DESIGN AND MICROFABRICATION OF A SMART 3D SCAFFOLD FOR TISSUE ENGINEERING WITH VASCULAR AND IMMUNO-PROTECTION CAPABILITIES

Settore disciplinare: (FIS/07)

DOTTORANO
Gerald James Bakeine MD.

COORDINATORE DEL COLLEGIO DEI DOCENTI
Chiar.mo Prof. Fernando Tommasini
Università degli Studi di Trieste

TUTORE
Dott. Massimo Tormen
CNR-INFM Laboratorio Nazionale TASC

RELATORE
Chiar.mo Prof. Ubaldo Prati
Università degli studi Magna Græcia di Catanzaro

2007
1. INTRODUCTION ........................................................................................................... 7

2. SCIENTIFIC BACKGROUND ................................................................................... 12

2.1. Cell and Tissue Biology .......................................................................................... 12
  2.1.1. Extracellular Matrix (ECM) .................................................................................. 12
  2.1.2. Bone ...................................................................................................................... 14
  2.1.3. Artery ..................................................................................................................... 16
  2.1.4. Liver ....................................................................................................................... 18
  2.1.5. Pancreas ............................................................................................................... 19

2.2. Tissue - Biomaterials Interactions ......................................................................... 20
  2.2.1. Cell adhesion to artificial substrates ................................................................. 20
  2.2.2. Host reaction to biomaterials ............................................................................. 21
        2.2.2.1. Acute Inflammation ..................................................................................... 24
        2.2.2.2. Chronic inflammation ................................................................................. 26
        2.2.2.3. Granulation tissue ...................................................................................... 28
        2.2.2.4. Foreign body reaction ............................................................................... 29
        2.2.2.5. Fibrosis and fibrous encapsulation ............................................................ 30
  2.2.3. Mathematical Model of Oxygen Diffusion Across a Biomembrane ................ 33

3. TISSUE ENGINEERING ......................................................................................... 37

3.1. Conventional Scaffolds ......................................................................................... 38

3.2. Cell Sources in Tissue Engineering: The Promise of Stem Cells ....................... 40

3.3. Scaffold and Stem Cells ....................................................................................... 41

3.4. Angiogenesis ......................................................................................................... 42

4. NANOTECHNOLOGY IN BIOMATERIALS SCIENCE ....................................... 45

4.1. Nanotechnogoly in biomaterials ........................................................................... 45

4.2. Current research efforts to improve biomedical performance at the nanoscale ...... 48

4.3. Ceramic nanomaterials ......................................................................................... 49
        4.3.1. Increased osteoblast functions .......................................................................... 50
        4.3.2. Increased osteoclast function .......................................................................... 52
        4.3.3. Decreased competitive cell functions ............................................................ 53
        4.3.4. Increased osteoblast functions on nanofibrous materials ............................... 54

4.4. Metal nanomaterials .............................................................................................. 55

4.5. Polymeric nanomaterials ....................................................................................... 56

4.6. Composite nanomaterials ..................................................................................... 58
5. AIMS OBJECTIVES AND METHODS ..............................................60

5.1. Rational of study .............................................................................................................60
5.2. Scope of Thesis ................................................................................................................61
5.3. Aims of the study.............................................................................................................61
5.4. Objectives.........................................................................................................................61

5.5. Methods............................................................................................................................62
5.5.1. Scaffold surface geometry design ................................................................................62
5.5.2. Surface Design Optimisation........................................................................................62
5.5.3. Cell Culture on Biomaterial..........................................................................................63
5.5.4. Scaffold Design............................................................................................................64
5.5.5. Scaffold Microfabrication Protocol..............................................................................64
5.5.6. Host response to Biomaterial......................................................................................69
5.5.6.1. Controlled Host Response at Biomaterial/Scaffold-Host Interface using "smart"
surface geometry....................................................................................................................69
5.5.6.2. Matrigel®............................................................................................................69
5.5.6.3. Controlled Host Response at Biomaterial/Scaffold-Host Interface using surface
chemistry ........................................71

6. RESULTS .........................................................................................................................73

6.1. Biomaterial surface geometry characterization ...........................................................73
6.2. Cell response to biomaterial and surface geometry .......................................................75
6.2.1. Neuroblastoma cells (mouse PC12 cell line).............................................................75
6.2.2. Embryonic Stem Cells (ECSs) [mouse TBV-2 cell line] .............................................75
6.2.3. Muscle myoblast cells (mouse C2C12 cell line)..........................................................81
6.3. Host response to Biomaterial ..........................................................................................83
6.3.1. Controlled Host Response at Biomaterial/Scaffold-Host Interface using surface
chemistry ...................................................................................................................................83
6.3.2. Controlled Host Response at Biomaterial/Scaffold-Host Interface using "smart"
surface geometry........................................86

6.4. Scaffold prototype ..........................................................................................................88

7. DISCUSSION ......................................................................................................................94

7.1. Scaffold Design and Architectural Hierarchy ..................................................................95
7.1.1. The Superstructure: The overall shape of the scaffold ..............................................95
7.1.2. At the Micro-Scale: ......................................................................................................95
7.1.3. The hydraulic aspect as of design ..............................................................................96
7.1.4. At the Nano-Scale ..................................................................................................... 100

7.2. Bioactively controlled host response (wound healing) at the host scaffold interface
101
# Introduction

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3. Microfabrication Protocol</td>
<td>102</td>
</tr>
<tr>
<td>7.4. Conclusion</td>
<td>103</td>
</tr>
<tr>
<td>7.5. Applications</td>
<td>103</td>
</tr>
<tr>
<td>7.6. Future areas of research</td>
<td>104</td>
</tr>
<tr>
<td>7.7. Acknowledgments</td>
<td>106</td>
</tr>
<tr>
<td>7.8. References</td>
<td>107</td>
</tr>
</tbody>
</table>
Abstract

Tissue loss or end-stage organ failure caused by injury or other types of damage is one of the most devastating and costly problems in human health care.

Although surgical strategies have been developed to deal with these problems, and significant advances have been achieved in organ transplantation, it is extremely limited by a critical donor shortage and the necessity of lifelong immunosuppression and its serious complications. In the USA, more than 6,000 people die each year as a result of shortage of donor organs.

Tissue engineering [TE] is seen by many as the only way to address this shortage. TE is an interdisciplinary field that draws from materials science, cell biology, biotechnology and chemistry, and strives to offer a new solution to tissue loss or organ failure through the use of synthetic, hybrid, or natural materials that have been designed and fabricated into a 3-dimensional scaffolds that provide support and allow cell attachment, proliferation, differentiation and function.

However, Skin and cartilage are the only two tissues grown under laboratory conditions that have achieved successful clinical application. The main reason for this is that cartilage doesn’t require blood vessels or nerves and skin is sustained by nutrients that diffuse through the thickness of the cells that make up the graft. Attempts to grow more biologically challenging tissue and organs have been had mixed results. The obstacles and challenges that have to be overcome include:

1. Graft loss/failure due to at the cyto-incompatibility at the graft-biomaterial interface and bio-incompatibility host-biomaterial interface.
2. Inadequate neovascularization and nutrient channels to support cell survival deep in the interior of the scaffolds.
3. Immuno-rejection of allogenic graft.
4. Lack of healthy easily accessible cells for use in tissue engineering

To this end we have developed “smart" biomaterials with nano-scale architecture to elicit desirable cell response (cytocompatibility) at the cell-biomaterial interface.
and desirable host response (biocompatibility) at the host biomaterial interface. We then designed and microfabricated an original scaffold that incorporated the “smart” architecture and microfluidic network to permit the flow of nutrient-rich media deep in the interior. The scaffold consists of two microporous hemi-membranes that are superimposed and aligned in such a way that the micropores are laterally offset. Sandwiched between these hemi-membranes is a microfluidic channel network that runs perpendicular to the micropore axis and permits interconnectivity between the laterally offset micropores. By decreasing the size of the channels from the micro- to the nano-scale the scaffold acquires a another “smart” characteristic as a immuno-isolating membrane.

To examine the scaffold’s potential for tissue regeneration, muscle myoblast cells (mouse C2C12 cell-line), neuroblastomas (mouse PC12 cell-line) and embryonic stem cells (mouse TBV-2 cell-line) were seeded and cultured on the scaffolds. Biocompatibility was evaluated by subcutaneous implantation of the scaffold in mice. Results show that myoblast and neuroblastomas attached, proliferated and differentiated. The exponential cell proliferation associated with in vitro embryonic stem cell culture was controlled. In vivo studies demonstrated scaffold-host integration as evidenced by vascular colonisation of the scaffold.

By developing the ability to construct and control the following scaffold parameters; microporous architecture; microfluidic interconnectivity and canal size; the external and internal shape of the scaffold and it’s multi-scaled surface architectures, the “smart” scaffold developed in our laboratories have great potential as an ideal scaffold for tissue engineering.
ABSTRACT (Italiano)

Nella cura del benessere degli uomini, tra i problemi di maggior impatto e costo si trovano la perdita di tessuti e di funzionalità degli organi, causati da ferite o incidenti di altro genere.

Sebbene siano state sviluppate efficaci strategie chirurgiche per ovviare a tali problemi, e nonostante i significativi avanzamenti tecnici raggiunti nel campo del trapianto degli organi, il ricorso a tale soluzione è fortemente limitato dal basso numero di donatori di organi e dalla necessità di durature terapie immuno-soppressive, con il loro portato di serie complicazioni. Basti pensare che a causa della mancanza di un adeguato numero di donatori, solo negli USA ogni anno muoiono più di 6000 persone.

Secondo il parere di molti, la strada più promettente per affrontare questo problema è la Ingegneria dei Tessuti Biologici (ovvero Tissue Engineering [TE], dall’acronimo dell’equivalente inglese). Si tratta di un approccio interdisciplinare, che combinando conoscenze e tecnologie dai campi della Scienza dei Materiali, Biologia Cellulare, Biotecnologia e Chimica offre la possibilità, in principio, di trovare soluzioni innovative per la sostituzione di tessuti o organi non più funzionali; la strada maestra in questo campo è la progettazione e costruzione di strutture tri-dimensionali (scaffold) capaci di provvedere al supporto funzionale di cellule cresciute in vitro, integrando materiali sintetici, naturali e ibridi.

Tuttavia, al momento soltanto pelle e cartilagini sono state cresciute in condizioni controllate da laboratorio e hanno raggiunto lo stadio della applicazione clinica. Il motivo fondamentale per tale successo, e per la limitazione a questi due soli casi, è che la cartilagine non necessita di vascolarizzazione o innervazione per sostentarsi, e la pelle ottiene i suoi nutrienti per mezzo della diffusione attraverso gli strati di cellule che la sostengono e ne garantiscono l'attaccoimento.

Tentativi per crescere altri tipi di tessuti e organi hanno incontrato gravi difficoltà;
1. perdita di adesione a causa della cito-incompatibilità all’interfaccia di connessione e della bio-incompatibilità tra i materiali dell’impianto e il corpo ospitante
2. scarsa o inadeguata vascolarizzazione dell’impianto, tale per cui le cellule all’interno della struttura 3-D non vengono raggiunte dai nutrienti necessari per una sopravvivenza a lungo termine
3. rigetto immunologico del tessuto trapianto
4. mancanza di facile accesso alle cellule necessarie per la costruzione degli impianti

Con lo scopo di rispondere a queste richieste, abbiamo sviluppato bio-materiali “smart” contraddistinti da una architettura a livello nano-metrico, capaci di stimolare la desiderata risposta cellulare (compatibilità citologica) all’interfaccia con il bio-materiale stesso, e capace di promuovere la bio-compatibilità con il corpo ospitante. Usando le tecniche della micro-fabbricazione, abbiamo quindi progettato e realizzato una struttura 3-D originale (lo scaffold) che incorpora tale architettura “smart” e il sistema micro-fluidico che garantisce l’apporto del flusso dei nutrienti al suo interno. Tale struttura consiste da membrane sovrapposte ed allineate in modo che lateralmente si aprano dei pori di comunicazione con dimensioni micrometriche. Compreso tra queste membrane si trova il sistema microfluidico, capace di garantire l’interconnessione tra i pori con un sistema di canali che scorre perpendicolarmente all’asse dei pori stessi.

Una ulteriore caratteristica di questo sistema è che riducendo la scala dei canali microfluidici al livello di scala nano-metrica, lo “scaffold” acquisisce proprietà immuno-isolanti.

Per lo studio della sue potenzialità nel campo della rigenerazione tissutale, abbiamo cresciuto nello “scaffold” mioblasti muscolari (linea cellulare C2C12 del topo), neuroblastomi (linea cellulare PC12 del topo) e cellule staminali embrionali (TBV-2 del topo). La biocompatibilità è stata valutata impiantando lo “scaffold” in topi da laboratorio. I risultati mostrano come i mioblastomi e i neuroblastomi aderiscono allo “scaffold”, proliferano e si differenziano. Abbiamo controllato la proliferazione esponenziale associata con la cultura in vitro delle cellule staminali.
embrionali e lo studio delle condizioni *in vivo* dimostra la integrazione dello “scaffold” nel corpo dell’ospite, come risultato della riuscita vascolarizzazione della sua struttura.

Con il controllo della architettura micro-porosa; interconnettività microfluidica e dimensione dei canali; della geometria esterna ed interna e della sua struttura e conformazione superficiale a scala nano-metrica, lo “scaffold” risultante, sviluppato nei nostri laboratori, mostra enormi potenzialità come struttura ideale per la Ingegneria dei Tessuti Biologici.
CHAPTER

1. Introduction

Key words: microfabrication | 3D scaffold | surface structure and topology | microfluidic canal network | cell vitality, differentiation and function | neoangiogenesis | host-scaffold integration | renewable cell source

Tissue loss or end-stage organ failure caused by injury or other types of damage is one of the most devastating and costly problems in human health care. Surgical strategies that have been developed to deal with these problems include organ transplantation from one individual to another, tissue transfer from a healthy site to the diseased site in the same individual, and replacement by using mechanical devices such as joint prosthesis or dialysis machine. Though significant advances have been achieved in terms of health care by these therapeutic options, many limitations and unsolved issues remain. Organ transplantation is extremely limited by a critical donor shortage [1] and the necessity of lifelong immunosuppression and its serious complications. In the USA, more than 6,000 people die each year as a result of shortage of donor organs. In 1990, the difference between the number of organs donated and the number of patients waiting for organs was 9,000 [2]. Today the waiting list of transplant candidates is 94,871 [1].

Tissue engineering [TE] is seen by many as the only way to address this shortage. TE is an interdisciplinary field that draws from materials science, cell biology, biotechnology and chemistry, and strives to offer a new solution to tissue loss or organ failure through the use of synthetic, hybrid, or natural materials that have been designed or fabricated into a 3-dimensional structure (scaffolds).
Artificially engineered scaffolds are a key requirement in TE. The fundamental requirements of these scaffolds are that they: provide mechanical support, are biocompatible, degrade over a predetermined period of time, can be moulded into the desired shape, are easy to sterilize and allow cell attachment proliferation, differentiation and function.

Biomedical scaffolds have been used since ancient times - for example, ancient Incas successfully used gold (Au) plates to repair cranial defects [3]. Until the last few decades of the 20th century, the criteria used in choosing materials for scaffolds has fundamentally changed very little and usually scaffolds were chosen that were functional because of their inertness. Since the discovery in the 1960s that some glass ceramics actively bond to living bone, the focus has shifted away from inert materials and towards materials that are bioactive – those that deliberately elicit a specific response from the body. Currently, most scaffolds provide a three dimensional environment in which tissue can grow and develop, so that it is able to reproduce the functions of the tissue it is intended to replace.

To date, many tissues have been developed in the laboratory, and laboratory-grown tissues are readily available on market shelves. For example, Laboratory expanded skin cells are provided by CellTran Ltd (UK) for wound healing in patients with extensive burns and chronic wounds. Cartilage is another tissue that is grown routinely by Mercy Tissue Engineering (Australia) to provide cartilage for knee implants. However, Skin and cartilage are the only two tissues grown under laboratory conditions that have achieved successful clinical application. The main reason for this is that cartilage doesn't require blood vessels or nerves and skin is sustained by nutrients that diffuse through the thickness of the cells that make up the graft.

Attempts have been made to grow more biologically challenging tissue and organs. Up to now, investigators have attempted to grow bone [4], liver [5, 6], arteries [7], bladder [8], pancreas [9], nerves [10], heart valves [7], corneas [11], and various soft tissues [12] but with disappointing clinical results.

Many obstacles and challenges remain. The fundamental problems involved are
5. Graft loss/failure due to bio-incompatibility at the graft-biomaterial and host-biomaterial interface.
6. Inadequate neovascularization of living tissue in the 3-dimensional structure. Leading to graft loss.
7. Immuno-rejection of allogenic graft.

Fig 1.1 demonstrates a graphic representation of failure points the in current TE assembly chain.

It is apparently evident that there is no one-stop-solution. A successful product of tissue engineering or organ fabrication will only possible when an adequate source of healthy, expandable cells, cell-friendly and host–friendly scaffolds, vascular and nervous reconnection and immuno-protection are achieved.

The mission of this PhD research program was to formulate a comprehensive strategy to optimise graft survival and function through all-embracing solutions to the identified problems in the tissue engineering production chain so as to achieve clinical-grade tissue engineered implants.
As a starting point, we looked at nature’s solutions to tissue building and function; in particular cellular microenvironment. The cell’s microenvironment is an architectural masterpiece with precise structural organisation at macro- micro- and nano-scales. We believe that complex cellular processes like proliferation, differentiation, organisation and function are precisely controlled and guided by this hierarchal 3D architecture. Therefore it can be deduced that full tissue viability and function cannot be artificially recovered without rebuilding the ultrastructures of the tissue itself.

We report the development of silicon and gold substrates with novel structured surface formulations at two scale lengths i.e., at the tens to hundreds of microns lengths as supporting scaffolds and at the submicron scale for surface properties, that were able to elicit controlled cell proliferation, differentiation and function. This cytocompatibility was tested and validated by in vitro cell culture studies using muscle myoblast cells (mouse C2C12 cell-line), neuroblastomas (mouse PC12 cell-line) and embryonic stem cells (mouse TBV-2 cell-line).

We further report the development a scaffold with an original design that incorporated these smart nanostructured features. In vivo studies demonstrated optimal biocompatibility evidenced by marked neovascularisation at the scaffold-host interface. This was further optimized by coating the scaffold with an angiogenic stimulating gel matrix at implantation.

Structural Organization of Research Project.
The research program was carried out following a Master protocol for the development of medical devices. The research work was carried out at the following institutions; National Laboratory of Advance Technology and NanoScience (TASC) Trieste, Consortium of Moleculare BioMedicine (CMB) Trieste, Department of Comparative Anatomy and Cell Biology, University of Pavia, and Department of Human Anatomy, faculty of Medicine, University of Pavia. Fig 2.2 shows a graphic presentation of the Master Protocol highlighting the multi-disciplinary input and the path followed.
Fig 1.2 Graphic representation of Master Protocol highlighting the disciplines involved and the research path being undertaken.
2. Scientific Background

Designing highly functional scaffolds for tissue engineering requires a fundamental understanding of cell/tissue biology and biomaterials science. This chapter provides basic working knowledge on concepts in these disciplines. The structural processes that control cell viability and tissue function are highlighted and tissue-biomaterial interactions are reviewed.

2.1. Cell and Tissue Biology

2.1.1. Extracellular Matrix (ECM)

It is well known that specific biological and chemical composition of tissues is critical for tissue function. However, the structural organization is just as important, if not more so, as this unique frame defines the three-dimensional shape of the tissue at multiple length scales. Typically, this frame consists of a complex network of structural and bioactive macromolecules such as proteins, proteoglycans, and glycosaminoglycans known in general as the extracellular matrix (ECM). The ECM has multiple functions but, in general include:

1. Mechanical support for cell anchorage
2. Determination of cell orientation;
3. Control of cell growth;
4. Maintenance of cell differentiation;
5. Scaffolding for orderly tissue renewal;
6. Establishment of tissue microenvironment;

7. Sequestration, storage and presentation of soluble regulatory molecules.

Matrix components markedly influence the maintenance of cellular phenotypes, affecting cell shape, polarity, and differentiated function through receptors for specific extracellular matrix molecules on cell surfaces. Cells communicate with various matrix components using surface-receptors and peripheral and integral membrane proteins; the resultant changes in cytoskeletal organisation and possibly in production of other second messengers can modify gene expression. Conversely, cells produce and secrete matrix molecules, often vectorially. These functions are accomplished by reciprocal instructions between cells and matrix, an interaction termed “dynamic reciprocity”.

Extracellular matrices are generally specialized for particular function, such as strength (tendon), filtration (kidney glomerulus), or adhesion (basement membrane generally). The extracellular matrix consists of large molecules linked together into insoluble composite. The extra cellular matrix is composed of (1) fibres (collagen and elastin) and (2) a largely amorphous interfibrillary matrix (mainly proteoglycans, noncollagenous glycoproteins, solutes, and water). There are two types of matrices: the interstitial matrix and the basal lamina. The major component of each is collagen, a cell-binding adhesive glycoprotein, and proteoglycans. The interstitial matrix is produced by mesenchymal cells and contains fibrillar collagens, fibronectin, hyaluronic acid, and fibril-associated proteoglycans. The basal lamina is produced by overlying parenchymal cells. Basal laminae contain a meshlike collagen framework, laminin and a large heparan sulfate proteoglycan. To produce additional mechanical strength, the extracellular matrix becomes calcified during the formation of bones and teeth. Although matrix turnover is quite slow in normal mature tissues; type-specific extracellular matrix components are turned over and remodelled in response to appropriate stimuli, such as tissue damage and repair.

The fibrillar components of the extracellular matrix include collagen and elastin fibres. Collagen represents a family of closely related genetically, biochemically, and functionally distinct glycoproteins, of which at least fifteen different types have been identified. Fibrillar collagen, also called interstitial collagens,
II, III and V), have periodic cross-striations. Type I, the most abundant, is present in most connective tissue. Type II collagen is a major component of hyaline cartilage. The fibrillar collagens provide a major component of tissue strength. Most of the remaining collagen types are nonfibrillar, the most common of which is type IV, a major constituent of all basement membranes. Elastin fibres confer an elastic flexibility to tissues.

In the amorphous matrix, glycosaminoglycans (GAGs), with the exception of hyaluronic acid, are found covalently bound to proteins (as proteoglycans). Proteoglycans serve as major structural elements of the extracellular matrix; some proteoglycans are bound to plasma membranes and appear to be involved in adhesiveness and receptor binding. A set of large noncollagenous glycoproteins is important in binding cells to the extracellular matrix, including fibronectins (the best understood of the noncollagenous glycoproteins), laminins, chondronectin and osteonecins. Fibronectins, found almost ubiquitously in the extracellular matrix are synthesized by many different cell types. The circulating form, plasma fibronectin, is produced mainly by hepatocytes. Fibronectin’s adhesive character makes it a crucial component of blood clots and pathways followed by migrating cells. Thus fibronectin-rich pathways guide and promote the migration of many cells during embryonic development and wound healing.

Basement membranes provide mechanical support for resident cell, cell as semipermeable barriers between tissue components, and act as regulators of cellular attachment, migration, and differentiation. They consist of discrete zone of amorphous, noncollagenous glycoprotein matrix (including laminin), proteoglycans, and type IV collagen.

In order to illustrate further the basic concept of ECM and how hierarchical organisation in tissues give rise to a wide variety of biological functions three examples of tissue are discussed.

2.1.2. Bone

Bone tissue is an extremely strong and dense material that is well suited to its role in providing load-bearing support and protection for the body. It is largely composed of mineral, which is in the form of tiny crystals of impure form of
hydroxyapatite, and collagen. The specific composition and organisation of the components dictate the mechanical properties of bone [10, 13]. The mineral portion is believed to be primarily responsible for the stiffness of the tissue, while the collagen dictates the post-yield properties.

Bone tissue can be divided into two distinct structural types: (i) compact, or cortical bone which is quite dense (porosity of 5-25%) [14], and (ii) cancellous, or trabecular bone, which is highly porous (porosity of 40-90%) [4]. Cortical bone is largely found in mid-shafts of long bones and is composed of longitudinally oriented cylindrical units known as osteons, which are typically 200μm in diameter, 10-20mm long, and are composed of concentric lamellae 3-7μm thick [2]. It is the orientation of the osteons that give rise to the strong mechanical anisotropiy found in cortical bone (i.e. cortical bone is much stiffer and stronger along the direction of the osteons compared with the direction transverse to the osteons).
At the centre of the osteons are Haversian canals that contain blood vessels; these vessels supply the tissue with nutrients via microchannels known as canaliculi; a crucial feature for such dense tissue. In contrast, the structure of cancellous bone consists of a network of interconnecting struts known as trabeculae 50-300µm thick) [2]. The pore space of this network is filled with bone marrow. Trabecular bone is also anisotropic, and it is believed that the principal direction of the trabecular network, which is the direction of greatest stiffness and strength, corresponds to the direction of habitual loading. Cancellous bone is found at the ends of long bones (surrounded by a layer of cortical bone) and in bones such as the skull and vertebra.

2.1.3. Artery

The arterial wall is also a crucial load-bearing tissue that derives its unique mechanical properties from its complex organisation of structural proteins and cells. However, since it must accommodate the pulsatile output from the heart, it must be distensible and, as a result, cannot achieve mechanical strength in the same manner as bone tissue. The arterial wall is composed of three distinct layers; (i) the intima, which comprises a single layer of endothelial cells that lines the inner surface of the vessel; (ii) the media, which is the primary load-bearing layer of the vessel; and (iii) the adventitia, which anchors the vessel to the surrounding tissue (fig. 2.2). The media is primarily responsible for maintaining the vascular tone, or degree of constriction relative to its maximally dilated state, and hence the vessel diameter. The media is composed of alternating layers of smooth muscle cells (SMCs) embedded in collagen and elastin lamellae, the number of which depend on the size of the vessel and its anatomical location [15]. Collagen provides the high-strain stiffness and strength to the tissue, while the elastin offers low-strain elasticity, which prevents the wall from permanently deforming as a result of repeated distension. The collagen and SMCs are arranged in a helical pattern around the circumference of the vessel, with the direction of the pitch alternating between successive layers. This helical arrangement not only provides enhanced circumferential load-bearing properties to the tissue, but also imparts torsional stability [16-19] Furthermore, this
arrangement of SMCs allows more efficient control of vessel tone (which in turn dictates blood pressure and shear stress) as this configuration leads to SMC contraction that primarily results in a reduction of the vessel lumen diameter rather than its length.

The high degree of organisation in the arterial wall does not solely serve a mechanical purpose. The intima, which can be considered as the biological focal point of the vessel wall, is crucial for providing a nonthrombogenic blood-contacting surface (the underlying media is highly thrombogenic), regulating vascular tone, and controlling nutrient and water transport across the vessel wall. The adventitia functions primarily to anchor the blood vessel to its surrounding tissue, but also supports the microvasculature and nerves that connect to the outer layers of the large arteries.

Fig 2.2 Anatomy of blood vessels [artery (Lt). and vein (Rt.)] demonstrating its constituent layers and 3D architecture.
2.1.4. Liver

Unlike the previous two examples, liver tissue has not inherent mechanical function, but organisation of its components is crucial for its ability to function properly. The liver is a complex organ that plays a major role in metabolism and performs a number of functions including glycogen storage, plasma protein synthesis, and drug detoxification [20]. The functional unit of the liver is a hexagonal lobule of approximately 2mm in diameter centred around a vein (terminal hepatic venule) with arteries and veins providing blood from the periphery of the lobule (fig. 2.3). The blood passes from the arteries into sinusoids (ducts lined with epithelial cells) where nutrients, metabolites, and toxins pass through the epithelium and are processed by the underlying hepatocytes. The processed blood is collected in the central before leaving the liver. Furthermore, bile ducts run throughout the tissue collecting bile secreted by the hepatocytes. The complex organisation of the individual, as well as their large-scale organisation throughout the liver in combination with its vascular network, is crucial to the organs’ function.

Fig 2.3 Functional anatomy and 3D architecture of liver hexagonal lobule
2.1.5. Pancreas

The pancreas is an elongated organ, light tan or pinkish in colour, which lies in close proximity to the duodenum. It is covered with a very thin connective tissue capsule which extends inward as septa, partitioning the gland into lobules. The bulk of the pancreas is composed of pancreatic exocrine cells and their associated ducts. Embedded within this exocrine tissue are roughly one million small clusters of cells called the Islets of Langerhans, which are the endocrine cells of the pancreas and secrete insulin, glucagon and several other hormones. A graphic representation of islets architecture is illustrated in fig. 2.3.

![Islets of Langerhans diagram](image)

Fig 2.3 Functional anatomy and 3D architecture of the pancreas The pancreas is located in the abdomen, adjacent to the duodenum (the first portion of the small intestine). A cross-section of the pancreas shows the islet of Langerhans which is the functional unit of the endocrine pancreas. Encircled is the beta cell that synthesizes and secretes insulin. Beta cells are located adjacent to blood vessels and can easily respond to changes in blood glucose concentration by adjusting insulin production. Insulin facilitates uptake of glucose, the main fuel source, into cells of tissues such as muscle.
2.2. **Tissue - Biomaterials Interactions**

2.2.1. **Cell adhesion to artificial substrates**

The importance of proteins in biomaterials science stems primarily from their inherent tendency to deposit on surfaces as a tightly bound adsorbate, and the strong influence these deposits have on the subsequent cellular interactions with the surfaces. It is thought that the particular properties of surfaces, as well as the specific properties of individual proteins, together determine the organisation of the adsorbed protein layer, and that the nature of this layer in turn determines the cellular response to the adsorbed surfaces. Since the cellular responses largely determine the degree of biocompatibility of the material, the properties of proteins and their behaviour at interfaces need to be understood by those interested in biomaterials. Fig 2.4 illustrates the interaction of a cell with an adsorbed protein layer on a solid substrate.

In order to improve biomaterial performance and hence extend the lifetime of implants, it is essential to design surface characteristics that interface optimally with select proteins and subsequently with pertinent bone cell types. That is, immediately after implantation, proteins will adsorb from plasma to biomaterial surfaces to control cell attachment and eventual tissue regeneration (Figure 2.4) [22, 23]. Initial protein interactions that mediate cell function depend on many biomaterial properties, including chemistry, charge, wettability, and topography [22, 23]. Of significant influence for protein interactions is surface roughness and energy [24-27], and this represents the promise of nanophase materials in implant applications.
2.2.2. Host reaction to biomaterials

Inflammation, Wound Healing, and the Foreign Body Reaction

Inflammation, wound healing, and foreign body response are generally considered as parts of the tissue or cellular host response to injury. Table 2.1 lists the sequence of these events following injury.

Table 2.1 Sequence of Local Events Following Implantation

<table>
<thead>
<tr>
<th>Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Inflammation</td>
</tr>
<tr>
<td>Chronic inflammation</td>
</tr>
<tr>
<td>Granulation Tissue</td>
</tr>
<tr>
<td>Foreign Body Reaction</td>
</tr>
<tr>
<td>Fibrosis</td>
</tr>
</tbody>
</table>
From a biomaterials perspective, placing a biomaterial in the *in vivo* environment involves injection, insertion, or surgical implantation, all of which injure tissues or organs involved.

The placement procedure initiates a response to the injury by the body, and mechanisms are activated to maintain homeostasis. The degrees to which the homeostatic mechanisms are perturbed and the pathophysiologic conditions created and resolved are a measure of the host’s reaction to the biomaterial and may ultimately determine its biocompatibility. While it is convenient to separate homeostatic mechanisms into blood-material or tissue-material interactions, it must be remembered that the various components or mechanisms involved in homeostasis are present in both blood and tissue and are part of the physiologic continuum. Furthermore, it must be noted that host reactions can be tissue-dependent, organ-dependent, and species-dependent. Obviously, the extent of injury varies with the implantation procedure.

Inflammation is generally defined as the reaction of vascularised living tissue to local injury. Inflammation serves to contain, neutralize, dilute, or wall off injurious agent or process. In addition, it sets into motion a series of events that may heal and reconstitute the implant site through replacement of the injured tissue by regeneration of native parenchymal cells, formation of fibroblastic scar tissue, or a combination of these two processes.

Immediately following injury, there are changes in vascular flow, calibre, and permeability. Fluid, proteins, and blood cells escapes from the vascular system into the injured tissue in a process called exudation. Following changes in the vascular system, which also include changes induced in blood and its components, cellular events occur and characterise the inflammatory response. The effect of this injury and/or biomaterial *in situ* on plasma or cells can produce chemical factors that mediate many of the vascular and cellular responses of inflammation.

Regardless of the tissue or organ into which a biomaterial is implanted, the initial inflammatory response is activated by injury to vascularised connective tissue. Since blood and its components are involved in the initial inflammatory response, blood clot formation and/or thrombosis also occur. Blood coagulation and
thrombosis are generally considered humoral responses and may be influenced by other homeostatic mechanisms such as the extrinsic and intrinsic coagulation systems, the complement system, the fibrinolytic system, the kinin-generating system, and platelets.

The predominant cell type present in inflammatory repose varies with the age of the injury. In general, neutrophils predominate during the first several days following injury and then are replaced by monocytes as the predominant cell type. Three factors account for this change in cell type: neutrophils are shorted lived and disintegrate and disappear after 24-48 hours; neutrophil emigration is of short duration; and chemotactic factors for neutrophil migration are activated early in the inflammatory response. Following emigration from the vasculature, monocytes differentiate into macrophages and these cells are very long lived (up to months). Monocyte emigration may continue for days to weeks, depending on the injury and implanted biomaterial, and chemotactic factors for monocytes are activated over longer periods of time.

The sequence of events following implantation of a biomaterial is illustrated in fig 2.5.

Fig 2.5 Schematic representation of the time course of the foreign body reaction to an implanted “biocompatible” material.
The size, shape and chemical and physical properties of biomaterials may be responsible for the variations in the intensity and duration of the inflammatory or wound-healing process. Thus, intensity and/or time duration of the inflammatory reaction may characterize the biocompatibility of a biomaterial.

While injury initiates the inflammatory response, the chemicals released from plasma, cells and injured tissue mediate the response. Important classes of chemical mediators of inflammation are presented in Table 2.2. Several points must be noted in order to understand the inflammatory response and how it relates to biomaterials. First, although chemical mediators are classified on a structural or functional basis, different mediator systems interact and provide a system of checks and balances regarding their respective activities and functions. Second, chemical mediators are quickly inactivated or destroyed, suggesting that their action is predominantly local (i.e. at the implant site). Third, generally the lysosomal proteases and oxygen-derived free radicals produce the most significant damage or injury. These chemical mediators are also important in the degradation of biomaterials.

2.2.2.1. Acute Inflammation

Acute inflammation is of relatively short duration, lasting from minutes to days, depending on the extent of the injury. Its main characteristics are the exudation of fluid and plasma proteins (oedema) and the emigration of leukocytes (predominantly neutrophils). Neutrophils and other motile white cells emigrate or move from the blood vessels to the perivascular tissues and the injury (implant) site. Leukocytes emigration is assisted by “adhesion molecules” present on leukocyte and endothelial surfaces. The surface expression of these adhesion molecules can be induced, enhanced, or altered by inflammatory agents and chemical mediators. White cell emigration is controlled, in part, by chemotaxis, which is the unidirectional migration of cells along a chemical gradient. A wide variety of exogenous and endogenous substances have been identified as chemotactic agents. Specific receptors for chemotactic agents on the cell membranes of leukocytes are important in the emigration or movement of
leukocytes. These and other receptors may also play a role in the activation of leukocytes. Following localization of leukocytes at the injury site (implant) site, phagocytosis and the release of enzymes occur following neutrophils and macrophages. The major role of neutrophils in acute inflammation is to phagocytose micro-organisms and foreign materials. Phagocytosis is seen as a three-step process in which the injurious agent undergoes recognition and neutrophil attachment, engulfment, and killing or degradation. In regard to biomaterials, engulfment and degradation may or may not occur, depending on the properties of the biomaterial.

Although biomaterials are not generally phagocytosed by neutrophils or macrophages because of the disparity of size, (i.e., the surface of the biomaterial is greater than the size of the cell), certain events in phagocytosis may occur. The process of recognition and attachment is expedited when the injurious agent is coated by naturally serum factors called “opsonins.” The two major opsonins are immunoglobulin G (IgG) and the complement-activated fragment, C3b. both these plasma-derived proteins are known to adsorb to biomaterials, and neutrophils and macrophages have corresponding cell membrane receptors for these opsonization proteins. These receptors may also play a role in the activation of the attached neutrophil or macrophage. Owing to the disparity in size between the biomaterial surface and the attached cell, frustrated phagocytosis may occur. This process does not involve engulfment of the biomaterial but does cause the extracellular release of leukocyte products in an attempt to degrade the biomaterial.

Henson [28] has shown that neutrophils adherent to complement-coated and immunoglobulin-coated nonphagocytosable surfaces may release enzymes by direct extrusion or exocytosis from the cell. The amount of enzyme released during this process depends on the size of the polymer particle, with larger particles inducing greater amounts of enzyme release. This suggests that the specific mode of cell activation in the inflammatory response in tissue depends upon the size of the implant and that a material in a phagocytosable form (i.e., powder or particulate) may provoke a different degree of inflammatory response than the same material in a nonphagocytosable form (i.e., film).


### TABLE 2.2 Important Chemical mediators of Inflammation Derived from Plasma, Cells, and Injured Tissue

<table>
<thead>
<tr>
<th>Mediators</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vasoactive amines</strong></td>
<td>Histamines, serotonin</td>
</tr>
<tr>
<td><strong>Plasma proteases</strong></td>
<td></td>
</tr>
<tr>
<td>• Kinin system</td>
<td>Bradykinin, kallikrein</td>
</tr>
<tr>
<td>• Complement system</td>
<td>C3a, C5a, C3b, C5b – C9</td>
</tr>
<tr>
<td>• Coagulation/fibrinolytic system</td>
<td>Fibrin degradation products; activated Hageman factor (FXIIA)</td>
</tr>
<tr>
<td><strong>Arachidonic acid metabolites</strong></td>
<td></td>
</tr>
<tr>
<td>• Prostaglandins</td>
<td>PGI₂, TXA₂</td>
</tr>
<tr>
<td>• Leukotrienes</td>
<td>HETE, Leukotriene B₄</td>
</tr>
<tr>
<td><strong>Lysosomal proteases</strong></td>
<td>Collagenase, elastase</td>
</tr>
<tr>
<td><strong>Oxygen-derived free radicals</strong></td>
<td>H₂O₂, Superoxide anion</td>
</tr>
<tr>
<td><strong>Platelet activating factors</strong></td>
<td>Cell membrane lipids</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td>Interleukin 1 (IL-1); tumour necrosis factor (TNF)</td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
<td>Platelet derived growth factor (PDGF); fibroblast growth factor (FGF); transforming growth factor (TGF-α or TGF-β)</td>
</tr>
</tbody>
</table>

#### 2.2.2.2. Chronic inflammation

Chronic inflammation is less uniform histologically than acute inflammation. In genera, chronic inflammation is characterized by the presence of macrophages, monocytes, and lymphocytes, with the proliferation of blood vessels and connective tissue. It must be noted that many factors can modify the course and histological appearance of chronic inflammation.
Persistent inflammatory stimuli lead to chronic inflammation. While the chemical and physical properties of biomaterials in themselves may lead to chronic inflammation, motion in the implant site by the biomaterial may also produce chronic inflammation. The chronic inflammatory response to biomaterials is usually of short duration and is confined to implant site. The presence of mononuclear cells, including lymphocytes and plasma cells, is considered chronic inflammation, while the foreign body reaction with the development of granulation tissue is considered the normal wound healing response to implanted biomaterials.

Lymphocytes and plasma cells are involved principally in the immune reactions and are key mediators of antibody production and delayed hypersensitivity response. Their roles in non-immunologic injuries and inflammation are largely unknown. Little is known regarding humoral immune responses and cell-mediated immunity to synthetic biomaterials. The role of macrophages must be considered in the possible development of immune responses to synthetic biomaterials. Macrophages process and present the antigen to immunocompetent cells and thus are key mediators in the development of immune reactions.

Monocytes and macrophages belong to the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES). These systems consist of cells in the bone marrow, peripheral blood, and specialized tissues. The specialized cells in these tissue may be responsible for systemic effects in organs or tissue secondary to the release of components or products from implants through various tissue-material interaction (e.g., corrosion products, wear debris, degradation products) or the presence of implants (e.g., microcapsule or nanoparticle drug delivery systems).

The macrophage is probably the most important cell in chronic inflammation because of the great number of biologically active products it can produce. Important classes of products produced and secreted by macrophages include neutral proteases, chemotactic factors, arachidonic acid metabolites, reactive oxygen metabolites, complement components, coagulation factors, growth-promoting factors, and cytokines.
Growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), TFG-α/epidermal growth factor (EGF), and interleukin-1 (IL-1) or tumour necrosis factor (TNF) are important to the growth of fibroblasts and blood vessels and the regeneration of epithelial cells. Growth factors released by activated cells can stimulate production of a wide variety of cells; initiate cell migration; differentiation, and tissue remodelling, and may be involved in various stages of wound healing.

2.2.2.3. Granulation tissue

Within one day following implantation of a biomaterial (i.e., injury), the healing response is initiated by the action of monocytes and macrophages. Fibroblasts and vascular endothelial cells in the implant site proliferate and begin to form granulation tissue, which is the specialized type of tissue that is the hallmark of healing inflammation. Granulation tissue derives its name from the pink, soft granular appearance on the surface of healing wounds and its characteristic histological features include the proliferation of new small blood vessels and fibroblasts. Depending on the extent of injury, granulation tissue may be seen as early as 3-5 days following implantation of the biomaterial.

The new small vessels are formed by budding or spouting of pre-existing vessels in the process known as neovascularisation or angiogenesis. This process involves proliferation, maturation, and organization of endothelial cells into capillary tubes. Fibroblasts also proliferate in developing granulation tissue and are active in synthesizing collagen and proteoglycans. In the early stages of granulation tissue development, proteoglycans predominate but later collagen, especially type II collagen, predominates and forms the fibrous capsule. Some fibroblasts in developing granulation tissue may have the features of smooth muscle cells. These cells are called myofibroblasts and are considered to be responsible for the wound contraction seen during the development of granulation tissue. Macrophages are almost always present in granulation tissue. Other cells may also be present if chemotactic stimuli are generated.
The wound healing response is generally dependent on the extent or degree of injury or defect created by the implantation procedure. Wound healing by primary union or first intention is the healing of clean, surgical incisions in which the wound edges have been approximated by surgical sutures. Healing under these conditions occurs without significant bacterial contamination and with a minimal loss of tissue. Wound healing by secondary union or second intention occurs when there is a large tissue defect that must be filled or there is extensive loss of cells and tissue. In wound healing by secondary intention, regeneration of parenchymal cells cannot completely reconstitute the original architecture and much larger amounts of granulation tissue are formed that result in larger areas of fibrosis or scar formation.

Granulation tissue is distinctly different from granulomas, which are small collections of modified macrophages called epithelioid cells that are usually surrounded by a rim of lymphocytes. Langhan's or foreign body-type giant cells may surround nonphagocytosable particulate materials in granulomas. Foreign body giant cells are formed by the fusion of monocytes and macrophages in an attempt to phagocytose the material.

2.2.2.4. Foreign body reaction.

The foreign body reaction to biomaterials is composed of foreign giant cells and the components of granulation tissue. These consist of macrophages, fibroblasts, and capillaries in varying amounts, depending upon the form and topography of the implanted material. Relatively flat and smooth surfaces such as those found on breast prostheses have a foreign body reaction that is composed of a layer of macrophages one to two cells thickness. Relatively rough surfaces such as those found on the outer surfaces of expanded poly(tetrafluoroethylene) (ePTFE) vascular prostheses have a foreign body reaction composed of macrophages and foreign body giant cells at the surface. Fabric materials generally have a surface response composed of macrophages and foreign body giant cells, with varying degrees of granulation tissue subjacent to the surface response.
As previously discussed, the form and topography of the surface of the biomaterial determines the composition of the foreign body reaction. With biocompatible materials, the composition of the foreign body reaction in the implant site may be controlled by surface properties of the biomaterial, the form of the implant, and the relationship between the surface area of the biomaterial and the volume of the implant. For example, high surface-to-volume implants such as fabrics or porous materials will have higher ratios of macrophages and foreign body giant cells in the implant site than smooth surface implants, which will have fibrosis as a significant component of the implant site.

The Foreign body reaction consisting mainly of macrophages and/or foreign giant cells may persist at the tissue-implant interface for the lifetime of the implant. Generally, fibrosis (i.e., fibrous encapsulation) surrounds the biomaterials or implant with its interfacial foreign body reaction, isolating the implant and foreign body reaction from local issue environment. Early in the inflammatory and wound healing response, the macrophages are activated upon adherence to the material surface.

While it is generally considered that the chemical and physical properties of the biomaterial are responsible for macrophage activation, the subsequent events regarding the activity of macrophages at the surface are not clear. Tissue macrophages, derived from circulating blood monocytes, may coalesce to form multinucleated foreign body giant cells. It is not uncommon to see very large foreign body giant cells containing large numbers of nuclei on the surface of biomaterials. While these foreign body giant cells may persist for the lifetime of the implant, it is not known if they remain activated, releasing their lysosomal constituents, or become quiescent.

2.2.2.5. Fibrosis and fibrous encapsulation

The end-stage healing response to biomaterials is generally fibrosis or fibrous encapsulation. However, there may be exceptions to this general statement (e.g., porous materials inoculated with parenchymal cells or porous materials implanted
into bone. As previously stated, the tissue response to implants is part dependent upon the extent of injury or defect created in the implantation procedure. Repair of implant sites can involve two distinct processes: regeneration, which is replacement of injured tissue by parenchymal cells of the same type, or replacement by connective tissue that constitutes the fibrous capsule. These processes are generally controlled by either (1) the proliferative capacity of the cells in the issue or organ receiving the implant and the extent of injury as it relates to destruction, or, (2) persistence of tissue the tissue framework of the implant site.

The regenerative capacity of cells allows them to be classified into three groups: labile, stable (or expanding) and permanent (or static) cells. Labile cells continue to proliferate throughout life, stable cells retain his capacity but do not normally replicate; and permanent cells cannot reproduce themselves after birth. Perfect repair with restitution of normal structure can theoretically only occur in tissue consisting of stable and labile cells, while all injuries to tissues composed of permanent cells may give rise to fibrosis and fibrous capsule formation with very little restitution of the normal tissue or organ structure. Tissues composed to permanent cells (e.g., nerve cells, skeletal muscle cells, cardiac muscle cells) most commonly undergo an organisation of inflammatory exudate, leading to fibrosis. Tissues composed of stable cells (e.g., parenchymal cells of the liver, kidney, and pancreas); mesenchymal cells (e.g., fibroblasts, smooth muscle cells, osteoblasts, and chondroblasts); and vascular endothelial and labile cells (e.g., epithelial cells and lymphoid and haematopoietic cells) may also follow this pathway to fibrosis or may undergo resolution of the inflammatory exudate, leading to restitution of the normal tissue structure.

The condition of the underlying framework or supporting stroma of the parenchymal cells following an injury plays an important role in the restoration of normal tissue structure. Retention of the framework may lead to restitution of the normal tissue structure while destruction of the framework most commonly leads to fibrosis. It is important to consider the species-dependent nature of the regenerative capacity of cells. For example, cells from the same organ or tissue...
but from different species may exhibit different regenerative capacities and/or connective tissue repair.

Following injury, cells may undergo adaptations of growth and differentiation. Important cellular adaptations are atrophy (decrease in cell size or function), hypertrophy (increase in cell size), hyperplasia (increase in cell number) and metaplasia (change in cell type). Other adaptations include a change by cells from producing one family of proteins to another (phenotypic change), or marked overproduction of protein. This may be the case in cells producing various types of collagens and extracellular matrix proteins in chronic inflammation and fibrosis. Causes of atrophy may include decreased workload (e.g., stress-shielding by implants), and diminished blood supply and inadequate nutrition. (e.g., fibrous capsules surrounding implants).

Local and systemic factors may play a role in the wound healing response to biomaterials or implants. Local factors include the site (tissue or organ) of implantation, adequacy of blood supply and potential for infection. Systemic factors may include nutrition, haematological derangements, gluco-cortical steroids, and pre-existing diseases such as atherosclerosis, diabetes, and infection.

Finally, the implantation of biomaterials or medical device may be best viewed at present from the perspective that the implant provides an impediment or hindrance to appropriate tissue or organ regeneration and healing. Given our current inability to control the sequence of events following injury in the implantation procedure, restitution of normal tissue structures with function is rare.

Current studies directed toward developing a better understanding of the modification of the inflammatory process, stimuli providing for appropriate proliferation of permanent and stable cells, and the appropriate application of growth factors may provide keys to the control inflammation, wound healing, and fibrous encapsulation of biomaterials.
2.2.3. Mathematical Model of Oxygen Diffusion Across a Biomembrane

Oxygen is very often the limiting nutrient in reconstructed tissues. The oxygen requirements vary largely with cell type. Among the cells commonly used in bioartificial systems, hepatocytes and pancreatic islet cells are particularly sensitive to the availability of oxygen. Oxygen is required for efficient cell attachment and the spreading to planar surfaces as well as microcarriers [29, 30]. Based on a simple mathematical model, we can estimate the critical distance at which the oxygen concentration at the cell surface becomes limiting (this concentration is arbitrarily set equal to $K_m$) if an unstirred aqueous layer is placed between the gas phase (air) and a confluent monolayer of cells [31]. In the case of a confluent monolayer of hepatocytes ($2 \times 10^5 \text{ cells/cm}^2$), this distance is 0.95 mm. Thus, in a first approximation, a successful bioartificial liver system containing hepatocytes will have to keep the diffusional distance between the oxygen-carrying medium and cells to below 1 mm (assuming a confluent cell monolayer on the surface). Figure 2.6 was used to estimate the maximum half thickness of a cell mass surrounded by a membrane or external diffusion barrier before the nutrient concentration in the centre falls below the Michaelis-Menten constant for nutrient uptake, a sign of nutrient limitation at the cellular level. Oxygen uptake parameters for different cell types are given by Fleischaker [32]. Values for hepatocytes and pancreatic islets have also been reported [31, 33, 34]. For illustrative purposes cylindrical hepatocytes aggregates were used as an example. The following was assumed: no external barrier ($R_1/R_0 = 1$); medium saturated with air at 37°C at the aggregate surface (160 mmHg = 190 nmol/cm³); diffusivity of oxygen in aggregates ($D_o$) similar to that of water ($2 \times 10^{-5} \text{ cm}^2/\text{s}$); a packed cell mass (given a cell diameter of approximately 20 µm, this corresponds to $1.25 \times 10^8 \text{ cells/cm}^3$). The oxygen uptake parameters for hepatocytes were $\mu_{\text{max}} = 0.4 \text{ nmol/10}^6 \text{cells/s}$ (thus 50 nmol/cm³/s for the above cell concentration) and $K_m = 0.5 \text{ mmHg}$ (e.g., 0.6 nmol/cm³). We obtain $C_{\text{r}}/K_m = C_o/K_m = 320$, $(\mu_{\text{max}} R^2)/D_o K_m = 724, 1370$ and 2010 for the slab, cylindrical and spherical geometries, respectively, and thus the corresponding maximum half thickness obtained are $R$.
= 132, 181, and 220 µm, respectively. We now consider the case where there is an 100µm thick membrane around a cylindrical cell aggregate assuming that the diffusivity of oxygen in the membrane (D₁) is the same as in the cell mass. An aspect ratio (α) must be assumed and values of Cₒ/Kₘ, (µmaxR²)/DₒKₘ), and Rₒ are calculated. R₁ is then obtained from the assumed aspect ratio. Calculations must be performed with several aspect ratios until the difference R₁ – Rₒ equates the membrane thickness. It was found that α =0.6 generates Cₒ/Kₘ = 141, (µmaxR²)/(DₒKₘ) = 622, Rₒ = 122 µm. For a cylinder, = Dₒ/D₁ 1n(R₁/Rₒ), and thus in this case R₁ = 222µm. Thus, the maximum half-thickness of the cell mass is 122µm, as compared to 181 µm in the absence of the membrane.

These estimates can be used as first guidelines to design a bioartificial liver, and they clearly suggest that the thickness of the cell mass must be limited to a few hundred microns to prevent the formation of an anoxic core.
Figure 2.6 Collection to predict the maximum half-thickness $R$ of a cell mass surrounded by a shell of thickness $R_1- R_0$ without nutrient limitation, assuming that diffusion is the only transport mechanism involved. A: The nutrient concentration at the surface of the cell mass normalized to the Michaelis-Menten constant for the nutrient by cells ($C_0/K_m$) is obtained from the normalized bulk nutrient concentration ($C_1/K_m$), and the aspect ratio of the system ($R_1/R_0$). $D_0$, $D_1$ are the diffusivities for nutrient within the cell mass and the external diffusion barrier, respectively. The partition coefficient between the cell mass and the surrounding shell is assumed to be equal to 1.

B. The half thickness $R$ for which $C/K_m = 1$ in the centre ($R=0$) is obtained from the value of the y-axis corresponding to $C_0/K_m$, knowing, in addition to the parameters listed above, the maximum nutrient uptake rate by cells ($\mu_{\text{max}}$). If $K_m$ is unknown, a zero order approximation may be used, in which case $K_m$ is set arbitrarily so that $C_1/K_m$ falls in the linear portion of the curve in A. In B, $R_o$ is obtained using the line labelled “0th order approximation,” and corresponds to the half thickness $R$ for which $C = 0$ at the centre ($R = 0$).
Table 2.2 Maximum Oxygen Uptake Rates of Cells Used in Tissue Reconstruction

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>$\mu_{\text{max}} \text{ (nmol/10}^6\text{cells/s)}$</th>
<th>$K_{0.5}^a \text{ (mmHg)}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4/2/0-derived mouse hybridoma</td>
<td>0.053</td>
<td>0.28</td>
<td>[35]</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>0.38 (day 1, single gel)</td>
<td>5.6</td>
<td>Oxyvice paper</td>
</tr>
<tr>
<td></td>
<td>0.25 (day 3, single gel)</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Pancreatic Islet cells</td>
<td>25.9 nmol/cm$^3$/s (100mg/dl glucose)</td>
<td>0.44</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>46.1 nmol/cm$^3$/s (300mg/dl glucose)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Oxygen tension for which the oxygen uptake rate equals half the maximum.
3. Tissue Engineering

This chapter reviews the state-of-the art in tissue engineering. Current obstacles and challenges are highlighted.

Tissue engineering is “an interdisciplinary field that applies the principles of engineering and of life science towards the development of biological substitutes that restore, maintain, or improve tissue or organ function [36]”. In a classical sense, tissue engineering implies the use of tissue or organs specific cells for seeding a scaffold ex vivo and holds the promise of one day replacing living tissue with living tissue designed and fabricated to meet the individual defects [36, 37]. This approach is convincing based on several observations for the behaviour of tissues and cells: Most tissues undergo remodeling, isolated cells tend to form the appropriate tissue structures in vitro under favourable conditions, isolated cells require a template to guide their organization into a proper architecture.

Tissue engineering focuses on providing a 3D environment, or scaffold, for cell attachment and growth, and assumes that by simulating the in vivo environment, cells can be coaxed into creating a desired tissue type or function. Therefore scaffold design must mimic, as far as possible, the structure and biological function of the gold standard scaffold; the extracellular matrix (ECM).

The ideal requirements of these scaffolds are that they provide mechanical support, are biocompatible, are non biodegradable over a predetermined period of time, can be molded into the desired shape, are easy to sterilize and can allow cell attachment, viability, proliferation and differentiation. The ultimate aim of
tissue engineering is to make a 3D cell containing scaffold that can be implanted in the body to cure or repair a defect.

3.1. Conventional Scaffolds

Biomedical implants have been used since ancient times – for example, a Brazilian group recently reported that the ancient Incas successfully used gold plates to repair cranial defects [3]. Until the last few decades of the 20th century, the criteria used in choosing materials for implants has fundamentally changed very little and usually implant materials were chosen that were functional because of their inertness. Since the discovery in the 1960s that some glass ceramics actively bond to living bone, however, the focus has shifted away from inert materials and toward materials that are bioactive – those that deliberately elicit a specified response from the body. Currently, most scaffolds provide a three-dimensional environment in which tissue can grow and develop, so that it is able to reproduce the functions of the tissue it is intended to replace. Some scaffolds may be designed to be implanted without any cellular component [38] – instead they are designed to encourage tissue ingrowth and de novo tissue synthesis in vivo – while most are intended to have some kind of cellular component engineered in vitro before implantation. The latter strategies require that cells have access to nutrients and space to grow. For this reason most scaffolds, regardless from which material they are made, are constructed with some kind of porous network and cultured with cells in a manner that encourages nutrient transport. For instance, inorganic materials such as bioactive glass and calcium phosphates have been extensively been used for bone tissue engineering because of the similarities to and their ability to bond with bone’s natural mineral backbone. Bioactive glasses can be sintered in powder form to create porous networks [39], or, when in solution, can simply be “foamed” using soap and gelled to make sol-gels [40]. Similarly, porosity can be engineered into polymers, such as polyesters (which have the advantage of being biodegradable), either by foaming the polymer solution [41] or by molding the dissolved polymer around lumps of another material such as salt, allowing the polymer to harden and then
leaching out the salt with water [42]. Porous networks can also be engineered into natural molecules – for example, collagen gels can be freeze-dried before cell seeding [43]. Alternatively, hydrogels can be used as scaffolds for cell growth and cell delivery. Since the gelling process is often nontoxic, cells can be introduced into the solution prior to gelation. In the case of alginate, a natural polymer made up of chains of guluronic and manuronic acid, calcium is usually added to cell/gel solutions, which crosslinks these chains and hardens the gel [25]. Likewise, collagen gels can be hardened by altering the pH of the solution [44, 45] and poly(ethene) glycol can be solidified using light [46]. Hydrogels have different mechanical properties from other scaffolds, so the material must be selected on the basis of its properties, keeping in mind the intended application.

But all of these scaffolds have their disadvantages. Inorganic scaffolds such as ceramics and glasses tend to be too brittle and weak to be used in load-bearing applications, and even bioactive glasses, discovered more than 30 years ago, are limited to non-load-bearing applications such as the replacement of small bones in the middle ear [47]. Artificial polymers, on the other hand, may be viewed by the body as foreign material because they lack sticky surface molecules for cell adhesion. Their degradation products are, in the case of polyesters, acidic, and though not directly toxic, may create a possibly unphysiological acidic microenvironment. This is particularly important and often overlooked in bone tissue engineering – the natural mechanism by which bone is degraded in vivo by osteoclasts involves the formation of an acidic microenvironment! Collagen may be a better bet in this case, as the natural mechanism of bone formation involves the mineralization of a collagen scaffold created by osteoblasts – unsurprisingly collagen scaffolds are readily mineralized in tissue engineering experiments [48]. Another problem with porous scaffolds is that because cells are seeded onto the internal porous matrix of the scaffold it becomes arguable whether the cells experience a truly three dimensional environment – they merely ‘see’ a slightly curved two dimensional surface. This can be solved to some extent by decreasing the pore size and adding surface texture, or embedding cells in a soft
extracellular matrix, but then problems arise as to how to keep deeply embedded cells supplied with nutrients.

### 3.2. Cell Sources in Tissue Engineering: The Promise of Stem Cells

Cells play a crucial role to tissue regeneration and repair due to their characteristics of proliferation and differentiation, cell-to-cell interaction, biomolecular production, and extracellular matrix formation. The sources of cells used in tissue engineering can be autologous, allogeneic, or xenogeneic. Ideal donor cells for tissue engineering would be those that are easily accessible, that can easily expand without permanently altering the phenotype and function and without transmitting species-specific pathogens, that are multipotent to differentiate or transdifferentiate into a variety of tissue- or organ-specific cells with specialized function, and that have the least immunologic response. Some cells, such as keratinocytes, fibroblasts, chondrocytes, endothelial cells, smooth muscle cells or skeletal muscle satellite cells, proliferate rapidly. They are good tissue-specific cell sources for tissue engineering. However, other cells, such as hepatocytes or adult cardiomyocytes, proliferate slowly or not at all. Therefore, alternative sources of cells are needed.

Recent advances in stem cell biology have had a marked impact on the progress of tissue engineering [49]. Stem cells, which are capable of self-renewal and differentiation into various cell lineages, hold great promise for treating affected tissue in which the source of cells for repair is limited or not readily accessible. Cells derived from human embryonic blastocysts (after undifferentiated proliferation in vitro for 4-5 months) still maintain the developmental potential to form trophoblast and derivatives of all 3 embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous
epithelium (ectoderm) [50]. Although these cell lines should be useful in human regenerative medicine, the ethical and legal issues are still under debate.

3.3. Scaffold and Stem Cells

Stem cells, of course, are reliant on the extracellular environment not only to survive but also to develop into a functional tissue. So increasingly, tissue engineers are beginning to use the composition of scaffolds to persuade stem cells to differentiate. Arinzeh et al. [51] have shown that adjusting the ratio of hydroxyapatite to tri-calcium phosphate could influence the degree to which osteogenic differentiation of MSCs occurs, while others have begun to engineer bioactive factors into porous scaffolds. For instance, Kim et al. [52] have created a polyester scaffold that slowly exudes vitamin C and β-glycerophosphate and demonstrated an increase in osteogenesis from MSCs, while Yang et al. [53] have demonstrated increased osteogenic differentiation in a polylactic acid (PLA) scaffold spiked with a bone-specific growth factor. Alternatively, mechanical force can be used to stimulate differentiation – Altman et al. [54] have recently shown that applying a mechanical force to a collagen-gel scaffold can encourage MSCs to differentiate into ligament tissue.

Micro- and nanopatterned scaffolds have been investigated less well in regard to stem cells, although two recent studies highlight their attractiveness. Silva and colleagues [55] included a five amino acid, laminin-specific cell-binding domain (which binds to specific integrins on cell surfaces) at the hydrophilic head of their amphiphiles, and showed that neural stem cells could be induced to differentiate into neurons when cultured within the network. In contrast, cells grown in control scaffolds without the laminin-specific domain or on two-dimensional tissue culture plastic coated with laminin solution differentiated much less. This was hypothesized to be largely as a result of the density of the cell-binding ligands to which the cells were exposed, indicating clearly the importance of extracellular matrix in influencing cell function. In a similar study, Hosseinkhani et al. [56] replaced the laminin-specific domain in the amphiphilic molecule with the amino
acid sequence, arginine-glycine-aspartate (RGD), a common cell-binding domain in many extracellular matrix proteins, especially collagen. They then showed that the differentiation of MSCs to osteoblasts is significantly enhanced compared with amphiphilic nanofibers without this sequence on to two-dimensional controls.

So far, remarkably few studies have been published on the effect of three-dimensional environments and scaffolds on ESC differentiation. In two rare examples, Levenberg and colleagues [57, 58] have shown that human ESCs embedded in an extracellular matrix gel called Matrigel can be differentiated in three dimensions on conventional polyester scaffolds. In these cases, several structures that resemble primitive tissues were generated, depending on the content of the growth medium. The authors also show that tissues grown in three dimensions express higher levels of differentiation-associated proteins than those on coated two-dimensional surfaces. Interestingly, another group has recently reported that the chondrogenic differentiation of human ESCs in a PEG hydrogel is dependent upon whether or not the hydrogel contains adhesive RGD sites, illustrating the importance of the cell matrix and microenvironment in ESC differentiation [59].

The use of more novel, patterned scaffolds should provide ESC biologists with an important new tool to stimulate and model differentiation in vitro. In this way, scaffolds could play a role in directing tissue organization, not only with the aim of producing tissue for transplant but also for studying differentiation in vitro. Similarly, ESCs could be compartmentalized within scaffolds to study cell-cell interactions and their effect on cell differentiation and tissue formation. Such scaffolds will undoubtedly find exciting applications in the study of ESC differentiation.

### 3.4. Angiogenesis

One vital requirement for tissue engineered grafts to survive is the sufficient supply of oxygen and nutrients and removal of carbon dioxide and waste. Both
the supply and removal process involve the transport of substances in the tissue possibly in all forms, gas, liquid, and solid. Transport in tissue engineering has two main issues: to design tissue with a well-perfused transport network and to create tissues that would have the function of transport, such as blood vessels. To properly perfuse large organs, such as kidney and liver, diffusion and convection alone cannot meet the requirements. A well-established vascular network is essential. However, techniques to grow a blood vessel network throughout the tissue have yet to be developed.

Using nanofabrication, different size scale textures and structures can be obtained from a few nanometers to tens of micrometers. These structures are good substrates for learning the endothelial cell behaviors on different structures. For example, Dike et al. showed that endothelial cells could switch between proliferation, apoptosis, and differentiation, when attached on different size scale textures and vascular-like tubular structures could form on certain texture range surfaces [60]. Kaihara et al. [37] applied micromachining to generate branched channels to promote vascular formation and ultimately to enhance the transport in tissue-engineered liver. Moldovan and Ferrari [61] developed a silicon-based “angiogenesis assistant device”, which is a nanofilter-based, drug delivery silicon capsules.

The nanofilter with controlled size ranging from 10 to 200 nm for angiogenic growth factor delivery is jointed with a millimeter-scale silicon frame with an endothelial cell coating for blood compatibility and vascularization. The nanofilter is created with micromachining and sacrificial layer techniques. This device would possess several advantages in both research and clinical applications, such as a controllable delivery if there is a combined sensor and remote control device in the design, a supply of endothelial cells for better blood compatibility, a delivery of angiogenic factors for improved vascularization, and ease of implantation.

The microscopic networked tubes with branches, which mimic the circulatory system in the body, can be designed by computer and fabricated with nanotechnology. Kaazempurmofrad's [62] approach is to create two half-pipes on
silicone, which serves as a mold for making degradable polymer tubes, using nanofabrication techniques. This technique has potential applications in tissue engineering liver and kidney, which require a significant amount of blood vessels to provide oxygen and nutrients to the engineered grafts.
CHAPTER

4

4. Nanotechnology in Biomaterials Science

This chapter discusses the state of the art of nanotechnology in biomaterials science. It highlights how nanotechnology is providing the science and technology for the development of next generation biomaterials which simulate the structural properties and chemical characteristics of natural extra cellular matrices. Current research efforts to create nanoscale surfaces features using ceramics, metals, polymers, and composites are reviewed.

4.1. Nanotechnogoly in biomaterials

The emergence of micro- and nanoscale science and engineering has provided new avenues for engineering materials with macromolecular and even down to molecular-scale precision, leading to diagnostic and therapeutic technologies that will revolutionize the way health care is administered. Biomaterials have evolved from off-the-shelf products (e.g., Dacron for vascular grafts) to materials that have been designed with molecular precision to exhibit the desired properties for a specific application, often mimicking biological systems [63, 64].

Controlling interactions at the level of natural building blocks, from proteins to cells, facilitates the novel exploration, manipulation, and application of living systems and biological phenomena. Nanostructured tissue scaffolds and biomaterials are being applied for improved tissue design, reconstruction, and reparative medicine [37, 65-67]. Nano- and micro-arrays have been established as the preferred method for carrying out genetic and other biological (e.g., drug discovery) analysis on a massive scale [68]. Natural nanopores [69, 70] and
synthetic nanopores of tailored dimensions [71, 72] are probing, characterizing, and sequencing biological macromolecules and have demonstrated the possibility to analyze the structure of individual macromolecules faster and cheaper [10]. Self-assembly is being applied to create new biomaterials with well-ordered structures at the nanoscale, such as nanofiber peptide and protein scaffolds [73]. In addition, polymer networks with precisely engineered binding sites have been created via molecular imprinting, where functional monomers are preassembled with a target molecule and then the structure is locked with network formation [74].

In medical diagnostics, the speed and precision with which a condition is detected directly impacts the prognosis of a patient. Point-of-care (POC) diagnostic devices, which enable diagnostic testing (in vivo or ex vivo) at the site of care, can enhance patient outcomes by substantially abbreviated analysis times as a result of the intrinsic advantages of the miniature device and by eliminating the need for sample transport to an on-site or off-site laboratory for testing. The development of micro or miniaturized total analysis systems (μ-TAS), also referred to as lab-on-a-chip devices, has profoundly impacted the corresponding development of POC diagnostic devices. These μ-TAS devices integrate microvalves, micropumps, micro-separations, microsensors, and other components to create miniature systems capable of analysis that typically requires an entire laboratory of instruments. Since being introduced as a novel concept for chemical sensing devices [75], μ-TAS devices have been applied as innovative biological devices [76] and POC diagnostic devices [77, 78]. With the further development of micro- and nanosensors, POC diagnostic devices will provide for improved medical management, leading eventually to self-regulated POC diagnostic devices that intermittently or continuously monitor the biological molecule of interest and deliver the therapeutic agents as required.

Additionally, nanoscale science and engineering have accelerated the development of novel drug delivery systems and led to enhanced control over how a given pharmaceutical is administered, helping biological potential to be transformed into medical reality [79]. Micro- and nanoscale devices have been fabricated using integrated circuit processing techniques and have been
demonstrated to allow for strict control over the temporal release of the drug. Silicon microchips that can provide controlled release of single or multiple chemical substances on demand via electrochemical dissolution of the thin anode membranes covering microreservoirs have been created [80]. The advantages of this microdevice are that it has a simple release mechanism, very accurate dosing, and ability to have complex release patterns, potential for local delivery, and possible biological drug stability enhancement by storing in a microvolume that can be precisely controlled. More recently, multi-pulse drug delivery from a resorbable polymeric microchip device was demonstrated [81].

In particular, the development of polymer systems that are able to interact with their environment in an “intelligent” manner has led to novel materials and applications. These intelligent materials are attractive options as functional components in micro- and nanodevices, due to the ease with which their recognition and actuation properties can be precisely tailored. In addition to advances in polymer nanotechnology for sensing and recognizing changes in micro-environments, advances have been made concerning tissue regeneration on ceramic and metallic nanomaterials.

Broadly speaking, nanotechnology embraces a system whose core of materials is in the range of nanometers \((10^{-9}\text{ m})\) [82-92]. The application of nanomaterials for medical diagnosis, treatment of failing organ systems, or prevention and cure of human diseases can generally be referred to as nanomedicine [51,52]. The branch of nanomedicine devoted to the development of biodegradable or nonbiodegradable prostheses fall within the purview of nanobiomedical science and engineering [51,52]. Although various definitions are attached to the word “nanomaterial” by different experts, the commonly accepted concept refers nanomaterials as that material with the basic structural unit in the range 1 to 100 nm (nanostructured), crystalline solids with grain sizes 1 to 100 nm (nanocrystals), individual layer or multilayer surface coatings in the range 1 to 100 nm (nanocoatings), extremely fine powders with an average particle size in the range 1 to 100 nm (nanopowders) and, fibers with a diameter in the range 1 to 100 nm (nanofibers) [42,43].
Since nature itself exists in the nanometer regime, especially tissues in the human body [53], it is clear that nanotechnology can play an integral role in tissue regeneration. Specifically, bone is composed of numerous nanostructures — like collagen and hydroxyapatite (HA) that, most importantly, provide a unique nanostructure for protein and bone cell interactions in the body [50]. Although the ability to mimic constituent components of bone is novel in itself, there are additional reasons to consider nanomaterials for tissue regeneration such as in orthopedic applications: their special surface properties compared to conventional (or micron constituent component structured) materials [87-90]. For example, a nanomaterial has increased numbers of atoms at the surface, grain boundaries or material defects at the surface, surface area, and altered electron distributions compared to conventional materials [90].

In summary, nanophase material surfaces are more reactive than their conventional counterparts. In this light, it is clear that proteins which influence cell interactions that lead to tissue regeneration will be quite different on nanophase compared with conventional implant surfaces.

Despite this, the evolution of tissue engineering has centred on the use of materials with non-biologically inspired micron surface features [93, 94], mostly changing in chemistry or micron roughness but not degree of nanometer roughness. In this context, it should not be surprising why the optimal tissue engineering material (in particular, to regenerate bone) has not been found.

4.2. Current research efforts to improve biomedical performance at the nanoscale.

Nanoscale materials currently being investigated for bone tissue engineering applications can be placed in the following categories: ceramics, metals, polymers, and composites thereof. Each type of material has distinct properties that can be advantageous for specific bone regrowth applications. For example, HA, a ceramic mineral present in bone, can also be made synthetically.
Ceramics, though, are not mechanically tough enough to be used in bulk for large-scale bone fractures. However, they have found applications for a long time as bioactive coatings due to their ionic bonding mechanisms favourable for osteoblast (or bone-forming cells) function [95]. Unlike ceramic materials, metals are not found in the body. Due to their mechanical strength and relative inactivity with biological substances, metals (specifically, Ti, Ti6Al4V, and CoCrMo) have been the materials of choice for large bone fractures [93, 94]. Polymers exhibit unique properties (such as viscosity, malleability, moldability) and possess mechanical strength that is comparable with many soft (not hard) tissues in the body [96]. To date, because of their excellent friction properties, polymers (like ultra-high-molecular-weight polyethylene) have been primarily used as articulating components of orthopaedic joint replacements [97]. Additionally, some polymers (particularly the polyester family) can be reabsorbed or degraded in the body, which opens the window for controllable repair of damaged bone that is actively being investigated in tissue engineering circles.

Lastly, composites of any or all of the above can be synthesized to provide a wide range of material properties to increase bone implant performance [59]; such ability to tailor composite properties to specific orthopaedic applications makes them attractive.

Owing to the numerous materials currently being used and investigated in orthopaedics, select efforts to create nanoscale surfaces in all of these categories: ceramics, metals, polymers, and composites are reviewed. Several current and potential materials that have shown promise in nanotechnology for bone biomedical applications as well as needed future directions will be emphasized.

4.3. Ceramic nanomaterials

Perhaps slightly more mature, is the application of nanophase ceramics in bone tissue engineering applications. The next series of sections will highlight the
improvement in bone regeneration that can be obtained through the use of ceramic nanotechnology.

4.3.1. Increased osteoblast functions

The first report correlating increased bone cell function with decreased material grain or particulate size into the nanometer regime dates back to 1998 and involves ceramics [98]. Such reports described how in vitro osteoblast (bone-forming cell) adhesion, proliferation, differentiation (as measured by intracellular and extracellular matrix protein synthesis such as alkaline phosphatase), and calcium deposition were enhanced on ceramics with particulate or grain sizes less than 100 nm [98-107]. Specifically, this was first demonstrated for a wide range of ceramic chemistries including titania, alumina, and HA [100]. For example, four, three, and two times the amount of calcium-mineral deposition was observed when osteoblasts were cultured for up to 28 days on nanophase compared with conventional alumina, titania, and HA, respectively [102]. It is important to note that for each respective nanophase and conventional ceramic mentioned in these first reports, similar chemistry and material phase were studied [98-107]. That is to say, only the degree of nanometer surface features were altered between respective nanophase and conventional alumina, titania, and HA. This is important since as previously discussed it is well known that alterations in surface chemistry will influence bone cell function [93-95, 97, 108, 109], but this was the first time changes in the degree of nanometer roughness alone were reported to enhance bone cell responses [98].

Although these studies provided preliminary evidence that osteoblast functions can be promoted on nanostructured materials compared with conventional materials regardless of ceramic chemistry, Elias et al. [110] further described a study where the topography of compacted carbon nanometer fibers were transferred to poly-lactic-glycolic acid (PLGA) using well-established silastic mold techniques. The same procedure was followed for compacts composed of conventional carbon fibers. The successful transfer of nanometer surface
features in compacted carbon nanometer fibers and micron surface features in conventional fiber compacts were compared. Importantly, osteoblast adhesion increased on PLGA molds made from nanometer fibers compared to conventional carbon fibers [110]. Increased osteoblast functions were also observed on the original nanometer fiber material compared with conventional carbon fiber compacts. In this manner, this study provided further evidence of the importance of nanometer surface features (and not chemistry) in promoting functions of bone-forming cells.

Equally interesting, a step-function increase in osteoblast performance has been reported at distinct ceramic grain sizes, specifically at alumina and titania spherical grain sizes below 60 nm [100]. This is intriguing since when creating alumina or titania ceramics with average grain sizes below 60 nm, a drastic increase in osteoblast function was observed compared to respective ceramics with grain sizes just 10 nm higher (i.e., those with average grain sizes of 70 nm) [100]. This critical grain size for improving osteoblast function is also of paramount importance since numerous other special properties (such as mechanical, electrical, catalytic) of materials have been reported when grain size is specifically reduced to below 100 nm [[82-90]. With this information, evidence has been provided to show for the first time that the ability of nanophase ceramics to promote bone cell function is indeed limited to grain sizes (or subsequent surface features) below 100 nm, specifically below 60 nm [100]. Thus, another novel size-dependent property of nanostructured ceramics has been elucidated by these studies.

Although an exact explanation as to why greater bone regeneration is observed on smaller grain size ceramics in the nanometer regime is not known to date, it is believed that the importance of this specific grain size in improving osteoblast function is connected with interactions of vitronectin (a protein known to mediate osteoblast adhesion with linear dimensions remarkably similar to the critical grain size of 60 nm mentioned above) [101, 106]. Moreover, as mentioned previously, several studies have indicated that vitronectin and other proteins important for osteoblast adhesion are more well spread and thus expose amino acid sequences to a greater extent when interacting with nanometer ceramics.
compared with conventional ceramics [101, 106]. It is also intriguing to note that numerous investigators have confirmed that the minimum distance between protein ligands (such as arginine-glycine-aspartic acid or RGD) necessary for cell attachment and spreading is in the nanometer regime (specifically from 10 to 440 nm depending on whether the study was completed with full proteins, protein fragments, or single RGD units) [111-116]. Therefore, an underlying substrate surface that mediates protein spreading (as opposed to protein folding) to expose such ligands, coupled with a nanometer surface roughness to further project such ligands to the cell, may promote cell adhesion due to this optimal ligand spacing.

4.3.2. Increased osteoclast function

In addition to studies highlighting enhanced osteoblast function on nanophase ceramics, increased functions of osteoclasts (bone-resorbing cells) have been reported on nanospherical compared with larger grain size alumina, titania, and HA [107]. Specifically, osteoclast synthesis of tartrate-resistant acid phosphatase (TRAP) and subsequent formation of resorption pits was up to two times greater on nanophase compared to conventional ceramics such as HA. Coordinated functions of osteoblasts and osteoclasts are imperative for the formation and maintenance of healthy new bone juxtaposed to an orthopaedic implant [54]. Frequently, newly formed bone juxtaposed to implants is not remodelled by osteoclasts and thus becomes unhealthy or necrotic [96]. At this time, the exact mechanism of greater functions of osteoclasts on nanophase ceramics is not known, but it may be tied to the well-documented increased solubility properties of nanophase compared with conventional materials [88]. In other words, due to larger numbers of grain boundaries at the surface of smaller grain size materials, increased diffusion of chemicals (such as TRAP) may be occurring to subsequently result in the formation of more resorption pits.

Collectively, results of promoted functions of osteoblasts coupled with greater functions of osteoclasts imply increased formation and maintenance of healthy bone juxtaposed to an implant surface composed of nanophase ceramics. In fact, although not compared with conventional grain size apatite-coated metals, some
studies have indeed demonstrated increased new bone formation on metals coated with nanophase apatite [71]. Incidentally, coating metals with nanophase HA has been problematic [117]. For example, owing to their small grain size, techniques which use high temperatures (like plasma spray deposition) are not an option since they will result in HA grain growth into the micron regime [117]. To circumvent such difficulties, some investigators have allowed nanophase HA to precipitate on metal surfaces; this can be time consuming and not very controllable [71]. In contrast, others have developed novel techniques which use high pressure-based processes that do not significantly create elevated temperatures to coat nanophase ceramics on metals so as to retain their bioactive properties [118].

4.3.3. Decreased competitive cell functions

Importantly, it has also been shown that competitive cells do not respond in the same manner to nanophase materials as osteoblasts and osteoclasts do [101, 102, 119]. In fact, decreased functions of fibroblasts (cells that contribute to fibrous encapsulation and callus formation events that may lead to implant loosening and failure [108] and of endothelial cells (cells that line the vasculature of the body)) have been observed on nanophase compared with conventional ceramics [101]. In fact, the ratio of osteoblast to fibroblast adhesion increased from 1:1 on conventional alumina to 3:1 on nanophase alumina [94]. Previously, such selectivity in bone cell function on materials has only been observed through delicate surface chemistry (e.g., through the immobilization of peptide sequences like Lys-Arg-Ser-Arg or KRSR) [120]. It has been argued that immobilized delicate surface chemistries may be compromised once implanted due to macromolecular interactions that render such epitopes non-functional in vivo. For these reasons, it is important to note that studies demonstrating select enhanced osteoblast and osteoclast functions with decreased functions of competitive cells on nanophase materials have been conducted on surfaces that have not been chemically modified by the immobilization of proteins, amino acids, peptides, or other entities [101, 110, 119]. Rather it is the unmodified, raw material surface that is specifically promoting bone cell functions.
Fibroblast function was also investigated in the same study that was previously mentioned in which Elias et al. transferred the topography of compacted carbon nanometer fibers compared to conventional fibers to PLGA using well-established silastic mold techniques [111]. Similar to the observed greater osteoblast adhesion already noted, decreased fibroblast adhesion was measured on PLGA molds synthesized from carbon nanometer fibers compared to conventional fibers [111]. Again, this was the same trend observed on the starting material of carbon nanometer fiber compacts compared to conventional fiber compacts [111]. Thus, this study demonstrated the importance of a nanometer surface roughness (and not chemical composition of the material) in decreasing functions of fibroblasts that may lead to undesirable fibrous encapsulation and callus formation events inhibiting osseointegration of orthopedic implants with surrounding bone.

4.3.4. Increased osteoblast functions on nanofibrous materials

Recently, researchers have further modified nanophase ceramics to simulate not only the nanometer dimension but also the aspect ratio of proteins and HA crystals found in the extra-cellular matrix of bone [119]. For example, consolidated substrates formulated from nanofibrous alumina (diameter = 2 nm, length > 50 nm) increased osteoblast functions in comparison with similar alumina substrates formulated from the aforementioned nanospherical particles [119]. Specifically, Price et al. [119] determined a twofold increase in osteoblast cell adhesion density on nanofiber vs conventional nanospherical alumina substrates, following only a 2-h culture. Greater subsequent functions leading to new bone synthesis has also been reported on nanofibrous compared to nano- and conventional spherical alumina [119]. Thus, perhaps not only is the nanometer grain size of components of bone important to mimic in materials, but the aspect ratio may also be key to simulate in synthetic materials to optimize bone cell response.
Another class of novel biologically inspired nanofiber materials that have been investigated for orthopedic applications are self-assembled helical rosette nanotubes [121]. These organic compounds are composed of guanine and cytosine DNA pairs that self-assemble when added to water to form unique nanostructures. These nanotubes have been reported to be 1.1 nm wide and up to several millimeters wide [121]. Compared to currently used titanium, recent studies have indicated that osteoblast function is increased on titanium coated with helical rosette nanotubes [121]. Although in these studies it has not been possible to separate the influence of nanometer dimensions from the effects of nanotube chemistry on cell functions, it is clear that these nanotubes are another category of novel nanostructured materials that can be used to promote bone formation. It is also intriguing to consider what role self-assembled nanofibers may play in orthopedics since bone itself is a self-assembled collection of nanofibers.

In this context it is important to mention that only nanophase materials can mimic the unique aspect ratio of HA and proteins found in the extracellular matrix of bone; it is not possible for micron-sized materials to simulate the unique nanometer constituent components of bone. As mentioned previously, results concerning the importance of nanofibrous materials in promoting functions of osteoblasts have been reported for carbon and polymer molds of carbon nanofibers [110]. These findings consistently testify to the unprecedented ability to create nanomaterials to mimic the dimensions of components of physiological bone to promote new bone formation.

### 4.4. Metal nanomaterials

Although much work has been conducted on nanophase ceramics for orthopedic applications to date, several recent studies have focused on the analysis of bone regeneration on nanophase metals. Metals investigated to date include titanium, Ti6Al4V, and CoCrMo [122]. While many have attempted to create nanostructured surface features using chemical etchants (such as HNO3) on titanium, results concerning increased bone synthesis have been mixed [97].
Moreover, through the use of chemical etchants it is unclear what the cells may be responding to — changes in chemistry or changes in topography. For this reason, as was done for the ceramics in this chapter, it is important to focus on studies that have attempted to minimize large differences in material chemistry and focus only on creating surfaces that alter in their degree of nanometer roughness.

One such study by Ejiofor et al. [122] utilized traditional powder metallurgy techniques without the use of heat to avoid changes in chemistry to fabricate different particle size groups of Ti, Ti6Al4V, and CoCrMo. Increased osteoblast adhesion, proliferation, synthesis of extracellular matrix proteins (like alkaline phosphatase and collagen), and deposition of calcium containing mineral was observed on respective nanophase compared to conventional metals [122]. This was the first study to demonstrate that the novel enhancements in bone regeneration previously seen in ceramics by decreasing grain size can be achieved in metals. Interestingly, when Ejiofor et al. [122] examined spatial attachment of osteoblasts on the surfaces of nanophase metals, they observed directed osteoblast attachment at metal grain boundaries. Because of this, the authors speculated that the increased osteoblast adhesion may be due to more grain boundaries at the surface of nanophase compared to conventional metals. As was the case with nanophase ceramics [101, 106], it is plausible that protein adsorption and conformation at nanophase metal grain boundaries may be greatly altered compared to non-grain boundary areas and conventional grain boundaries; in this manner, protein interactions at grain boundaries may be key for osteoblast adhesion.

### 4.5. Polymeric nanomaterials

For ceramics and metals, most studies conducted to date have created desirable nanometer surface features by decreasing the size of constituent components of the material, e.g., a grain, particle, or fiber. However, due to the versatility of polymers, many additional techniques exist to create nanometer surface
roughness values. In addition, polymers contribute even further to rehabilitating damaged tissue by possibly providing a degradable scaffold that dissolves within a controllable time while the native tissue reforms. Techniques utilized to fabricate nanometer features on polymers include e-beam lithography, polymer de-mixing, chemical etching, cast-mold techniques, and the use of spin-casting [122-130]. For those that have been applied to bone regeneration, chemical etching followed by mold casting and polymer de-mixing techniques have received the most attention [123, 124].

For chemical etching techniques, polymers investigated to date include PLGA, PU, and polycaprolactone [124, 126-128]. The idea proposed by Kay et al. has been to treat acidic polymers with basic solutions (i.e., NaOH) and basic polymers with acidic solutions (i.e., HNO3) to create nanosurface features [116]. Kay et al. observed greater osteoblast adhesion on PLGA treated with increasing concentrations and exposure times of NaOH only on two-dimensional films. As expected, data were also provided indicating larger degrees of nanometer surface roughness with increased concentrations and exposure times of NaOH on PLGA. Park et al. [126] took this one step further and fabricated three-dimensional tissue engineering scaffolds by NaOH treatment of PLGA. When comparing osteoblast functions on such scaffolds, even though similar porosity properties existed between nontreated and NaOH-treated PLGA (since similar amounts and sizes of NaCl crystals were used to create the pores through salt-leaching techniques), greater numbers of osteoblasts were counted on and in NaOH-treated PLGA [126]. Unfortunately, due to these fabrication techniques, it is unclear whether the altered PLGA chemistry or nano-etched surface promoted osteoblast adhesion; however, in light of the previous studies mentioned in this chapter, the authors of Ref. [126] suggested that the nanometer surface roughness of the NaOH-treated PLGA played an important role [126].

Studies have also been conducted on cell responses to polymers with changes in nanometer surface roughness without changes in chemistry. Specifically, Li et al. utilized polymer de-mixing techniques to create well-controlled nanometer islands of polystyrene and polybromo-styrene [129]. Although osteoblast functions have not been tested on these constructs to date, fibroblast morphology was
significantly influenced by incremental nanometer changes in polymer island dimensions. Again, this study points to the unprecedented control that can be gained over cell functions by synthesizing materials with nanometer surface features.

Although not related to orthopedic applications, vascular and bladder cell responses have also been promoted by altering the topography of polymeric materials in the nanometer regime [124, 126, 128, 130]. In these studies, chondrocytes [124], bladder [128], and vascular smooth muscle cell [127] adhesion and proliferation were greater on two-dimensional nanometer surfaces of biodegradable polymers such as PLGA, PU, and polycaprolactone; similar trends have recently been reported on three-dimensional PLGA scaffolds [130].

4.6. Composite nanomaterials

Owing to the previous information of increased osteoblast function on ceramics [107] and polymers [119], bone cell function on nanophase ceramic polymer composites have also been determined. Specifically, studies conducted to date show promoted osteoblast responses on composites of PLGA combined separately with nanophase alumina, titania, and HA (30:70 wt% PLGA/ceramic) [131]. For example, up to three times more osteoblasts adhered to PLGA when it contained nanophase compared to conventional titania particles [124]. Since similar porosity (both percentages and diameters) existed between PLGA with conventional titania compared to nanophase titania, another novel property of nanophase ceramic composites was elucidated in this study: increased osteoblast functions. This is in addition to numerous reports in the literature highlighting greater toughness of nanophase compared to conventional ceramic/polymer composites [83, 85, 86].

Moreover, promoted responses of osteoblasts have also been reported when carbon nanofibers were incorporated into polymer composites; specifically, three times the number of osteoblasts adhered on PU with increasing weight percentages of nanometer carbon fibers when compared with conventional
dimension carbon fibres[132]. As mentioned, reports in the literature have demonstrated higher osteoblast adhesion on nanophase carbon fibers in comparison with conventional carbon fibers (or titanium [ASTM F-67, Grade 2] [132], but this study demonstrated greater osteoblast adhesion with only a 2 wt% increase of carbon nanofibers in the PU matrix. Up to three and four times the number of osteoblasts that adhered on the 100:0 PU/CN wt%, adhered on the 90:10 and the 75:25 PU/CN wt% composites, respectively [132]. This exemplifies the unprecedented ability of nanophase materials to increase functions of bone cells whether used alone or in polymer composite form.
5

5. Aims objectives and methods

5.1. Rational of study

To date, most tissue engineering studies are focused on the investigations of macro-level structures (e.g., supercellular structures >100 µm and cellular structures >10 µm) to build the essential gross morphology and generate real-size organ systems. However, to ultimately engineer the functional units of the tissue, not only the supercellular and cellular scale structures but also the subcellular scale structures (0.1–10 µm) and nanostructures (1–100 nm) need to be constructed to control cellular environment, cell–molecular interactions, and cell–cell interactions. It is quite obvious that the full function of the tissues and organs cannot be recovered without rebuilding the ultrastructures of the tissue itself. The future of tissue engineering is highly dependent upon our profound knowledge of how subcellular and even smaller structures affect cell functions and how this knowledge will be transformed into technologies to fabricate biomimetic organ scale structures with subcellular resolution and nanoresolution, e.g., integration of functional cells into 3D architectures (scaffold) with nanoresolution structures for improved tissue functionality.

Therefore, engineering tissue toward the miniaturization at the nano-level is one of the most promising directions for tissue engineers. With the advent of nanotechnology, it is now possible to develop techniques to modify the surface structures and properties of biomaterials in order create the appropriate microenvironment for cells with predictable physical and biological properties.
5.2. Scope of Thesis

The scope of this thesis was to identify problems in current applied tissue engineering research and hence formulate problem based solutions exploiting the emerging micro-nanoscale technology to contribute to the development of better natural and synthetic materials and innovative scaffolds.

5.3. Aims of the study

A) To develop an innovative scaffold that mimics native cellular micro-environment, in order to guide and control cell viability and function, so that indefinite graft survival and clinically viable therapy can be achieved.

B) To explore and develop a renewable source of tissue cells for use in tissue engineering.

5.4. Objectives

In particular;

- To develop biomaterials with innovative surface geometry that promotes precise control of cell response at the graft-biomaterial and host-biomaterial interface.
- How to incorporate these surface geometries into supracellular 3D scaffolds, indispensable for tissue support and organization.
- To optimise tissue perfusion in the 3D scaffold.
- Recruitment of capillaries to penetrate the scaffold in order to maintain an oxygen gradient compatible with cell survival deep in the interior of the scaffold given that the graft is transplanted in ectopic sites, like subcutaneous or retroperitonium, without adequate vascularisation.
- To overcome inevitable foreign body reaction whereby the host encapsulates foreign bodies with fibrotic sac hereby completely isolating the graft from nutrients.
- Transform the scaffold into an immuno-protective device through scaling down the heights of the fluidic channels. The resulting semipermeable membrane should block the passage to large sized components of the immune system but allow the passage to smaller sized nutrients and hormones.

- To develop innovative surface geometry that promote precise control of stem cell proliferation and differentiation so as to have a renewable source of pure harvests of differentiated cells.

5.5. Methods

In this section we describe 3 classes of experiments that were performed with the purpose of investigating independently each of the research objectives mentioned above. This was in the framework of the Master protocol for the development of medical devices outlined in Chapter 1, fig 1.2.

5.5.1. Scaffold surface geometry design

Using a combination of standard and “smart” microfabrication techniques a number of innovative surface geometry with 2 scale lengths (micro- and nano-) were formulated. A selection of natural and synthetic polymers were used. A total of 12 samples with different combinations of surface geometry and substrate material were developed. The resulting surface geometry was characterised by Scanning Electron Microscopy (SEM). Samples were tilted at 69 degrees to show the depth and features.

5.5.2. Surface Design Optimisation.

In order to investigate cellular response to the surface design, in vitro and in vivo studies were carried out using candidate substrates in parallel to the microfabrication of new ones with improved design. This as to ensure that there was a result-orientated feedback at every stage of the design and microfabrication process, so that surface geometry and biomaterials that
promoted favourable cell response (coined “smart”) were maintained and optimised and those with poor results were dropped.

5.5.3. Cell Culture on Biomaterial.

Candidate substrates for biological studies were microfabricated in such a way that half of the surface was covered with the “smart” surface geometry under investigation. The other non-patterned half (but of the same material) acted as control. Neuroblastoma cells (mouse PC12 cell line), muscle myoblasts (mouse C2C12 cell line) and embryonic stem cells [ESCs (mouse non-transformed TBV-2 ES cell line)] were used for the study.

Neuroblastoma cells were seeded on the substrates and cultured in growth medium composed of Foetal Bovine Sera (FBS) 10% and Roswell Park Memorial Institute media (RPMI) 90% for 5 days. The substrates received no cell adhesion protein treatment and no growth factors were added to the culture medium during the period of study.

In order to get an expanded quantity of undifferentiated cells, ESCs were grown on murine embryonic fibroblast (MEF) feeder in ES medium (Knockout Dulbecco’s modified eagle medium supplemented with 15% FBS, 0.1 mM non-essential amino acids, 50mM (2)b-mercaptoethanol, 2 mM L-alanyl-L-glutamine, 100U/ml Penicillin, 100mg/ml Streptomycin and 1,400 U/ml of Leukemia inhibitory factor (ESGRO-LIF) which acted as the cell differentiation. Cell replating was done every three to four days.

The expanded cells were then seeded on candidate substrates using the above culture medium but without Leukemia inhibitory factor (ESGRO-LIF).

The specific culture protocol of muscle myoblasts is described elsewhere. All experiments reported above were performed in sterile conditions and according to regulations laid down by the Experiments Committee of the University of Pavia and The International School of Advanced Studies (SISSA) Trieste.

Myoblast cells response to the candidate biomaterials was evaluated using PCR assay for gene protein expression. Scanning Electron microscopy was used to
evaluate Neuroblastomas and Embryonic Stem Cell response. Parameters evaluated were cell adhesion to substrate, cell survival and proliferation, cell differentiation.

Only the candidate biomaterials that passed this biocompatibility performance benchmark progressed to the next phase of research protocol: scaffold microfabrication as was outlined in the Master Protocol in fig 1.2.

5.5.4. Scaffold Design

In order to address the problems of inadequate scaffold perfusion, fibrotic overgrowth, poor vascularization and immune rejection, we coined an original scaffold design with “smart” structural characteristics that would enhance scaffold perfusion and elicit desirable host response. A detailed description of the blue print and the rational of the design is presented in the discussion.

5.5.5. Scaffold Microfabrication Protocol

Described below is the microfabrication process based on the scaffold design we developed using the biomaterials and integrating the “smart” surface geometry that passed the performance benchmark using in vitro cell cultures.

**Step 1** etching of position markers

A double-polished silicon wafer was used. 100nm of Cr was plated on the backside by metallic evaporation. This was followed by standard spin coating of positive resist (S1828) at 1500 rpm, soft baking (100°C for 3min), and photolithography using a photomask with alignment markers. This was followed by development of the resist and chromium wet etching in a solution of [35ml CH$_3$COOH+ 600ml H$_2$O +200g (NH$_4$)$_2$Ce(NO$_3$)$_6$] With chromium acting as the mask the markers were completely etched from back to front of the wafer using Inductively Coupled Plasma (ICP). Bosh® process was used. A schematic summary of this process is outlined in fig 5.1.
**Aims** objectives and methods

Fig 5.1 (a) through to (d) side view of a schematic representation of step 1 of the microfabrication process: etching of markers.

**Step 2**
On the frontside a base-plating layer Cr/A (Cr10nm Au 20nm) was deposited by metallic evaporation. This was to facilitate the subsequent electroplating. Positive resist PMMA (220K AR-649.04) was spin coated on the Cr/Au base-plating. At spin coating speeds up to 6000 rpm we were able to scale down the resist thickness to the order of ten nanometres. After soft backing at a temperature of 175°C for 3 minutes, another layer of S1828 positive resist was spin coated at 1500rpm followed by soft baking a temperature of 100°C for 3 minutes. Photolithography was performed using a photomask with a grid pattern. After developing S1828, the grid pattern was then effectively transferred to PMMA resist by reactive ion etching (RIE) using oxygen plasma. S1828 was then stripped off by blank UV exposure and developed leaving a patterned PMMA layer.

This pattern in the form of a grid network was to act as a spacer during the electrolytic growth of the gold hemi-membrane, and as a sacrificial layer whose chemical stripping at the end of the microfabrication process would result in a microfluidic network connecting the micropores of the gold and silicon hemi-membrane. A schematic summary of this process is outlined in fig 5.2.
Fig 5.2 Side view of a schematic representation of step 2 of microfabrication process (A-F) for creating a grid network of a sacrificial layer in PMMA.

**Step 3.**
Positive resist (S1828) was spin coated and photolithography was done using the pore mask 1 after alignment with the markers on the silicon wafer. The resist was developed to leave behind an array of 100µm diameter pillars sitting on the PMMA grid thus forming the mould which was to act as a spacer for the nanopores during electrolytic gold plating [fig. 5.3 (a-b)].

**Step 4 Gold Electroplating**
After plasma surface cleaning at low power (50W, 0.17W/cm²) for efficient electroplating, gold electroplating was carried out to obtain the complementary pattern of the array of pillars in S1828 to height of 2µm. (fig 6.3 c). The resulting structure was the gold hemi-membrane.
Fig. 5.3 Side-view of a schematic presentation step 3 of microfabrication process of the pillar patterned sacrificial layer that acts as a spacer for micropores during electrolytic gold plating. (c) Magnified insert of the resulting nanofluidic channels exhibiting nanometric and micrometric size in the vertical and lateral direction respectively. The nanometric control is obtained by the deposition of a sacrificial layer of precise nanometric thickness.

**Step 5** Micropore etching on the backside.
Positive resist (S1828) was spin coated on the backside and then using the position markers, pore mask 2 was aligned to the silicon wafer and exposed. The resist was developed followed by chromium wet etching. 100µm diameter pores were then plasma etched with ICP in the silicon substrate until coming in contact with the Cr/Au base plating which acted as the etch stop. The base plating was then etched away using Cr and Au wet etching solutions respectively, to gain access to the PMMA grid below. The structure was then immersed in warm acetone that stripped the PMMA grid network and S1828 pillars spacers to create a semi-permeable microporous scaffold whose micropores were interconnected by a microfluidic network (fig. 5.4).
Aims objectives and methods

Fig. 5.4 (a) through to (g). Side-view of a Schematic presentation step 5 of microfabrication process: backside membrane pores created by ICP on silicon substrate. (g) Magnified insert of scaffold prototype. The stripping of spacer resist in warm acetone results in a network of nanofluidic cannals connecting the laterally displaced micropores to produce a semipermeable scaffold.
5.5.6. Host response to Biomaterial

5.5.6.1. Controlled Host Response at Biomaterial/Scaffold-Host Interface using “smart” surface geometry

Candidate gold and silicon microfabricated scaffold chips with “smart” surface geometry with micropores of varying diameters on one surface were evaluated using an animal model.

The chips were surgically introduced into 4 subcutaneous pockets made in the mice abdomen. The upper right contained a silicon chip and the lower right pocket, a gold chip. The chips’ microporous surface was coated with a commercially available neoangiogenic proteinous gel matrix, Matrigel®. Gold and silicon chips in pockets on the left without matrigel® acted as controls. A total of 6 mice were used. On day 5, 10 and 15, two mice were sacrificed and the chips harvested for histology analysis. A graphic representation of the surgical procedure is illustrated in figures 5.5 – 5.7.

5.5.6.2. Matrigel®

BD Matrigel™ Matrix is a solubulized basement membrane preparation extracted from EHS mouse sarcoma, a tumor rich in Extracellular Matric (ECM) proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin. At room temperature, BD Matrigel™ Matrix polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane. Cells behave as they do in vivo when they are cultured on BD Matrigel™ Matrix. It provides a physiologically relevant environment for studies of cell morphology, biochemical function, migration or invasion, and gene expression.
Fig. 5.5 Coating scaffold chips with matrigel® which was maintained in liquid form at 4°C. The chip on the left had micropores with 100µm diameter while that on the right had pores with a diameter of 1.25mm. Blue arrow indicates needle of insulin syringe.

Fig. 5.6 Surgical implantation of scaffold chip in a subcutaneous pocket of the mouse abdomen.
5.5.6.3. Controlled Host Response at Biomaterial/Scaffold-Host Interface using surface chemistry

In a parallel study a suspension of latex microcapsules (Polysciences Inc.) in normal saline (0.9%) were used to investigate the hosts body reaction to a biomaterial with different surface chemistry. The following chemical functional groups were use;

*Polybead Amino microspheres.*

1.00 μm diameter mono disperse latex particles (in 2.5% aqueous suspension) containing primary amine surface functional groups.

*Polybead Carboxy-sulphate microspheres.*
Aims objectives and methods

1.00 μm diameter mono disperse surfactant free polystyrene particles with primary carboxyl-sulphate surface functional groups.

*Polybead Carboxyl microspheres.*

4.50 μm diameter mono disperse polystyrene particles containing surface carboxyl groups

*Polybead Hydroxylate microspheres*

3.00 μm diameter mono disperse polystyrene particles containing surface hydroxyl groups.

*Polybead Sulfate microspheres.*

2.00 μm diameter mono disperse polystyrene with surface sulfate groups.

Polybead Poly(methyl methacrylate) or PMMA microspheres

1-10 μm diameter particles with surface carboxylic acid groups.

Alginate poly-L-lysine (200 μm diameter) capsules were used as controls.

Male Lewis rats (*Charles River, Italia*), weighing 300-400g were used in this study.

For each candidate material, a suspension of empty candidate capsules in 0.9% normal saline were implanted, via a midline incision, in the retroperitoneal space over the ileopsoas muscle (50μl) and renal subcapsular space (10μl). Two rats were used for each candidate material. Kidney and muscle containing all the capsules were explanted after 4 weeks for digital image histological analysis

All surgical procedures reported above were performed under general anaesthesia with diethyl ether and in sterile conditions according to regulations laid down by the Animal Experiments Committee of the University of Pavia.
CHAPTER

6

6. Results

6.1. Biomaterial surface geometry characterization

Reported below are the surface geometry of the biomaterials that demonstrated amplified cellular response. Fig 6.1 (a) shows Pattern 1, a silicone substrate with irregularly-regular pattern of concave craters of diameters ranging from 1µm to 50nm. While fig. 6.1(b) shows Pattern 2, irregularly-regular pattern of trenches (1µm width) whose surface is covered with nano-grain geometry (100nm-20nm in size) in a silicon substrate. Fig 6.1 (c) shows Pattern 3, an irregularly regular pattern of nano-rods of varying diameter (100nm-200nm) and varying heights (50nm –100nm) in silicon. Fig 6.1 (d) shows Pattern 4, a gold substrate with irregularly regular nano-grains ranging from 10nm 100nm in size. Fig 6.1(e) shows Pattern 5, finger-like polymer strands on a metallic base (chromium). They too had an irregularly-regular pattern and were 200nm diameter and 200nm in height.

In spite of the differences in surface geometry and the different biomaterials from which they were microfabricated, it was observed that all candidate biomaterials that showed amplified cellular response were characterized by surface geometry with combined micro- and nano-scale lengths.
Fig 6.1. (a)–(e) Scanning Electron Micrograph (SEM) of surface geometry with 2 combined scale lengths (nano-on-micro) that elicited favourable cellular response. (a) pattern 1 (b) Pattern 2, (c) pattern 3, (d) Pattern 4, (e) Pattern 5.
6.2. Cell response to biomaterial and surface geometry.

6.2.1. Neuroblastoma cells (mouse PC12 cell line)

After a 5 day cell culture period, cell survival was proliferation was observed on all biomaterials made from polymer (Photo resist S1828), silicon and gold. However the following patterns elicited additional striking cellular response; Neuroblastomas (PC12) seeded on pattern 1 (fig 6.1) described above showed firm adhesion and morphological change from the immature spherical shaped cells to differentiating cells with growth cones (fig. 6.2). A similar response was observed when PC 12 cells were seeded on Pattern 2 (fig 6.3). However, pattern 5 promoted full differentiation into neuron cells with well developed axons with an interconnecting network of axons. (fig. 6.4)

No change in morphology or differentiation was observed on the non-patterned half of the biomaterial and the plastic floor of the culture wells that acted as controls. In conventional culture studies, Neuroblastoma cells differentiate only when Nerve Growth Factor (NGF) is added to the culture medium.

6.2.2. Embryonic Stem Cells (ECSs) [mouse TBV-2 cell line]

ESCs were cultured on gold substrates with micro canals of varying widths but of the same height and nano-grain surface geometry Exponential cell proliferation with coalescing cell colonies was observed on both micro patterned (fig. 7.5) and non-patterned gold substrates and the plastic wall of the culture wells that acted as controls at the end of the 5 day culture period. However when the microcanals dimensions were reduced to 4µm wide, 8µm period and 1µm height cell survival and adhesion to substrate was still observed but exponential proliferation and coalescing of colonies was blocked (fig. 7.6).
Fig 6.2 Scanning Electron micrograph (SEM) of a microfabricated “smart” pattern 1 on silicon substrate (a). The substrate was seeded with neuroblastomas (PCR12) and cultured for 5 days in basic culture medium without Nerve Growth Factor. (B) shows cell survival, adhesion, growth cone spreading as indicated by morphological change indicated by red arrows (b).
Fig. 6.3 Scanning Electron micrograph (SEM) of a microfabricated “smart” pattern 2 on silicon substrate (a). The substrate was seeded with neuroblastomas (PCR 12) and cultured for 5 days in basic culture medium without Nerve Growth Factor. (b) shows cell survival, proliferation and adhesion indicated by red arrows.
Fig 6.4. Scanning Electron micrograph (SEM) of a microfabricated “smart” pattern 3 on silicon substrate (a). The substrate was seeded with neuroblastomas (PCR 12) and cultured for 5 days in basic culture medium without Nerve Growth Factor. (b) shows change in cell morphology and differentiation into neurons (red arrows) with axons (blue arrows).
Fig 6.5 Scanning Electron micrograph (SEM) of a gold microfabricated micro-pattern canals (20µ width, 40µ period) with “smart” nano-grain surface geometry. The substrate was seeded with Embryonic Stem Cells (ESCs) on fibroblast feeder cells in ES culture medium. B) Magnification shows exponential cell proliferation with coalescing of colonies which tend to flatten out and follow the direction of the canals.
Fig 6.6 Scanning Electron micrograph (SEM) of gold microfabricated micro-pattern canals (4µ width, 8µ period) with “smart” nano-grain geometry. The substrate was seeded with Embryonic Stem Cells (ESCs) on fibroblast feeder cells in ES culture medium for 5 days. (b) magnification shows cell survival but with step-function decrease of exponential proliferation.
6.2.3. Muscle myoblast cells (mouse C2C12 cell line)

Gold and silicon substrates with different surface geometry were seeded with C2C12, an embryonic muscle cell line and cultured in basic culture medium without growth factors for 5 days. RNA polymerase Chain Reaction test was carried out to detect the presence of RNA for $\alpha$-actin and MLC3F expression. $\alpha$-Actin and MLC3F proteins are expressed during cell adhesion/proliferation and differentiation respectively. The PCR results charts are presented in fig 6.7 below. Yellow boxes depict the results of gold substrates and the Red boxes silicon substrates. In fig 6.7(a) all substrates promoted cell proliferation and except gold substrates G and C (blue arrows). Substrate C pattern was 4$\mu$m wide canals with 8$\mu$m period while Substrate G was coated with matrigel® before being seeded with cells. While Figure 6.7(b) demonstrates that all silicone substrates promoted myoblast differentiation into myocytes (blue arrows). No cell differentiation was observed on gold substrates (yellow box).
Fig 6.7 RNA polymerase Chain Reaction charts showing presence RNA that expresses α-actin and MLC3F proteins. α-Actin and MLC3F proteins are secreted during cell proliferation and differentiation respectively. Yellow boxes depict the gold substrates and the Red boxes the silicon substrates. (a) all substrates promoted cell proliferation and except gold substrates G and C (blue arrows). (b) demonstrates that all silicone substrates promoted myoblast
differentiation into myocytes (blue arrows). No cell differentiation was observed on gold substrates (yellow box).

6.3. Host response to Biomaterial

6.3.1. Controlled Host Response at Biomaterial/Scaffold-Host Interface using surface chemistry.

Renal subcapsular implantation site
After one month of implantation in the renal subcapsular space, the six types of microcapsules were explanted and investigated for fibrosis. Results of all six capsules are expressed graphically in fig. 6.8. Carboxylsulfate microbeads provoked the least 14.73% (SD 8.4)(Fig3a). Amino beads provoked the most fibrosis with 38.79% (SD 9.93). Alginate poly lysine capsules that acted as controls provoked 36.61% (SD 9.5) fibrosis.

Retro-peritoneal iliopsoas implantation site
As described above the six types of microcapsules were explanted from the ileopsoas muscle and analysed. Results of all six capsules are expressed graphically in fig. 6.9. Carboxylate microcapsules provoked the least fibrosis 24.09.% (SD 16.0) while Amino microcapsules provoked the most 49.9% (SD12.2) Alginate poly lysine capsules that acted as controls provoked 38.72.% (SD 4.14). A Fig 6.10a–6.10d are selected histological samples demonstrating isolation of the micropheres by pericapsular fibrosis with no pericapsular neovascularization observed.
Results

% RENAL SUBCAPSULAR FIBROSIS

<table>
<thead>
<tr>
<th>Material</th>
<th>% Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Amino</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Carboxyl-sulfitae</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Carboxylate</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Sulfate</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Hydroxylate</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Alginate PLL</td>
<td>12 ± 2</td>
</tr>
</tbody>
</table>

Fig 6.8 Renal Subcapsular fibrosis provoked by microcapsules

% FIBROSIS AT MUSCLE IMPLANT SITE

<table>
<thead>
<tr>
<th>Material</th>
<th>% Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Amino</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Carboxyl-sulfitae</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>Carboxylate</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Sulfate</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Hydroxylate</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Alginate PLL</td>
<td>22 ± 2</td>
</tr>
</tbody>
</table>

Fig 6.9 Fibrosis at muscle implant site provoked by microcapsules

**Fig. 6.10a**
Histological section of renal subcapsular inoculation of “AMINO” microspheres demonstrating pericapsulare fibrosis (black arrows)(100X; 1200X, Masson stain).
Results

Fig. 6.10b Histological section of renal subcapsular inoculation of “Alginate PLL” microsphere demonstrating pericapsular fibrosis (red arrows) (100X; 450X, Masson stain).

Fig. 6.10c Sirius Red stained histological section of retroperitoneal muscle inoculated with “Alginate PLL” microspheres demonstrating pericapsular fibrosis (arrows) (450X).

Fig. 6.10d Sirius Red stained histological section of retroperitoneal muscle inoculated with “Amino” microspheres demonstrating pericapsular fibrosis (arrows) (1220X).
6.3.2. Controlled Host Response at Biomaterial/Scaffold-Host Interface using “smart” surface geometry.

Both Gold and Silicon hemi-membranes had their micropores filled with matrigel and were subcutaneously implanted in the abdominal wall of mice and explanted on day 5 for histological analysis. Fig 7.11(a) broken line depicts the space that was occupied by scaffold which was removed to facilitate histology (it couldn’t be cur through). Red arrows demonstrate mild acute inflammatory reaction at the matrigel® - host interface which the host recognized as self. And severe acute inflammatory reaction on the silicon backside–host interface without micropores and matrigel® that acted as control.

![Image of histology](image)

Fig 6.11 (a) At day 5 of explant histology demonstrates mild acute inflammation at the host-matrigel® interface (red arrows) and severe acute inflammatory reaction at the non-patterned host-biomaterial interface that acted as control (white arrows).
Fig 6.12(a) At day 10 of explant, histology demonstrates infiltration of the matrigel® by chronic inflammatory cells and marked neoangiogenesis, red arrows.

Fig 6.12(b) At day 10 of explant that acted as control (without matrigel®), histology demonstrates chronic inflammatory reaction at the host-biomaterial interface (red arrows) and neoangiogenesis, (blue arrows)
Fig 6.13(c). At day 14 of explant, histology demonstrates granulation tissue with fibroblast and connective tissue with a high vascular index. The numerous ed dots depicts immature blood capillaries containing red blood cells (red arrows).

6.4. Scaffold prototype

The resulting scaffold was a microporous silicon scaffold assembled on a microporous gold semi-permeable membrane. The semi-permeability was guaranteed by nanofluidic network embedded between the gold and scaffold structure. The figures below are Scanning Electron Microscopy (SEM) characterisation of the device highlighting its macro- micro- and nano-architecture.
6.14 Scanning Electron micrograph of top view 2 scaffold prototypes demonstrating its external gold hemi-membrane’s 3D architecture, microporosity and the integration of "smart" surface geometries that promoted desirable cellular response. Inserts; magnified and tilted micrographs (69°) demonstrating 3D architectures and microfluidic canals.
Fig. 6.15. Red arrows demonstrate the mouths of the microfluidic canals that interconnect the laterally displaced micropores of the gold membrane and the silicon scaffold.
Fig 6.16 Scanning Electron micrograph of a broken gold hemi-membrane of the scaffold prototype (1) to reveal the trajectory of microfluidic canals that connect micropores (Red arrows). The sample is tilted at 69 degrees to show the depth of the islands (720nm) and channel features. Fig b. demonstrates the micro-nanoscale patterning 1 incorporated onto the scaffold surface.
Fig 6.17 Scanning Electron micrograph of the gold hemi-membrane of the scaffold prototype (2) demonstrating micro-nanoscale patterning incorporated onto the scaffold surface.
6.18 (a) Scanning Electron micrograph of the backside of scaffold prototypes demonstrating its internal silicon hemi-membrane’s 3D architecture and microporosity (a). Fig. (b), demonstrates the micro- nano-scale patterning 1 and 2 incorporated into the silicon micropore walls.
7. Discussion

In this chapter the concept and design of the scaffold are discussed. The scientific considerations that inspired the design and factors that influenced the construction of the prototype are outlined. The results and implications of the proof-of-concept biological investigations that were carried out are evaluated and future areas of study and potential applications are proposed.

Cell survival, differentiation and function is regulated by precise biochemical, biophysical and neural signals. Furthermore, physiological function of cells is achieved and maintained through an architectural hierarchy of supporting structures and complementary cells that together form tissue. (as was outlined in chapter 2). In order to have any success in artificial engineering of tissue, these fundamental principles must be respected.

This was against this background that we set out to design a scaffold that reflected these fundamental principals and for inspiration we looked at the structure of nature’s solution to tissue building: the extra-cellular matrix.

The final design of our scaffold was a compromise between the incorporation of biological requisites for cell survival and function on one hand and technical - logistical considerations on the other hand namely: choice of biomaterial, integration of surface geometry that gave the most desirable cellular response, engineering feasibility and structural integrity, production costs, access to technology and the capacity to mass produce.
7.1. Scaffold Design and Architectural Hierarchy

7.1.1. The Superstructure: The overall shape of the scaffold

The scaffold consists of a square chip with the following dimensions 1cm (L) x 1cm (W) x 350µm (H). It is composed of a 3 layers; a middle layer of microfluidic network embedded between two microporous membranes made of gold and silicon respectively. This miniaturized size of the scaffold have obvious advantages. It results in a reduced diffusion distance to the core of the scaffold and the large surface to volume ratio favours increased mass flow of nutrients and by products. Small chips minimize surgical trauma at the implantation site and hence promote healing by primary intention which is associated with minimal fibrotic overgrowth at the host-biomaterial interface (refer to fig 6.6).

7.1.2. At the Micro-Scale:

The two hemi membranes consist of a “smart” pattern of honeycomb-inspired micropores that confers them 3D architecture and porosity. The role 3D architecture in the promotion of tissue organisation, and cell function is well established. While porosity optimises perfusion and mass flow of biological fluids to the core of the scaffold. The choice of this design was further based on striking in vivo results where the microporous surface elicited vascular recruitment at the scaffold-host interface irrespective of the biomaterial used [fig 6.12(a)–6.12(b)]. This host response was further optimised by filling the micropores with matrigel. On The other hand encapsulating fibrotic overgrowth was elicited in the controls and in a parallel study using latex with different surface chemistry (Fig 6.8-6.10).
7.1.3. The hydraulic aspect as of design

The hemi-membranes are superimposed and aligned in such a way that the micropores are laterally offset (fig. 7.1). Sandwiched between these hemi-membranes is a microfluidic canal network that runs perpendicular to the micropore axis and permits interconnectivity between the laterally offset micropores (fig 7.2.) The canals section is rectangular, with a width in the order of 4 microns (limited by the resolution of the UV lithography), period of 8µm, and height which can scaled down to the range of 10nm, (corresponding to the thickness of a sacrificial layer) (7.3).

Fig 7.1. Schematic representation of micropores of two superimposed hemi-membranes (A) and when laterally offset (B).

Fig 7.2. Schematic representation of a microfluidic canal network embedded between two hemi membranes and interconnecting laterally displaced micropores.
The fluidic network design was inspired from the blood capillary network (fig.7.3a) and serves as nutrient channels to support cell survival deep in the interior of the scaffolds. By scaling down the height of the canals from the micro- to the nano-scale the scaffold acquires a “smart” characteristic as a semipermeable membrane and by
size exclusion can block the passage of large immune cells but permit the passage of nutrients and hormones.

The external hemi-membrane serves as a scaffold for vascular endothelial cells and neoangiogenic stimulating matrix and is made of gold (fig.7.1). Gold was the biomaterial of choice because it was demonstrated to be non toxic in our cell culture studies and above all maintained structural and physical stability in biological solutions and at implantation site over a long period of time. The pore size diameter of 100µm was observed as the minimum diameter that was associated with adequate blood capillary penetration of the scaffold.

The internal hemi-membrane serving as a scaffold for the tissue of transplantation interest was made of silicon (fig. 7.2) The choice of silicon as a the scaffold for graft cells was based on combined ease of microfabrication of microscale geometry and the integration of nanoscale geometry that promoted desired cell response. The Micropores are 100µm in diameter with a period of 220µm resulting in a pore density of 2066/cm² This pore density was a compromise between structural integrity and the need to ensure mass flow of solutes across the membrane. We observed that high pore density resulted in brittle, easily breakable and disintegration on long term implantation.
Fig. 7.4 Functional unit of Computer Aided Design (CAD) of the outer microporous membrane inspired from the honeycomb (green circles). The dotted circles correspond to the laterally offset position of the micropores the overlying inner membrane.

Fig. 7.5 Functional unit of Computer Aided Design (CAD) of the inner microporous membrane inspired from the honeycomb as well (green circles). The dotted circles correspond to the laterally offset position of the micropores of the underlying outer membrane as illustrated above in fig.1.
7.1.4. At the Nano-Scale

Biomaterial surface geometry and proof of concept

Identification of biomaterials that support desirable cell responses such as cellular attachment, survival, proliferation and differentiation is critical for tissue engineering and cell therapy. The observation of how C2C12 myoblasts and PC12 neuroblastomas cultured on our “smart” biomaterials differentiated into myocytes and neurons respectively without the addition of growth factors into the culture medium is a striking demonstration that in vitro soft tissue cell differentiation can be precisely controlled using surface geometry. It was further observed that cell differentiation occurred only on substrates structured at two scale lengths (illustrated in fig 6.1) i.e. structures at the tens to hundreds microns as supporting scaffold and at the submicron scale for surface geometry. This didn’t happen when the same substrate was used but with regular patterns like pillar or canal arrays at the micron scale length (refer to results; section 6.2.3). Webster et al and other investigators (refer to chapter 4) have reported how vitro osteoblast adhesion, proliferation, differentiation (as measured by intracellular and extracellular protein synthesis as alkaline phosphates), and calcium were...
enhanced on biomaterials with particulate or gain sizes less than 100nm. We can deduce that it is this hierarchal geometry that adds precision as a biophysical signal for cell differentiation. To the best of our knowledge, no one had until now reported inducing precisely controlled morphological differentiation using a biophysical signal.

Equally interestingly, we exploited this difference in cell response to surface geometry in our investigations on Embryonic Stem Cells (ESCs) as a renewable source of cells for tissue engineering. One of the major drawbacks of ESCs as a renewable source of cells for tissue engineering is the inability to have precise control of their proliferation and failure to control their differentiation into pure cultures of target tissue cells. The step-function decrease in ESCs' exponential proliferation by narrowing the period between the micro patterns demonstrates that we can control ESCs' proliferation using surface geometry. Although our preliminary findings are exciting, proliferation control must be coupled with precise differentiation control if the use of ESCs in tissue engineering is to have clinical relevance. Our research group is currently researching on innovative ways of optimising ESCs' differentiation and function.

7.2. Bioactively controlled host response (wound healing) at the host scaffold interface

By combining microporous surface (that inhibits excessive fibrotic overgrowth), nanopatterned surface (that promotes protein adsorption and hence vascular endothelial cells attachment) and the filling of the micropores with vascular recruiting gel, matrigel®, we actively elicited the desired host response: vascular recruitment and penetration at the scaffold host interface. The clinical implication of this is two fold:
1) optimised ectopic graft function
In their native environment functional/endocrine cells like hepatic and pancreatic
islets cells has a very rich vascular supply in order to carry out their high oxygen-
consuming functions. When transplanted they are usually returned to low oxygen
ectopic sites (refer to fig. 5.6) where even if they may survive they wouldn't be
able to carry out their endocrine function.

2) Host-scaffold integration
Orthopaedic and dental implants are associated with alarming failure rates. In the
USA, 12.8% of the total hip arthroplasties in 1997 were simply due to revision surgeries of previously implanted failed hip replacements. While recent studies have found that dental implants which have been used in over 300,000 cases have a success rate of 75% after 15 years. Although there are many reasons why implants fail, a central one is the lack of sufficient bone regeneration around the implant immediately after insertion. Shockingly, about one quarter of dental implant failures (those that fail between 3 and 6 months) are attributed to incomplete healing of the implant to juxtaposed bone. Therefore by designing surface characteristics that interface optimally with select proteins and subsequently with pertinent bone types, improved biomaterial performance can be achieved.

7.3. Microfabrication Protocol

Our microfabrication protocol was kept as simple as possible despite the fact that there are more elegant and refined protocols that could be formulated using state of the art technology like electron beam and x-ray lithography. But this had to be reconciled with logistical considerations such as mass production, technology transfer, and Industrial Investment given that industry is not willing to invest in high production cost using very expensive machines at that need highly specialized manpower.
7.4. Conclusion

By developing the ability to control microporous architecture, micro pore interconnectivity and size, the external and internal shape of the scaffold and it’s nanometer scaled surface architectures, the “smart” scaffold developed in our laboratories have great potential as an ideal scaffold for tissue engineering.

7.5. Applications

1. Micro 3D cell culture wells

One of the immediate spin-off applications of our research is the integration of our innovative surface geometry into cell culture dishes to produce functional micro 3D cell wells. By combining traditional cell culture techniques and surface geometry we can create an \textit{in vitro} micro environment that simulates the natural microenvironment of cells resulting in more physiologically accurate results. Furthermore, it would be cost effective since the miniaturized sizes and “smart” surface geometry results in the use of less consumables (cells, biological reagents) and time consuming complex culture protocols. Fig 7.7 illustrates a prototype of micro 3D cell culture wells for Pancreatic islets of Langerhans.
7.6. Future areas of research

1. Given the good results in toxicology, cytocompatibility and biocompatibility by the “smart” biomaterials and scaffolds developed from them. We will pass on to the next phase of the study: tissue engineering and transplantation using animal models. The areas of research to be addressed are as follows:
   - Metabolic diseases; diabetes: Pancreatic islet of Langerhans regeneration
   - Spinal Cord injuries: neurons regeneration
   - Orthopaedic and Dental injuries: bone regeneration
   - Connective tissue diseases (Rheumatoid arthritis): Cartilage regeneration

2. Integration of scaffold’s microfluidic network with external pump to create bioreactor to stem cell derived tissue research.

3: Immuno-isolation studies
We developed the technological capacity to make nanofluidic canals of down to 10 nm height and hence transform the scaffold into an immuno isolating
membrane by size exclusion of antibodies. However, further studies are needed in this direction to reconcile the conflicting interest of graft revascularisation and immune protection. This has important therapeutic implications given that one of the biggest drawbacks to graft transplant is the need of chronic immunosuppression therapy that is associated with grave side effects.
7.7. Acknowledgments

This research work was carried out in collaboration with the institutions listed below and I would like to acknowledge the participation and support of the following personalities:

National Laboratory of Advanced Technology and NanoScience (TASC) Trieste,
Interdisciplinary Laboratory of Lithography (LILIT) Group
Dott. Massimo Tormen
Dott. Luca Businaro
GianLuca Grenci
Mauro Prasciolu
Alessandro Pozzato
Simone Da Zilio
Radu Malureanu

International School of Advanced Studies (SISSA), Trieste
Consortium of Moleculare BioMedicine (CMB) Laboratories, Trieste,
Prof. Vicenti Torre,
Jelena Ban
Jummi Laishram
Rajesh Shahapure

Department of Comparative Anatomy and Cell Biology, University of Pavia,
Prof. Rosanna Nano
Prof. Angelica Facoetti
Alessia Bertolotti

Faculty of Medicine and Surgery; Department of Human Anatomy, University of Pavia
Prof. Gabriela Cusella De Angeli et al
....
....
7.8. References


