly placed on OCT (Polysciences,IL) for the purpose of embedding and then were frozen immediately. 10 μm thick sections were sliced using a Leica Cryostat (CM1850) and placed on microslides for H&E staining. The tissues were subjected to H&E stain and the foreign body response was observed for different surface functionalities. A Leica DMIL fluorescence microscope was used for observation and microscopic analyses.

Immunohistochemistry

Frozen tissues embedded in OCT compound, placed in plastic cryomoulds were retrieved from -80C. 10 μm thick sections were sliced using a Leica Cryostat (CM1850) and placed on microslides coated with poly-L-lysine solution, 0.1% w/v , in water (P8920, Sigma-Aldrich Co.,MO, USA). Sections were fixed in ice-cold acetone for 5

min and were allowed to dry. They are then rinsed in washing buffer, phosphate buffered saline (PBS, ph 7.4) for 5 min x 3. Sections were then blocked using 1% BSA solution by incubating for 40min at 37C. This was followed by rinsing in PBS,5min x 3. Sections were then incubated with the primary antibody,CD11b, using the appropriate dilution for 2 hrs at 37C. This was followed by rinsing in PBS for 5 min x 3. The sections were then incubated using the secondary antibody, Texas Red, at a dilution of 1:500 for 1hr at 37C. This was finally followed by rinsing in PBS, 5min x 4.

DAPI Staining

Following Immunohistochemistry, the samples were incubated in DAPI (Cambio Ltd., UK) at a dilution of 1:200, at room temperature for 5min. This was followed by rinsing in washing buffer, PBS for 5min x 3. Prolong gold antifade reagent (Molecular Probes, OR) was mounted on the samples after the experiment to preserve the fluorescence and DAPI staining. Finally, a cover slip was placed on top to retain the antifade.

Inflammatory cells are observed to stain positive for CD11b when subjected to Immunohistochemistry (Fig 3.3). CD11b cell surface antigen, also known as MAC-1, a differentiation antigen expressed by tissue macrophages, granulocytes.31,43,44 DAPI staining was done as a reference for quantitative analyses of cells that stained positive for CD11b. The microscopic images obtained were overlapped for this purpose (Fig 2.4) A graphical representation of the cell number for each specimen, using a histogram, is also shown (Fig 2.5)

2.4 Results

2.4.1 Influence of Surface Functionality on Foreign Body Response

We performed H&E histology stain on the 2 week old implanted skin tissue, to mark the location of the capsule, implant and surrounding tissue. Shown below is the H&E stain of the samples obtained for all 5 specimens (Fig 3.1). The extent of implant-associated fibrotic capsule thickness was measured in each case, as a reflection of the extent of collagen production and possibly fibrotic response. We observed that the thickness of the capsule varied between all the samples, with -OH showing the highest, followed by $–NH_2$, $-CF_3$, $-CH_3$ and -COOH. A graph showing the quantitative analysis of the thickness of the fibrous capsule around the implants 2 weeks after implantation is also shown (Fig 3.2).

The thickness of the capsule around different specimens showed a significant extent of variation. -OH has the maximum, with an average thickness of 288 ± 34.59 microns. $–NH_2$ follows with an average thickness of 78.75± 25.61 microns, while $-CF_3$ has an average thickness of 63.3±11.92 microns. The control $-CH_3$ has an average thickness 45.3± 17.44 microns, while -COOH has an average thickness of 43.5± 21.58 microns.

The end stage of healing response is usually fibrosis or fibrous encapsulation[16] Type-I collagen often predominates and forms the fibrous capsule that' surrounds the implant.[16,31] Generally, fibrous encapsulation surrounds the implant or biomaterial with its interfacial foreign body reaction from the local tissue environment. Thus, the

formation of this fibrous capsule is considered as a secondary adaptive response of the local tissues of the host to implantation.[16,31]

Our investigation shows that the chemical nature of the surface of a subcutaneous implant modulates the thickness of the fibrous capsule that is organized around the implant, with the OH group inducing a thicker capsule around the implant followed by that of the NH_2 group. Thus, the result confirms our hypothesis that the surface chemistry of the implant modulates the extent of host response and in turn the thickness of the fibrous capsule.

2.4.2 Effect of Surface Functionality on the Recruitment of CD11b+ Cells

We carried out immunostaining using the inflammatory cell marker CD11b, to assess the biomaterial implant-mediated inflammatory cell recruitment .Interestingly, we found large differences in the extent of inflammatory responses engendered by the different surfaces in the order, based on functionality : NH_2 > OH> CF_3 > COOH.

From Fig 3.5, it is observed that the number of inflammatory cells at the capsule for –OH shows the maximum, with a cell number of 287 ± 40.35 cells per field of view. –NH_2 follows with a cell number of 251± 32.58 cells per field of view, while –CF_3 shows a cell count of 204.75±56.31cells per field of view. The control –CH_3 shows a cell count of 113.75± 13.65 cells per field of view, while –COOH shows a cell count of 95.25±43.25 cells per field of view.

The structure of the fibrous capsule has been attributed, by previous studies, to both on the nature of the implant and on the inflammatory response caused by the implant.[52] Since biomaterial-mediated inflammatory responses have been related to

subsequent fibrotic responses and surface functionality influences the thickness of the capsule formed around the implant, we had reasons to believe that surface functionality may affect the degrees of inflammation and in turn the recruitment of inflammatory cells in response to the biomaterial implants.

2.5 Discussion

Inflammation is a result of the inflicting surgical trauma and the presence of the implanted material.[16,18] The inflammation process is closely linked to the subsequent repair/regeneration of tissues. The first phase of wound healing-acute inflammation-follows as neutrophils and monocytes migrate to the locus of the inflammatory stimulus.[31,30] Persistent inflammatory stimuli lead to chronic inflammation, which is characterized by the presence of monocytes, macrophages and lymphocytes with the proliferation of blood vessels and connective tissue.[16,18, 31,30]

Foreign body response essentially comprises of three primary stages, they are – cellular transmigration, chemotaxis towards implanted biomaterial (foreign body) and cellular adhesion on the biomaterial.

Cellular transmigration

Mast cell produces Histamine, which is important to the migration of phagocytic cells through the endothelial layer.[30, 31]Histamine release increase the expression of adhesion molecules though edema and hyperemia.[30] This increase in adhesion molecule expression leads to increase in diapedesis of phagocytes. Importance of histamine, and in turn mast cells, can be significantly observed by blocking the histamine receptors using drugs that diminish the accumulation of macrophages and monocytes.[30,31]

Chemotaxis towards the implant

Phagocytes accumulate on the surface of the implanted biomaterial, once out of the endothelial barrier.[16,18,30] The macrophage inflammatory protein 1α and monocyte chemoattractant protein 1 send signals prompting the phagocytes to travel to the implantation site. Adherence of proteins on the biomaterial surface determines the extent of inflammation.[16,18,30,31]

Cellular adhesion on implant

A biomaterial placed in the body is eventually covered by a layer of proteins, like – albumin, immunoglobulin, fibrinogen, etc. Of these, Fibrinogen plays an important role. It has been observed that surfaces that irreversibly bind fibrinogen prompt greater acute inflammatory responses.[18,30] Adsorbed fibrinogen is seen to expose epitopes that may act as signals to cells including macrophages and neutrophils helping them adhere to the surface of the implant. Researchers have observed that the P1 epitopes binds the Mac-1 Integrin, a protein present in phagocytic cells.[18,31] Studies reveal the fibrinogen adsorbed to the biomaterial surface send signals to the mast cells to release histamine.[18,30] This causes phagocytes to migrate towards the implant and cumulate inflammatory cells. This marks the beginning of chronic inflammation and fibrosis.[16,18]

It has been observed that the inflammatory cells on adherence to the surface, spread, undergo a change in morphology and finally denature.[16,18] There is a motivation to decrease the amount of inflammatory cells, as they release harmful degradative enzymes towards the biomaterial, leading to the failure of the implant.[16]

CD11b is an integrin molecule that binds to intercellular adhesion molecule-1 present on the endothelial surface. The expression of CD11b at the surface of the leukocytes is associated with enhanced adherence, chemotaxis, opsonization and aggregation of inflammatory cells.[31,43,44] Leukocytes from sites of active inflammation have been shown to increase surface density of Mac-1 compared with leukocytes from non-inflamed tissues.[31,43,44]

The chemical composition of the surface of the biomaterial may modify the local inflammatory response, the secretion of cytokines/fibrogenic factors, and development of the fibrous capsule. Implants displaying surface hydroxyl groups (OH), have known to be potent in activating the complement cascade.[18] Such complement activation would be expected to promote inflammatory responses, such as found in the PP (-CH_3) coated with EDA(–NH_2) , which may prompt the accumulation of inflammatory cells by complement activation. It has also been previously observed that surface adsorption and partial 'denaturation' of fibrinogen is critical in triggering tissue responses, specifically acute and chronic inflammation. Amounts of 'denatured' fibrinogen has been seen to be high for NH_2; followed by the CF_3 & the OH group.[18]

Overall, our data on inflammatory response, based on the CD11b, to the four types of chemical surfaces provides evidence that surface functionality influences the foreign body response in host tissues. Thus chemical groups such as NH_2, present on the surface of implanted material, are capable of inducing acute inflammatory reaction.

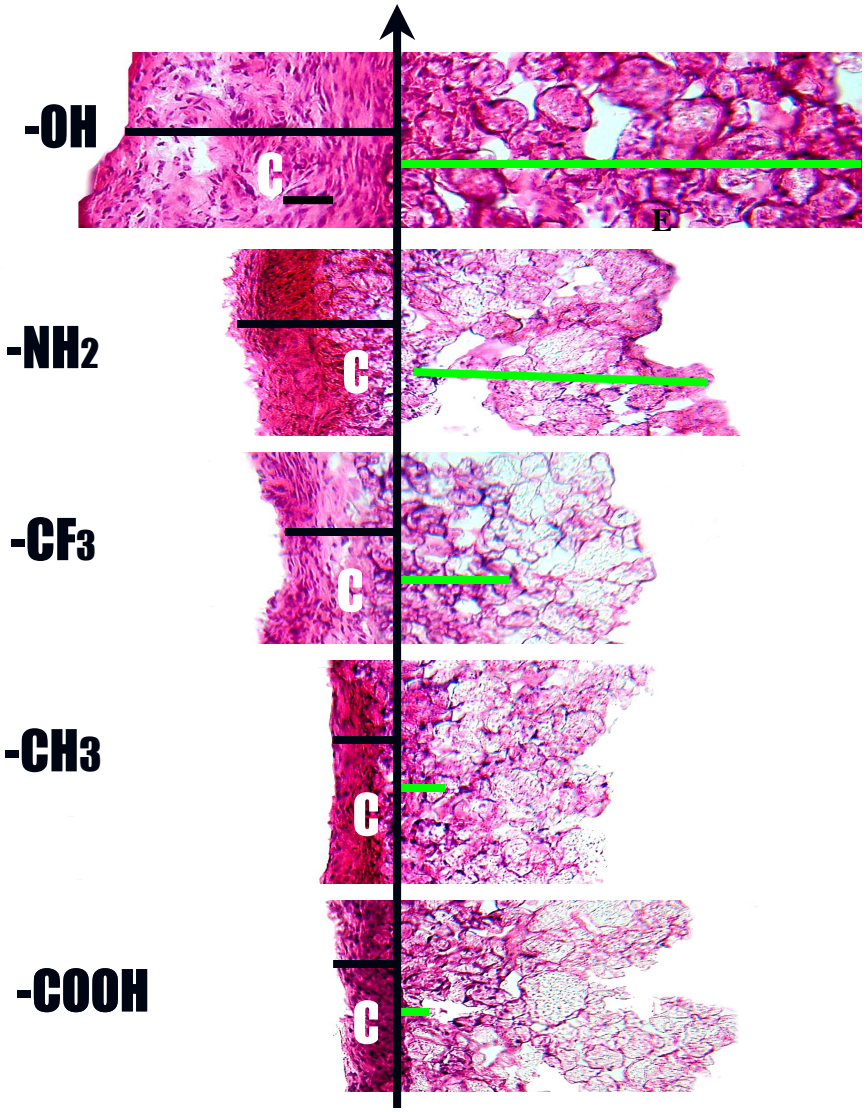

Figure 2.2 H&E stain of 2 week old skin tissue loaded with implant with different surface functionalities The presence of inflammatory cells ; defined by the purple stained nuclei, at the capsule **C**(marked by▬)represents the foreign body response triggered by the implant, for PP (A), EO2V (B), EDA (C), C6F14 (D) and VAA (E).Infiltration of the cells into the particles is marked by ▬ (Magnification 20x)

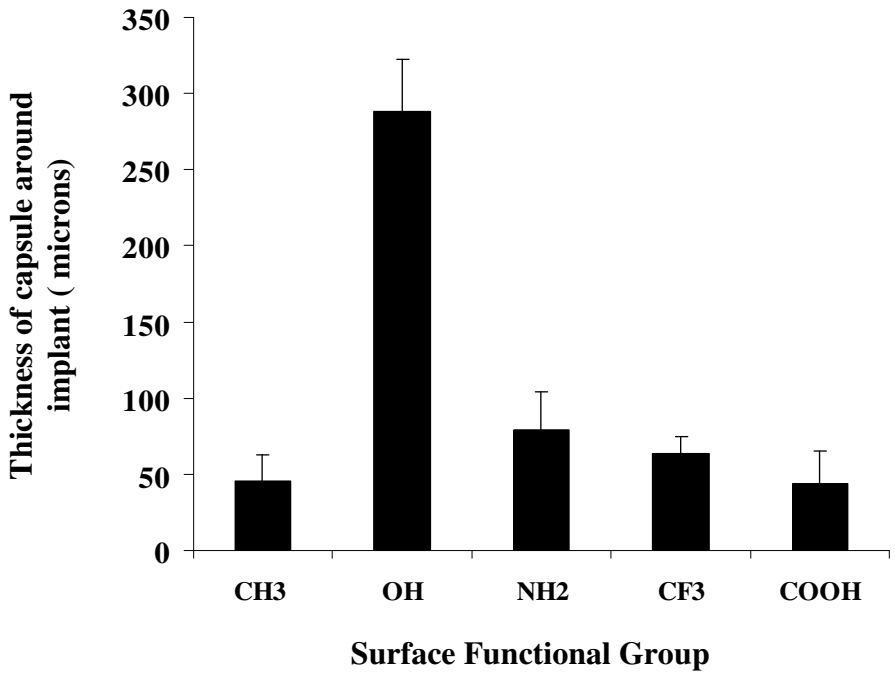

Figure 2.3 A graph depicting the thickness of the capsule mediated by the implantations. Polypropylene (PP) coated with different surface functionalities were subcutaneously implanted in Balb/C mice. The animals were sacrificed at 2 weeks post implantations. Values shown reflect the average thickness of the capsule. Vertical lines denote ± 1 SD (n= 4 for –CH_3, -NH_2, -OH, -CF_3 and -COOH)

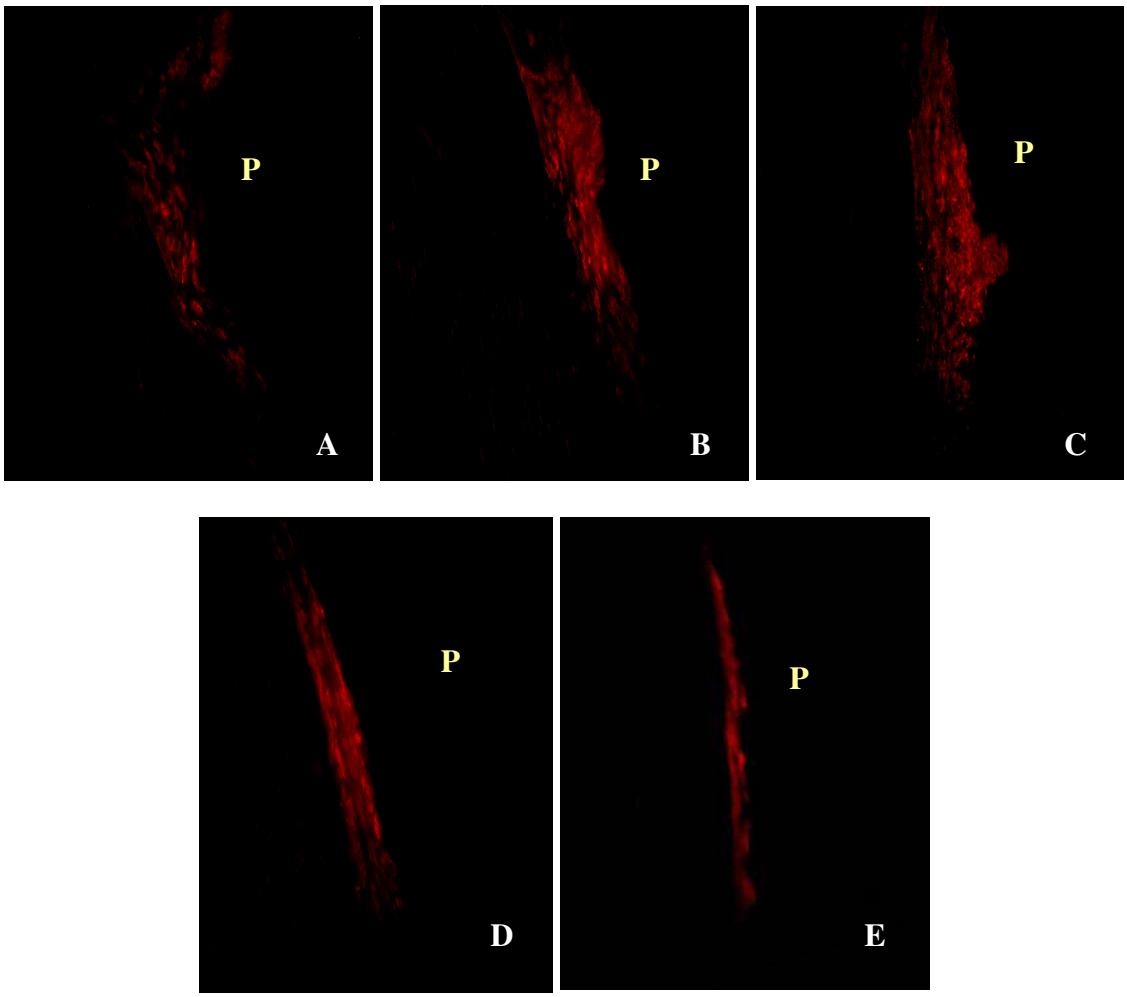

Figure 2.4 Immunohistochemical stain of 2 week old skin tissue loaded with implant with different surface functionalities showing the presence of inflammatory cell marker CD11b at the capsule triggered by the implant(P), for –CH_3 (A), -OH (B), –NH_2 (C), –CF_3 (D) and -COOH (E). (Magnification 40x)

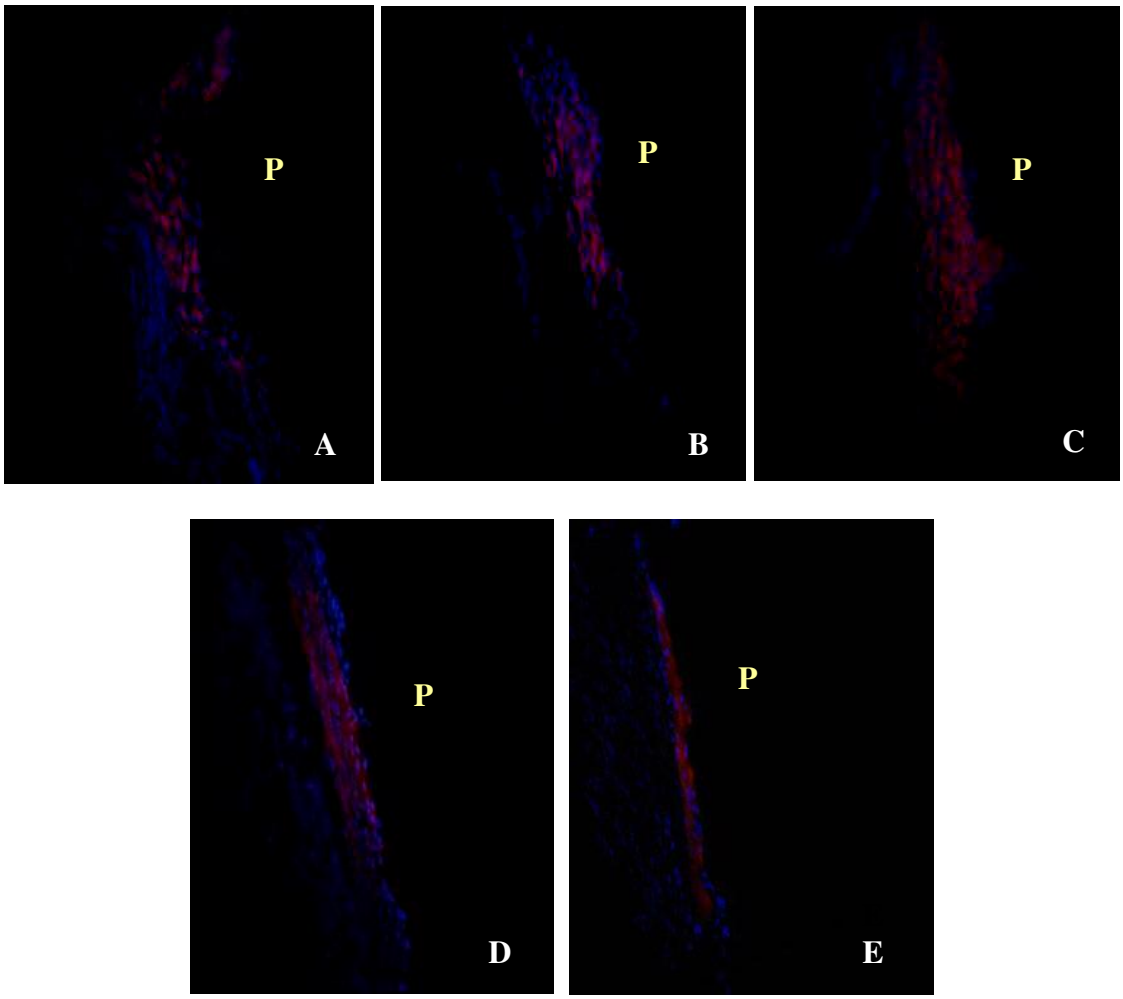

Figure 2.5 Overlapped images of Immunohistochemical stain and DAPI stain of 2 week old skin tissue loaded with implant with different surface functionalities showing the presence of inflammatory cell marker at the capsule triggered by the implant(P), for $–CH_3$ (A), -OH (B), $–NH_2$ (C), $–CF_3$ (D) and -COOH (E). (Magnification 40x)

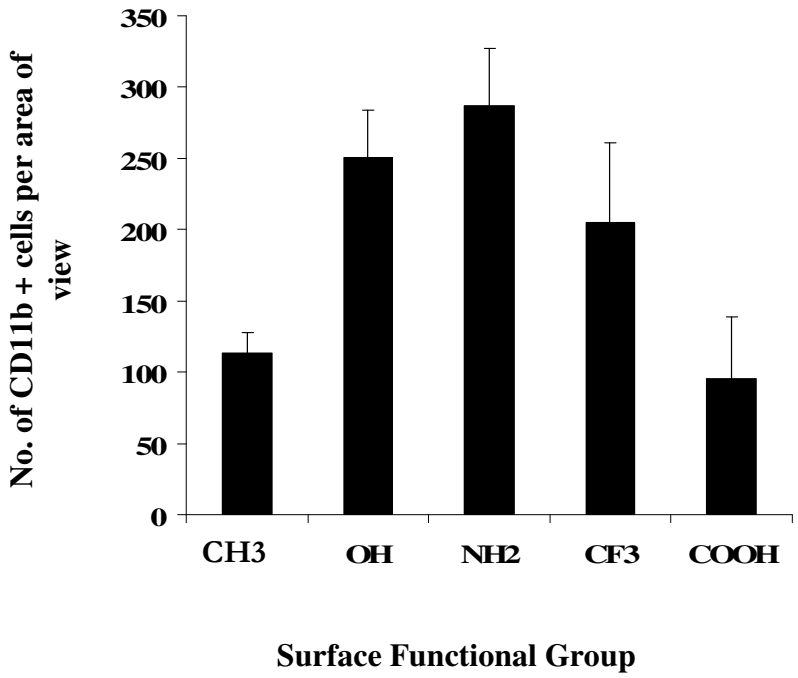

Figure 2.6 Accumulation of CD11b positive cells at the capsule. Polypropylene (PP) coated with different surface functionalities were subcutaneously implanted in Balb/C mice. The animals were sacrificed at 2 weeks post implantations. Values shown reflect the average number of CD11b+ cells seen at the capsule for different surface functionalities. Vertical lines denote ±1 SD (n= 4 for –CH_3, -NH_2, -OH, -CF_3 and -COOH)

CHAPTER 3

SURFACE CHEMISTRY OF IMPLANT INFLUENCES BIOMATERIAL MEDIATED FOREIGN BODY RESPONSE

3.1 Introduction

3.1.1 Foreign Body Response and Stem Cell Recruitment

With the knowledge that biomaterial implants trigger varied extents of inflammatory responses, extensive research has been underway in our laboratory using mice subcutaneous implantation model to study the process governing foreign body reactions to polymer particles. It has been accidentally discovered, using a panel of cell surface markers, that migratory stem cells accumulated in the fibrotic capsule or implants.[31] As the stem cell recruitment coincided with the migration of inflammatory cells, it has been assumed that stem cells would be actively recruited by inflammatory signals released owing to a biomaterial implantation, irrespective of the nature or composition of the implant.

Stem cells are cells that exhibit both self renewal and the ability to give rise to differentiated progeny. A stem cell can be classified as either embryonic stem cells (ESC) or an adult stem cell (ASC). Embryonic stem cells are primordial, undifferentiated cells derived from the developing embryo. Specifically, they are isolated from the inert cell mass of an embryo in the blastocyst stage, at which point the embryo has not yet implanted in the uterine lining.[32,33] The embryonic stem cells are

pluripotent –that is, they are able to generate all types of differentiated cells that make up the body.[32,33]

Adult, or somatic stem cells, by contrast, are undifferentiated cells found in more mature tissues. Adult stem cells are an attractive source for cell therapy, as they occur naturally in the various tissue compartments.[34,35] Although their exact point of origin is unclear, it is presumed that adult stem cells arise at point in fetal development from embryonic stem cells. Adult stem reside in different tissue compartments throughout the body, each filling its own stem cells "niche".[32,35] They are self renewing and either unipotent or multipotent, giving rise to cell types specific to their tissue of origin. Adult stem cells have been isolated from peripheral blood[32], cord blood[3], skin[32,38], liver[32,39], etc. Adult stem cells are divided into Hematopoietic stem cells (HSCs) and Mesenchymal Stem Cells (MSCs) based on their ability to differentiate.

Hematopoietic Stem cells

Hematopoietic stem cells (HSCs) are adult stem cells that give rise to blood and immune cells. They are cells isolated from the blood or bone marrow that can renew themselves, can differentiate to a variety of specialized cells, can mobilize out of the bone marrow into circulating blood, and can undergo programmed cell death, called apoptosis—a process by which cells that are detrimental or unneeded self-destruct.[32,40] Bone marrow is considered the abundant source of HSC; recently discovered sources include the umbilical cord, peripheral blood and Wharton's jelly of the placenta.[32,36].

Mesenchymal Stem Cells

In addition to the hematopoietic stem cells (HSCs), the bone marrow also contains the mesenchymal stem cells (MSCs). Mesenchymal stem cells are non-hematopoietic, stromal cells that exhibit multilineage differentiation capacity being capable to give rise to diverse tissues, including bone, cartilage, adipose tissue, tendon and muscle.[39,41] They can rapidly divide and form colonies. Expanded cells could be guided to differentiate along multiple phenotypic pathways through specific media containing growth factors.[32,39,41] They are essential components of the hematopoietic microenvironment and play an important role in the hematopoietic physiology.[32,39,41]

Identification of adult stem cells is based on cell morphology, plasticity and cell surface markers which are found in the tissue where they belong.[32] Scientists have long been seeking a good way to identify stem cells. The majority of researchers who lay claim to having identified adult stem cells rely on two of these characteristics—appropriate cell morphology, and the demonstration that the resulting, differentiated cell types display surface markers that identify them as belonging to the tissue.[32] While stem cells are best defined functionally, a number of molecular markers have been used to characterize various stem cell populations.[32]

To determine the presence and the extent of stem cells, in this Thesis, we employed three markers, as below:

Src Homology 2- B (SH2-B)

The SH2-B, an Src homology 2 (SH2) and pleckstrin homology domain-containing adaptor protein takes part in cell movement regulation and morphology. It forms a part of the signaling network. Alternative splicing of the SH2-B mRNA produces at least four isoforms (α, β, γ, and δ) that differ in their C termini after the SH2 domain: therefore, all isoforms are expected to bind to similar tyrosine kinases via their SH2 domains.[31,45,46] These isoforms are found to be expressed in the lung, brain, liver, skeletal muscle, and fat. SH2-B binds via its SH2 domain to insulin receptor in response to insulin; however, its physiological role remains unclear. SH2-Bβ is composed of a pleckstrin homology domain, an SH2 domain, and multiple phosphorylation sites.[45,46] binds to JAK2 (Janus Kinase 2) via its SH2 domain, resulting in potentiation of JAK2 activation in response to growth hormone in cultured cells. SH2-Bβ also binds via its SH2 domain to multiple receptor tyrosine kinases including receptors for insulin, insulin-like growth factor 1, platelet-derived growth factor, fibroblast growth factor, and nerve growth factor receptor TrkA.[45-47]

Stem cell Factor (SCF)

Stem cell factor (SCF) binds to c-Kit and is an important mediator of survival, growth, and function of hematopoietic progenitor cells and mast cells. C-Kit is an RTK encoded by the c-*kit* proto-oncogene.[48,49] The c-Kit ligand is stem cell factor (SCF). The absence of either SCF or c-Kit is lethal, and decreases in expression or function of ligand or receptor results in macrocytic anemia, mast cell deficiency, aberrations in pigmentation, and sterility.[48,49] The SCF ligand is seen to be important for the

development of germ cells, melanocyte precursors and hematopoietic cells. It is also known to promote the maturation and proliferation of early mast cells. SCF exists in fibroblasts and brain in its isoform, large form, and in spleen, testis, placenta and cerebellum in its isoform, small form.[31,48,49]

Nanog

Nanog is a divergent homeodomain protein that directs propagation of undifferentiated ES cells. It plays a significant role in maintaining the pluripotency of ESCs as well as its self-renewal by regulating the gene expressed for proliferation and differentiation of these stem cells.[31,50,51] It has been observed that Nanog is highly expressed in undifferentiated embryonic stem cells and undergoes down regulation during differentiation.[50,51]

3.1.2 Previous Observation

We accidentally discovered the presence of stem cells, using a panel of cell surface markers, in a previous study undertaken in our laboratory involving the implantation of microparticles in the subcutaneous tissue of mice.[31] The tissues were explanted and the cells associated were studied at different time points of implantation. In order to identify the presence of stem cells, some stem cell markers claimed to characterize stem cells were used. It was observed that the stem cells were concentrated and existent at the host tissue-implant interface (near the capsule). It was also observed that the number of implant associated stem cells increased with implantation time.[31] Due to the coexistence of stem cells and inflammatory cells, we attributed the foreign body response as the primary factor for triggering stem cell recruitment.

However, we do not know if the surface chemistry of the implant influenced the recruitment of the stem cells recruited at the host tissue-implant interface. Thus, we need to conduct more experiments to establish whether stem cells could be recruited, in all biomaterials, irrespective of surface chemistry.

3.2 Second Hypothesis

Surface chemistry of the implant influences foreign body response and in turn modulates the recruitment of stem cells to the site of implantation.

3.3 Experimental Section

3.3.1 Materials and Methods

Ethylene diamine, Di (ethylene glycol) vinyl Acetate and Vinyl Acetic acid were obtained from Sigma Chemical Company (St.Louis,MO) and Perflurohexanes from PCR Chemical (Gainesville,FL). This was done in collaboration with Dr Richard B Timmons Laboratory at the University of Texas at Arlington.

The following table enlists the antibodies used in this study.

Table 3.1 List of Antibodies used in this study

Antibody Name	Animal Source	Antibody Type	Company	Dilution Used
SH2	Mouse	IgG_1	Pharmigen	1: 100
SCF	Rabbit	IgG	Santa Cruz	1: 100
Nanog	Rabbit	IgG	Chemicon	1: 500

Primary antibodies against SCF were purchased from Santa Cruz Biotechnology Inc. SH2B was purchased from BD Biosciences Pharmigen. Nanog was purchased from Chemicon International, Inc. Secondary antibodies (1:200) labeled with FITC or Texas Red was from Jackson ImmunoResearch laboratories, Inc. (West Grove, PA). DAPI

(1:200) for nuclear staining was obtained from Cambio Ltd., UK. Prolong gold antifade reagent (Molecular Probes, OR) was mounted on the samples after the experiment to preserve the fluorescence. Microscopy analysis were done using the Leica Standard Microscope (Leica Microsystems, Wetzlar, GmbH) equipped with a Nikon E500 Camera(8.4V,0.9A, Nikon Corp., Japan)

3.3.2 Animal Implantation Model

In order to test the hypothesis, we employed animal models in which test specimens of Polypropylene subject to Plasma polymerization and coated with Ethylene Diamine (-NH_2), Di (ethylene glycol)-vinylether (-OH), Perflurohexane(-CF_3) and Vinyl Acetic Acid (-COOH), were implanted subcutaneously in the back of the Balb/C mice. Uncoated Polypropylene (-CH_3) was employed as a control for the study and also implanted subcutaneously using a similar animal model of Balb /C mice. 2 weeks after implantation, the implant-bearing mice were sacrificed and the implants and the surrounding tissues were recovered for analyses. To assess the biomaterial implant-mediated cell recruitment and tissue responses, the explants were fixed and frozen sectioned. The stem cell recruitment for different specimens was observed by the presence of various stem cell markers.

3.4.3 Immunohistochemistry

For immunostaining, the tissue samples were incubated with the primary antibody, (Nanog, SCF and SH2B), individually, for two hours at 37C. The primary antibody was conjugated with FITC for one hour at 37C. The tissue samples were then rinsed in Phosphate Buffered Saline (PBS) for 15 min. This was followed by DAPI

staining for 5 min. PBS was used to rinse for 5min. Antifade was mounted on the slide and a cover slip was placed for microscopy.

3.4 Results & Discussion

To prove our hypothesis that biomaterial implantation modulates the recruitment of stem cells, we performed a routine H&E histology stain to mark the location of the capsule, implant and surrounding tissue. We carried out immunostaining using various stem cell markers for the implant bearing skin tissue samples mentioned and have included the figure below (Fig 3.1, Fig 3.3, Fig 3.5). Histograms depicting the variation in stem cell number amongst the 5 specimens are also shown (Fig 3.2, Fig 3.4, Fig 3.6)

3.4.1 Assessment of Stem Cell Recruitment using Nanog

Nanog is a divergent homeodomain protein that directs propagation of undifferentiated ES cells. It plays a significant role in maintaining the pluripotency of ESCs as well as its self-renewal by regulating the gene expressed for proliferation and differentiation of these stem cells. It has been observed that Nanog is highly expressed in undifferentiated embryonic stem cells and undergoes down regulation during differentiation.[31,50,51]

From the pictures shown (Fig 3.1) we can observe that there does seem to be a difference in the expression of Nanog between the 5 specimens. We also carried out DAPI staining for quantitative analyses of the inflammatory cells obtained from immunostaining (pictures not shown here)

Histogram representing the stem cells outside the capsule at the tissue-implant interface (Fig 3.2), shows -COOH with the maximum cell number of 280.25 ±28.02 cells per field of view. $–CF_3$ follows with a cell number of 208.5± 15.15 cells per field of view, while $–NH_2$ shows a cell count of 106±12.40 cells per field of view. -OH shows the least cell number of 39.5±8.54 cells per field of view. The control $–CH_3$ shows a cell count of 224.1± 12.35 cells per field of view.

3.4.2 Assessment of Stem Cell Recruitment using Stem Cell Factor (SCF)

A second marker that we used to identify the stem cells is Stem Cell Factor (SCF). Stem cell factor (SCF) binds to c-Kit and is an important mediator of survival, growth, and function of hematopoietic progenitor cells and mast cells. The SCF ligand is seen to be important for the development of germ cells, melanocyte precursors and hematopoietic cells.[31,48,49] It is also known to promote the maturation and proliferation of early mast cells. SCF exists in fibroblasts and brain in its isoform, large form, and in spleen, testis, placenta and cerebellum in its isoform, small form.[31,48,49]

From the Fig 3.3 we see that the expression of SCF by the cells varies across the 5 specimens considered in the study. Perhaps the surface chemistry of the implants influenced the recruitment leading to the variation in the expression of the SCF marker. DAPI staining for quantitative analyses of the stem cells obtained from immunostaining was done and microscopic images were overlapped for cell counting.

A histogram depicting the expression of SCF in cells recruited in response to each specimen as shown in Fig 3.4. reveals that, -COOH shows the maximum, with a cell number of 124.25 ± 5.74 cells per field of view. $–CF_3$ follows with a cell number

of 68.25 ± 6.60 cells per field of view, while –NH_2 shows a cell count of 49.75 ±4.11 cells per field of view. –OH shows the least cell number of 41.5 ±10.3 cells per field of view. The control –CH_3 shows a cell count of 90.01± 8.76 cells per field of view.

3.4.3 Assessment of Stem Cell Recruitment using SH2-B

A third marker that we used to identify the stem cells is SH2-B, a Src homology 2 (SH2) and pleckstrin homology domain-containing adaptor protein that takes part in cell movement regulation and morphology. It forms a part of the signaling network. Alternative splicing of the SH2-B mRNA produces at least four isoforms (α, β, γ, and δ) that differ in their C termini after the SH2 domain; therefore, all isoforms are expected to bind to similar tyrosine kinases via their SH2 domains. These isoforms are found to be expressed in the lung, brain, liver, skeletal muscle, and fat. SH2-B binds via its SH2 domain to insulin receptor in response to insulin; however, its physiological role remains unclear.[31,45,46,47] SH2-Bβ is composed of a pleckstrin homology domain, an SH2 domain, and multiple phosphorylation sites. It binds to JAK2 (Janus Kinase 2) via its SH2 domain, resulting in potentiation of JAK2 activation in response to growth hormone in cultured cells SH2-Bβ also binds via its SH2 domain to multiple receptor tyrosine kinases including receptors for insulin, insulin-like growth factor 1, platelet-derived growth factor, fibroblast growth factor, and nerve growth factor receptor TrkA[45-47]

From the Fig 3.5 we see that the expression of SH2-B by the cells varies across the 5 specimens considered in the study. The surface chemistry of the implants may be

responsible for this difference in the expression of the SCF marker. DAPI staining for quantitative analyses was also carried out (not shown here).

A histogram depicting the expression of SH2-B positive cells recruited in response to the implantation of each specimen as shown in Fig 3.6, shows -COOH to have maximum number of stem cells around the tissue-implant interface, with a cell number of 317.75 ± 11.81 cells per field of view. $–CF_3$ follows with a cell number of 231.10 ± 51.97 cells per field of view, while $–NH_2$ shows a cell count of 141.25 ±29.84 cells per field of view. –OH shows the least cell number of 53±21.18 cells per field of view. The control $–CH_3$ shows a cell count of 193.25± 43.25 cells per field of view.

The presence of various stem cell markers as seen by the strength of the fluorescent signal, around the capsule, shows that the cells with stem cell markers are actively recruited to the implantation site. It is surprising to note that the number of stem cells seen at various surfaces show a difference. In other words the distribution and/recruitment of stem cells around the implant is not the same throughout the surface functionalities used in the study. Surface with -COOH group shows the maximum stem cell marker + cells that with –OH and $-NH_2$ show comparatively much lesser positive cells, for all three markers tested in this study.

It is suggested that the inflammatory milieu characterizing many pathologies, may act as a pathway that activates stem cell molecular programs during injury suggesting that inflammation may be viewed not simply as playing an adverse role but also as providing stimuli that recruit cells with a regenerative homeostasis-promoting capacity.[53] Based on a previous study , conducted in our laboratory, we could attribute

Foreign Body response as one of the factors that triggers stem cell recruitment [31]. Other possible factors though to influence stem cell recruitment could be over activation of neutrophils and macrophages, inhibitory by-products of the inflammatory cascade or the influence of a chemokine or protein.

We observe from the data obtained that surface functionality may be a candidate for being a potential factor that influences recruitment of stem cells in response to an implanted biomaterial. Our results indicate that -NH_2 shows lesser stem cells than that of –CF_3 with all three stem cell factors. This suggests that factors associated with surface chemistry may be the factor to cause this difference in stem cell number amongst various groups. Accordingly, we believe that surface property; in particular surface functionality might be one of the stimulating factors for migration and adherence of stem cells to the implant.

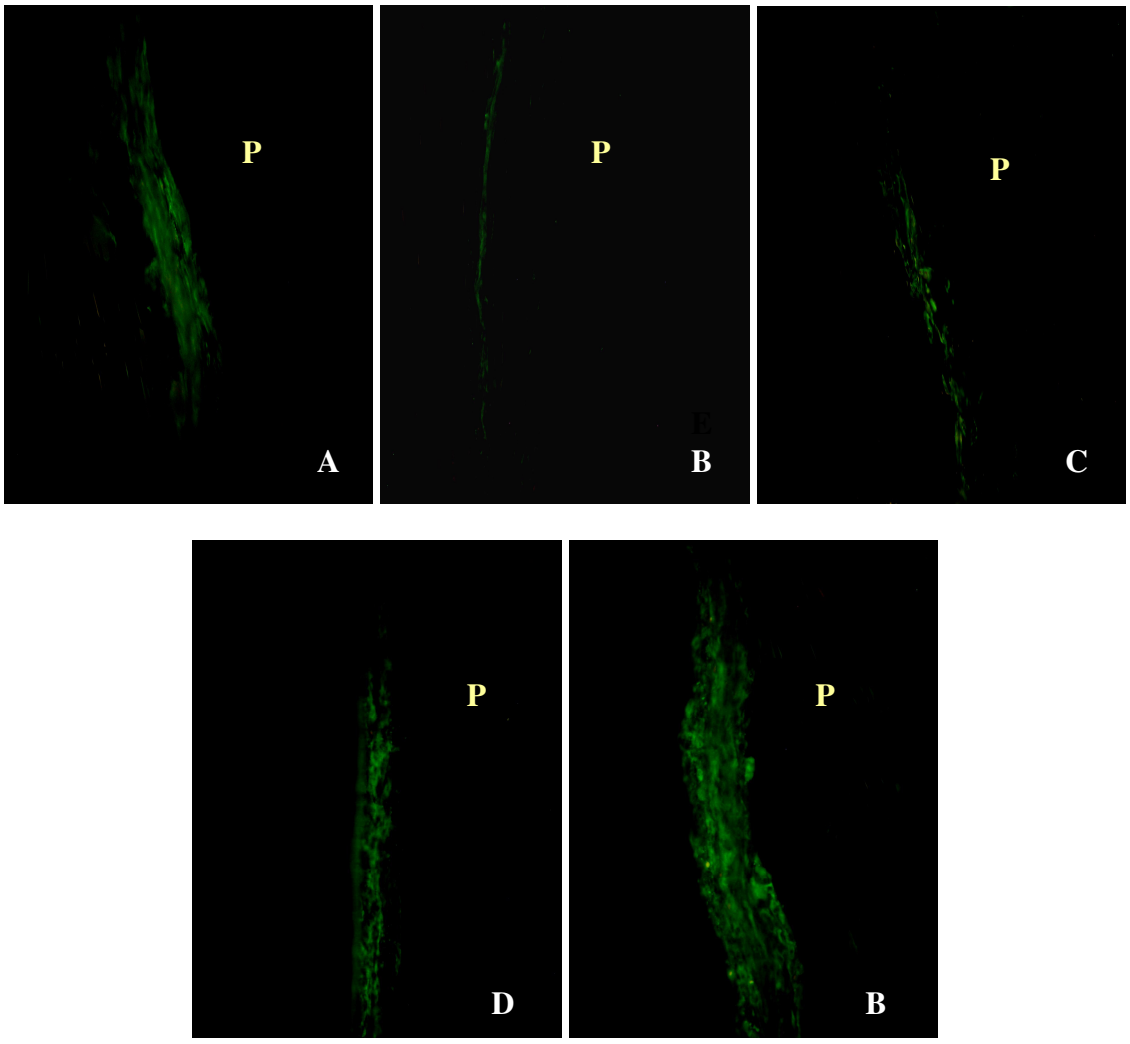

Figure 3.1 Immunohistochemical stain of 2 week old skin tissue loaded with implant with different surface functionalities showing the presence of stem cell marker Nanog at the capsule surrounded by the implant(P), for $–CH_3$ (A), -OH (B), $–NH_2$ (C), $–CF_3$ (D) and -COOH (E). (Magnification 40x)

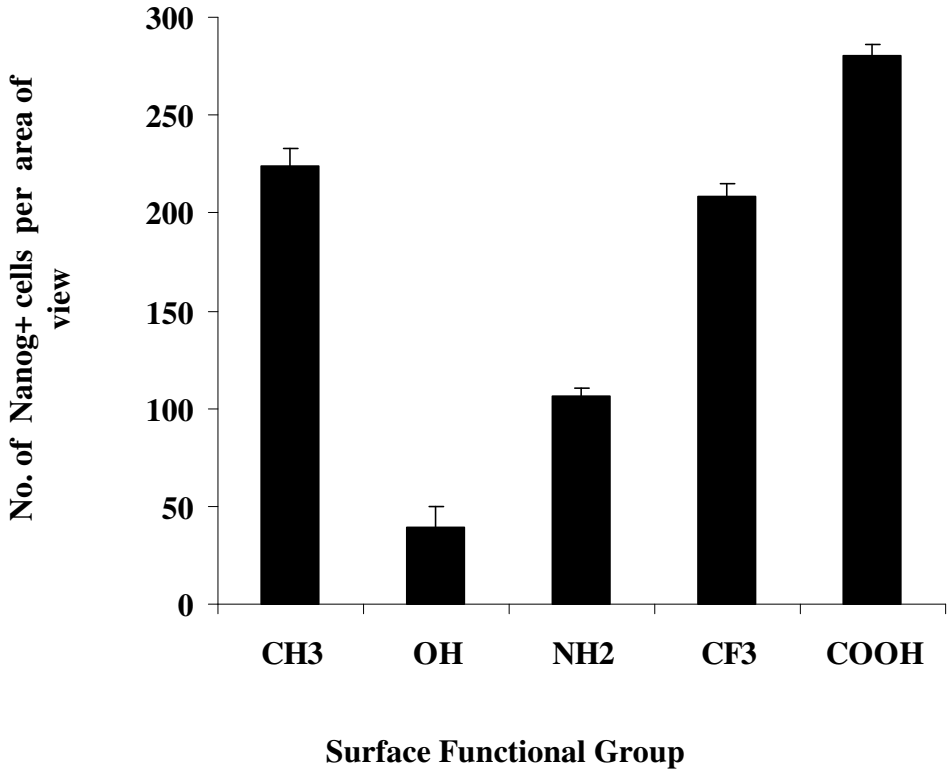

Figure 3.2 Accumulation of Nanog positive cells at the capsule. Polypropylene (PP) coated with different surface functionalities were subcutaneously implanted in Balb/C mice. The animals were sacrificed at 2 weeks post implantations. Values shown reflect the average number of Nanog+ cells seen at the capsule for different surface functionalities. Vertical lines denote ± 1 SD (n= 4 for $–CH_3$, $-NH_2$, -OH, $-CF_3$ and -COOH)

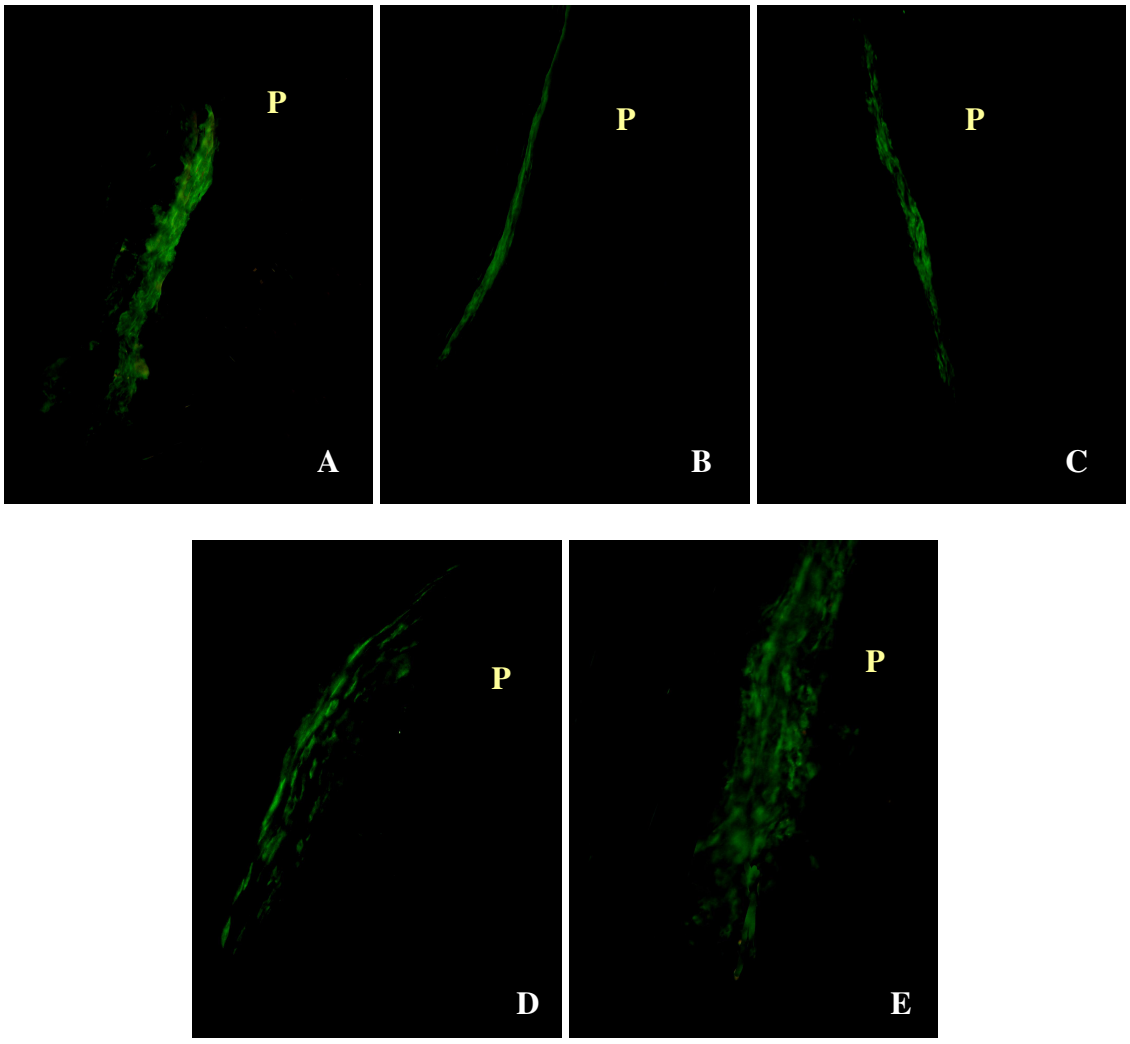

Figure 3.3 Immunohistochemical stain of 2 week old skin tissue loaded with implant with different surface functionalities showing the presence of stem cell marker SCF at the capsule surrounded by the implant(P), for $–CH_3$ (A), -OH (B), $–NH_2$ (C), $–CF_3$ (D) and -COOH (E). (Magnification 40x)

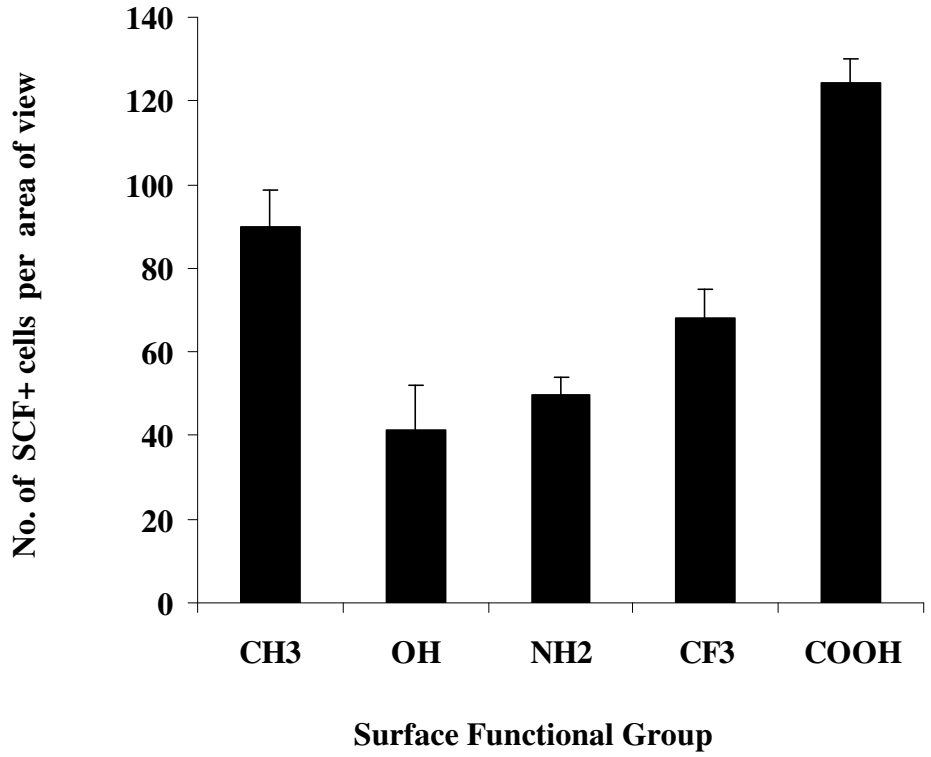

Figure 3.4 Accumulation of SCF positive cells at the capsule. Polypropylene (PP) coated with different surface functionalities were subcutaneously implanted in Balb/C mice. The animals were sacrificed at 2 weeks post implantations. Values shown reflect the average number of SCF+ cells seen at the capsule for different surface functionalities. Vertical lines denote ± 1 SD (n= 4 for–CH_3, -NH_2, -OH, -CF_3 and -COOH)

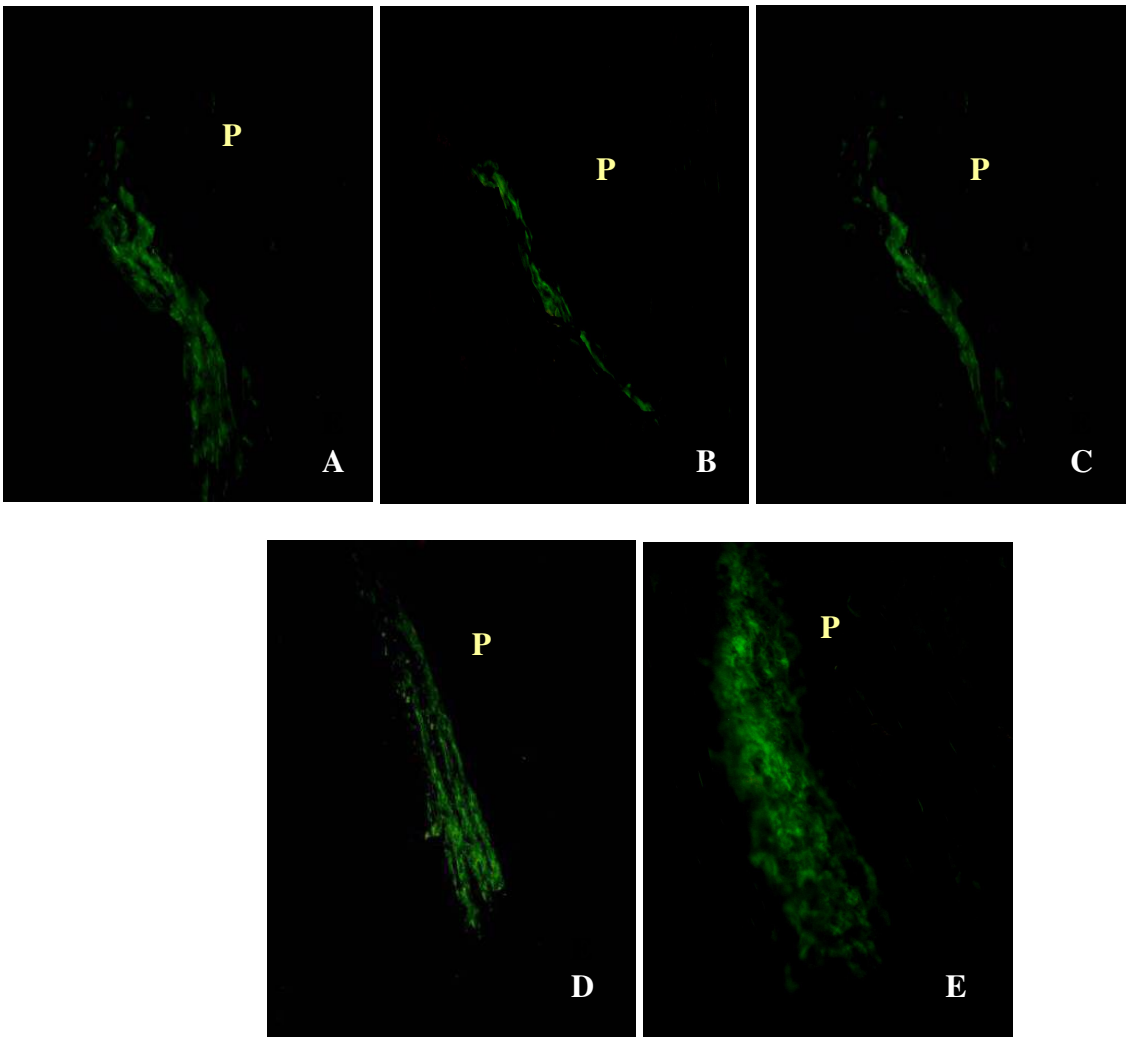

Figure 3.5 Immunohistochemical stain of 2 week old skin tissue loaded with implant with different surface functionalities showing the presence of stem cell marker SH2B at the capsule surrounded by the implant(P), for $–CH_3$ (A), -OH (B), $–NH_2$ (C), $–CF_3$ (D) and -COOH (E). (Magnification 40x)

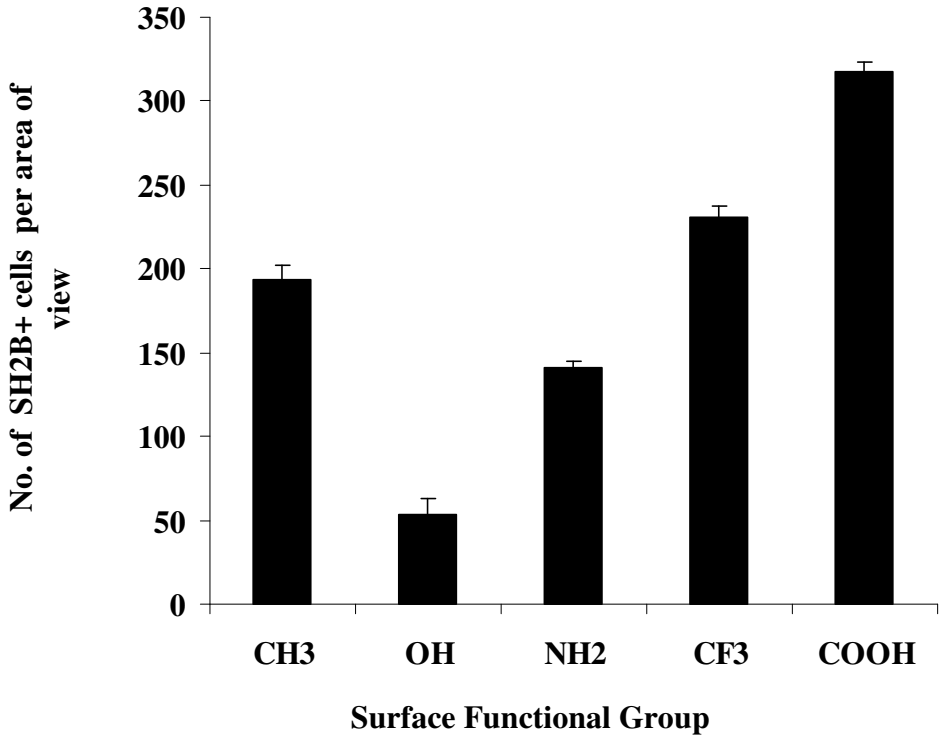

Figure 3.6 Accumulation of SH2-B positive cells at the capsule. Polypropylene (PP) coated with different surface functionalities were subcutaneously implanted in Balb/C mice. The animals were sacrificed at 2 weeks post implantations. Values shown reflect the average number of Nanog+ cells seen at the capsule for different surface functionalities. Vertical lines denote ± 1 SD (n= 4 for –CH_3, -NH_2, -OH, -CF_3 and -COOH)

CHAPTER 4

RELATION BETWEEN FOREIGN BODY RESPONSE AND STEM CELL RECRUITMENT

4.1 Introduction

4.1.1 Inflammatory Response Triggers Stem CellR ecruitment

With the knowledge that biomaterial implants trigger varied extents of inflammatory responses, extensive research has been underway in our laboratory using mice subcutaneous implantation model to study the process governing foreign body reactions to polymer particles. It has been accidentally discovered, using a panel of cell surface markers that migratory stem cells accumulated in the fibrotic capsule or implants.[31] As the stem cell recruitment coincided with the migration of inflammatory cells, it has been assumed that stem cells would be actively recruited by inflammatory signals released owing to a biomaterial implant, irrespective of the nature or composition of the implant.

4.1.2 Previous Observation

While conducting experiments to determine the role of surface chemistry in stem cell recruitment , we determined the presence of both the CD11b + (inflammatory marker) cells (Fig 2.3, Fig 2.4 and Fig 2.5) and SH2B+ (stem cell marker) cells (Fig.3.5 and Fig.3.6). We also found that they exist side by side outside the capsule, with the inflammatory cells being in the closer proximity of the capsule. This tells us that

perhaps both the inflammatory cells and stem cells migrate to the implantation site . This suggests that both these cells respond to the trauma caused by the biomaterial implantation, possibly in the same way, hence it is likely that stem cells are synonymous with inflammatory cells.

4.2 Third Hypothesis

Stem cells are a type of inflammatory cells.

4.3 Experimental Section

4.3.1 Data Analysis

In order to test this hypothesis we employed the skin tissue samples bearing the implant explanted at the end of 2 weeks for histological analyses. The tissue samples, placed side by side, were incubated with inflammatory cell marker, CD11b and stem cell marker SH2B for two hours at 37C. CD11b was conjugated with Texas Red, a red fluorescent tag while SH2B was conjugated with Fluorescein Isothyocyanate (FITC), a green fluorescent tag, for one hour at 37C. The tissue samples were then rinsed in Phosphate Buffered Saline (PBS) for 15 min. This was followed by DAPI staining for 5 min. PBS was used to rinse for 5min. Antifade was mounted on the slide and a cover slip was placed for microscopy.

4.4 Results and Discussion

Using the DAPI staining as the reference the microscopic images obtained were compared and merged for analyses as observed.

4.4.1 Inflammatory Cells Do Not Have Stem Cell Markers

Our data shows the cells on and around the capsule expressing the inflammatory marker CD11b, while the layer of cells around the capsule, towards the muscle expressing the Stem cell marker SH2B. No overlap is observed, indicating that the inflammatory and stem cells exist side by side and do not infiltrate into each other, around the capsule. This also rules out the possibility that Inflammatory cells may respond to stem cell markers. Further, no infiltration of stem cells was seen inside the capsule (not shown here).

4.4.2 Increase in CD11b+Cells Accompanied by Decrease in SH2B+ Cells

Cell number quantification and comparison between the CD11b+ cells and SH2B+ cells, interestingly , reveals an inverse relation between the two. Our results indicate that an increase in CD11b+ cells is accompanied by a corresponding decrease in SH2B+ cells. All of this tells us that even though stem cells respond to the trauma caused by the implantation of a biomaterial, they are distinct and different from the inflammatory cells.

Overall, our results indicate that CD11+ cells are not identical with the SH2B+ cells. The lack of inflammatory marker expression on stem cells led us to believe that stem cells are not a group of inflammatory cells. Thus showing that stem cells are distinct and different from inflammatory cells. Since both the cells are seen to migrate to the implantation site we are led to believe that there exists a possibility of a relation between stem cells recruitment and the inflammatory stimuli. Studies by Imitola et al shows human NSCs migrate *in vivo* (including from the contralateral hemisphere) toward an infarcted area (a representative CNS injury), where local astrocytes and endothelium up-regulate the inflammatory chemoattractant stromal cell-derived factor 1

(SDF-1)[53] It is also known that during the process of inflammation, certain byproducts are released.[16] We believe it is possible that these may also have a role to play in the inverse relation existing between CD11b+ and SH2B + cells.

Though a detailed mechanism underlying this type of implant surface mediated stem cell recruitment is yet to be determined, a better understanding of this complex dynamic may also permit us to devise more effective repair strategies by neutralizing those aspects of inflammation that are inimical to progenitor well being while enhancing those aspects that facilitate repair.

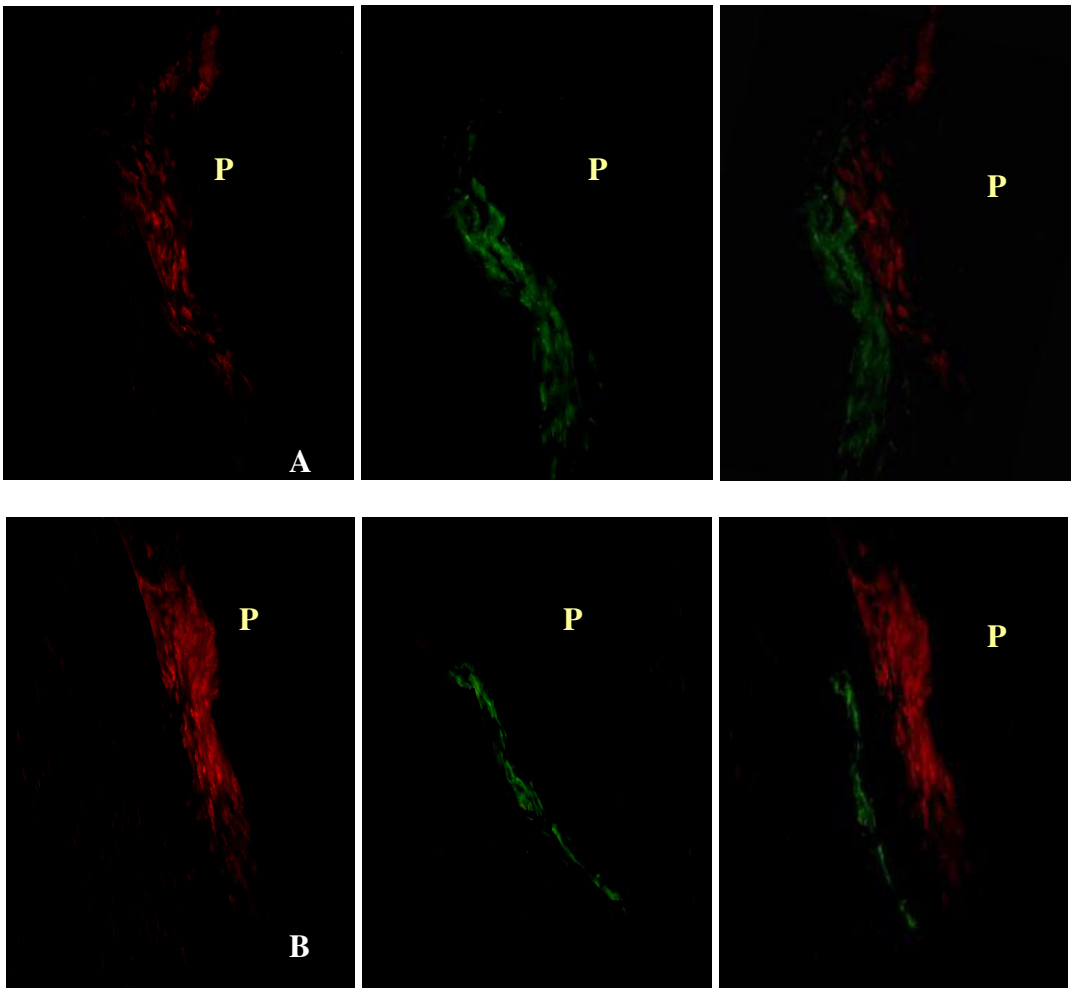

Figure 4.1 Overlapped Immunohistochemical stain images obtained from 2 week old skin tissue loaded with implant with different surface functionalities showing the presence of inflammatory cell marker CD11b (red) and stem cell marker SH2B (green) at the capsule surrounding the implant(P), for $-CH_3$ (A), -OH (B), (Magnification 40x) The pictures show that stem cells are significantly distinct and different from inflammatory cells.

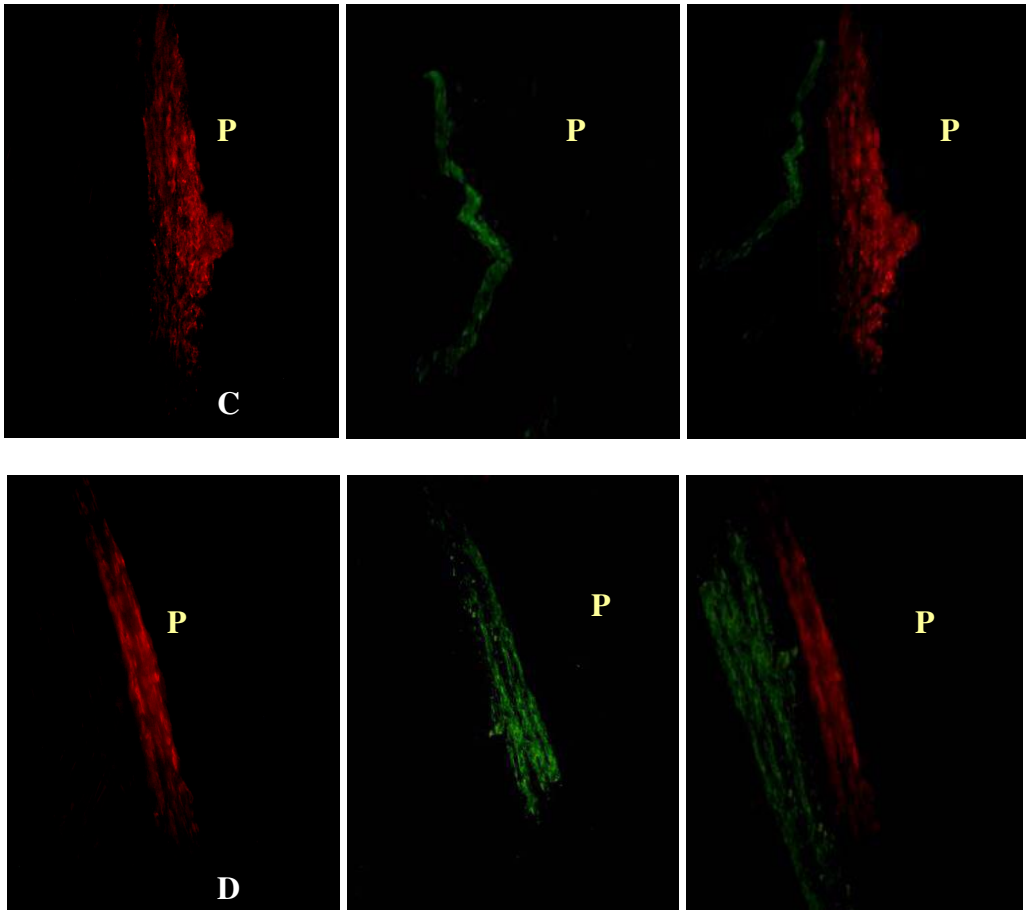

Figure 4.2 Overlapped Immunohistochemical stain images obtained from 2 week old skin tissue loaded with implant with different surface functionalities showing the presence of inflammatory cell marker CD11b (red) and stem cell marker SH2B (green) at the capsule surrounding the implant(P),–NH_2 (C) and –CF_3 (D). (Magnification 40x) The pictures show that stem cells are significantly distinct and different from inflammatory cells.

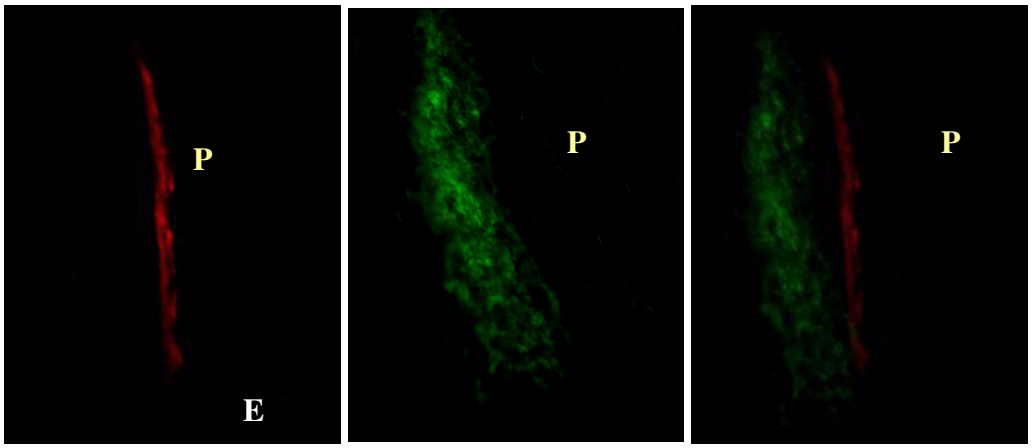

Figure 4.3 Overlapped Immunohistochemical stain images obtained from 2 week old skin tissue loaded with implant with different surface functionalities showing the presence of inflammatory cell marker CD11b (red) and stem cell marker SH2B (green) at the capsule surrounding the implant(P) -COOH (E). (Magnification 40x) The pictures show that stem cells are significantly distinct and different from inflammatory cells.

CHAPTER 5

SUMMARY

The physical and chemical characteristics of material surfaces are thought to play important roles in biomaterial-mediated tissue responses. The work described in this thesis concerns the contribution of the surface chemistry of an implant to the thickness of the capsule formed around it and the associated recruitment of inflammatory cells to the site of implantation. Interestingly, we have also uncovered that many cells stained for stem cell markers like SCF, Nanog and SH2B.

Implantation of Polypropylene, surface coated with various monomers using pulsed radio frequency plasma polymerization (RGFD), to obtain different functionalities, including $–NH_2$ from ethylene diamine, -OH from di(ethylene glycol) vinyl ether, $-CF_3$ from perflurohexane and –COOH from vinyl acetic acid, induced formation of fibrous capsules around the implant as well as triggered the recruitment of inflammatory cells with leukocyte marker CD11b. Our results indicate the possibility that recruited inflammatory cells may influence the formation of the fibrotic tissue associated with the type of the surface. We propose that the fibrous capsule increases in thickness around implants when chemical groups, such as –OH and $-NH_2$, capable of inducing acute inflammatory responses and recruiting large number of inflammatory cells, are present on the surface of the implant.

Stem cells are recruited to the site of implantation. The presence of various stem cell markers as seen by the strength of the fluorescent signal, around the capsule, shows that the cells with stem cell markers are actively recruited to the implantation site. Our results show that surface functionality-NH_2 shows lesser stem cells than that of $–CF_3$ with all three stem cell factors. This suggests that factors associated with surface chemistry cause this difference in stem cell number amongst various groups.

In addition, comparison of the staining with CD11b and SH2B+, using DAPI as reference, we observed the co-presence of CD11b+ and SH2B+ cells. The lack of inflammatory markers expression on stem cells, led us to believe that stem cells are not a group of inflammatory cells. Microscopic analysis along with comparative study represents that a decrease in stem cell is marked by the increase of inflammatory cells, ruling out the possibility that stem cells are inflammatory cells. Thus stem cells are distinct and different from the inflammatory cells , though they co-exist side by side at the capsule.

Through this study, we support the idea that the surface functional groups of the implants greatly influence the host tissue responses as well as the recruitment of stem cells to the site of implantation. Though a detailed mechanism underlying this type of implant surface mediated stem cell recruitment is yet to be determined, a more comprehensive understanding of the surface functional group: stem cell interaction may permit a purposeful insight into a potential technique for isolation of adult stem cells using the most biocompatible material. A better understanding of this complex dynamic may also permit us to devise more effective repair strategies by neutralizing those

aspects of inflammation that are inimical to progenitor well being while enhancing those aspects that facilitate repair.

CHAPTER 6

FUTURE PROSPECTS

Surface chemistry of an implant (particles) is seen to influence the inflammatory response mediated by the biomaterial inside the host body. Stem cells recruited to the site of the implant is also seen to be influenced by the surface functionality of the implant. The inverse relation observed between the inflammatory cells recruited to the implant and that of stem cell recruitment and its dependency on surface chemistry, indicates a possibility of fabricating particles with the most suitable surface functionality that can minimize inflammatory response to the highest extent possible, and at the same time stimulate high recruitment of stem cells. We believe such a surface would be a promising candidate for not only developing the most biocompatible particles for drug delivery but also provide a potential technique for isolation of adult stem cells required for therapy.

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BIOGRAPHICAL INFORMATION

Shwetha Kamath, (Sihi), was born in Mangalore, India, on the 25th January 1980. She graduated with a Bachelors Degree in Biomedical Engineering, from the Manipal Institute of Technology, Manipal in 2000. She continued her research in the field of Medical Imaging and pursued a novel project in Telemedicine, which came to be recognized by the President of India, Dr APJ Abdul Kalam. Following this, she joined GE Medical Systems, Bangalore, India, as a Lead for Cardiology Monitoring Systems. After getting married, she relocated to Dallas-Fort Worth, TX, in 2003 to join her husband.

Currently, a Graduate student at the University of Texas at Arlington, Sihi, intends to pursue her interests in research and development in the field of Tissue Engineering. With all course requirements fulfilled, she would soon be graduating with a Masters Degree in Bioengineering.

She firmly believes that Tissue Engineering/Regenerative Medicine will drive every imagination in traditional medicine to reality and touch lives of those who battle it each day owing to incurable diseases and disorders. It is this battle of saving lives that kindles her interest and motivates her to pursue a career in research and development in the field and contribute to the society at large.