CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

Implantable biomaterials are used in different parts of the human body such as in valves, blood vessels, shoulders, knees, hips and elbows to replace the diseased biological structures and restore its function. Among these, the incidence of implants to be used in joint replacements, especially in hip and knee is extremely high. It is estimated that over one million joint arthroplastic procedures are performed in patients every year worldwide [1]. Though these artificial joint replacements function properly for a while, the long term maintenance of osteointegration due to the aseptic loosening of implants is still a great source of concern in the biomedical field. This often results in discomfort, pain and need for a revision surgery to the patients. Revision surgery offers more technical difficulties than primary arthroplastic procedures because of the availability of less bone stock to work with, which ultimately would result in bone fracture. With the increasing requirement for joint arthroplasties, the need to increase the life expectancy of these endosseous devices is high [2].

Generally two types of arthroplastic procedures are being performed. Implants can be fixed properly with the aid of PMMA based bone cement or without the use of cement, which are referred as Cementless or Non-Cemented arthroplasty respectively. Cemented arthroplastic procedures are an ideal choice for older individuals with short life expectancy and low physical activity. However, for younger patients, cementless implants are the recommended choice nowadays, owing to the direct anchorage it offers to the surrounding bone via osteointegration [2]. Osteointegration depends on many different factors which include the choice of implant, implant surface properties, implant design and
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surgical skill [3]. The choice of a material or its combination for hard tissue replacements demands qualities such as biocompatibility, excellent corrosion resistance, acceptable strength to sustain the cyclic loading endured by the joint, a low elastic moduli and a high wear resistance. Some of the commonly used metals for joint replacement include commercially pure titanium (cPTi), titanium alloy with 6% aluminium and 4% vanadium (Ti-6Al-4V), cobalt alloy with 27-30% chromium and 5-7% molybdenum (Co-Cr-Mo) and stainless steel (SS) grades 316 and 316L. Of these metals, Ti and its alloys are the most commonly preferred materials of choice because of their excellent mechanical as well as biological properties [4]. Ti possesses a native 1 – 5 nm thick TiO2 layer on its surface which imparts excellent corrosion resistance and biocompatibility. It also holds remarkable advantages such as high specific strength, fatigue resistance and ease of processability. Lesser elastic moduli of Ti (110 GPa) compared to SS (210 GPa) and Co-Cr- Mo (240 GPa) is another advantage of it being widely utilized in the fabrication of femoral stem prostheses [5].

Although, Ti based materials meet most of the requirements for an ideal endoprosthetic device, its bioinert native surface oxide layer does not form a good chemical bonding with bones at the early stage after implantation. The early osteointegrative and osteoconductive properties of pristine Ti are inadequate, which limits its in vivo performance. Since an implant surface is mostly in contact with the surrounding bone tissue rather than the bulk fraction, the physicochemical properties of the surface greatly influence its degree of osteointegration. Hence the current research trend in orthopaedic field is to employ different surface modification strategies to improve the topographical features of Ti-based implants for augmented bone bonding [6]. Diverse strategies have been reported in literature to improve the surface characteristics of Ti implants which include surface roughening by sandblasting [7], immobilizing TiO2 nanofibers onto Ti surface by electrospinning [8], applying a thin film coating of hydroxyapatite by plasma spraying [9], generating porous nanotubular structures by anodization [10] and hydrothermal modifications [11]. Even though, modified Ti substrates were found to enhance better rate of cellular response than unmodified Ti, adequate osteointegration has not been achieved yet. Hence, the
need for superior surface modification techniques that yield enhanced osteointegration are highly demanding [12].

One of the promising approaches to improve the tissue integration response towards Ti implants is to surface functionalize the implant surfaces with bioactive moieties [13]. The bone ECM mostly contains protein fibrils such as collagen and elastin, organized to form a structural scaffolding of nano and microfibers, which in turn serves as nucleation sites for bone growth and mineralization. Hence, a scaffolding approach that mimics the ECM morphology would be beneficial in aiding appropriate cellular response [14]. The idea of reproducing such a native bone ECM-like architectural composition on the implant surface has led us to develop a bio-polymeric scaffolding on metallic Ti using bone stimulating agents, for increased cellular adhesion. We have chosen fibrin and alginate for fabricating the scaffolds, both of which have been used individually as biodegradable scaffolds in biomaterial applications. Fibrin being an endogenous protein has limitations in its strength as well as degradation rate when devised as a scaffold. To address this limitation, a natural biodegradable polymer, viz., alginate was incorporated jointly with fibrin for the proposed scaffolding on metallic Ti. To ensure an enhanced stability of the scaffolding on the metal, a bidentate co-ordination chemistry using dopamine hydrochloride was adopted for immobilization of fibrin-alginate. After a comprehensive evaluation of the material, its ability to enhance the osteogenic differentiation of mesenchymal stem cells (MSCs) into functional osteoblasts was assessed. This thesis work, for the first time, has evaluated whether such a bio-polymeric scaffolding approach on metallic Ti surface could improve the early osteointegration and bone formation on Ti implants in a rabbit intramedullary model in comparison to a control polished Ti (CTi).

1.2 REVIEW OF LITERATURE

1.2.1 Total Joint Arthroplasty

Total joint arthroplasty or total joint replacement is an orthopaedic procedure in which a dysfunctional joint is replaced with an orthopaedic
prosthesis [15]. Joint replacement surgeries are more commonly performed on hips. Hip replacement (hip arthroplasty) surgeries are often executed to relieve the pain and discomfort to patients suffering from degenerative joint disorders such as osteoarthritis, rheumatoid arthritis, avascular necrosis, bone fractures and tumours [16]. Hip replacement is a surgical procedure in which the defective cartilage and bone surrounding the joint is removed and replaced with a prosthesis. Based on the severity of the damaged tissue, it can be carried out as a total hip replacement (THA) or a hemi replacement (hemiarthroplasty). It has been estimated that THA is one of the widely performed surgeries in the orthopaedic field. About 90% of the population above the age of 40 suffers from regenerative joint disorders, and in most industrialised countries THA has an incidence higher than 150 procedures per 100,000 inhabitants an year and this trend is expected to increase tremendously in the coming years due to ageing population. THA comes under the category of a successful, safe and cost-effective medical intervention which has proved remarkably successful in eliminating pain and restoring function in hips severely involved with diseases such as osteoarthritis [17]. A typical joint prosthesis as shown in Figure 1.1 includes a metallic femoral stem, a femoral head, an acetabular metallic cup and an articulating cup which is usually made up of an ultra high molecular weight polyethylene (UHMWPE).

![Figure 1.1: Modular hip prosthesis design consisting of femoral stem, femoral head, and acetabular cup with an outer shell and an inner liner (adapted from Total Hip Arthroplasty [18]).](image)

A typical joint prosthesis as shown in Figure 1.1 includes a metallic femoral stem, a femoral head, an acetabular metallic cup and an articulating cup which is usually made up of an ultra high molecular weight polyethylene (UHMWPE).
Both the acetabulum and the femoral head are replaced in a total hip replacement (total hip arthroplasty) procedure, whereas only the femoral head is replaced in a hemiarthroplasty. In a THA, the surgeons excise the head and the proximal neck of femur and an artificial canal is created in the proximal medullary region of femur. A metallic femoral prosthesis composed of a stem and a small diameter head is inserted into the canal. Followed by this an acetabular cup is anchored in the pelvis and is composed of an outer shell in which a liner is inserted that provides the load bearing articulating surface. The outer acetabular shell and the stem have to be properly fixed onto the iliac bone of the pelvis and femur to provide a better bone integration. Based on the fixation methods in bone tissues, prosthesis are broadly classified as cemented and cementless endosseous prostheses [18].

1.2.2 Cemented Arthroplasty

For yielding a successful arthroplastic procedural outcome, the implants have to be properly anchored with the surrounding tissue. In a cemented arthroplasty, the implants are fixed with the aid of a Polymethyl methacrylate (PMMA) based bone cement (Figure 1.2). Immediately after the placement of femoral stem, bone cement is injected into the medullary cavity with the help of a syringe. The cement hardens within a few minutes after injection and thereby offers a firm fixation of implants within the bone. This method offers the disadvantage of necrotic damage to the surrounding living bone by the heat liberated during polymerization of the cement. However, the clinical outcomes of cemented arthroplasties are reported to be excellent in older patients with a relatively short life expectancy and low physical activity. For younger individuals regardless of the cementing methods employed, mechanical loosening due to localized resorption of bone around the implant is more commonly observed which hamper with the implants long term performance. The higher levels of physical activity result in the formation of wear particles due to the micro motion between the implant and the cement, thereby resulting in the biological loosening of implants due to aggressive osteolysis. About two third of the revision surgeries
reported are due to aseptic loosening of implants, with younger patients showing a higher incidence than older individuals [19, 20].

Figure 1.2: A typical representation of cemented arthroplastic design fixed with PMMA cement to hold the prosthesis in place [20].

1.2.3 Cementless (Non-cemented) Arthroplasty

Compared to cementing, cementless procedures offer higher survival rates in younger patients. This technique was developed as an alternative to cemented wherein, cement debris formation plays a significant role in promoting bone lysis and loosening. Cementless prostheses are inserted with a “press-fit” method enabling a bony ingrowth into the material resulting in a biologic fixation rather than a PMMA based anchorage as depicted in Figure 1.3. This method relies on the mechanism of osteointegration and interference fit between the implant and the femur. Cementless implants are the first choice of treatment option in young patients with high physical activity and bone quality where a revision surgical procedure in the future will be more likely. Preliminary statistics show relatively low revision rates and excellent implant stability associated with noncemented designs. The ingrowth of bone into implant mainly depends on the optimum surface architecture and composition of implant. Surfaces with good roughness, porosity, osteoconductive and osteoinductive coatings have shown to improve the
early bone-bonding ability of prostheses. In comparison to cementing techniques, cementless implants offering direct anchorage with bone via osteointegration is a more adopted technique nowadays [19-22].

Figure 1.3: A typical uncemented prosthesis with porous architecture on surface that allows bone in growth into the surface and helps in anchoring of implant to the bone [20].

1.2.4 Osteointegration

Osteointegration is a term coined by the famous Swedish Professor Per-Ingvar Branemark in the early 1960s. It is defined as the direct bonding of bone with the implant surfaces without any intervening fibrous tissue formation. In general, an implant is said to be osseointegrated when there is limited movement between the implant and bone under normal conditions of loading following a defined healing period. This clinical state is the outcome of bone apposition onto an implant surface without the formation of a fibrous tissue encapsulation [23]. However, the characteristic features of implant surface greatly influence the interface interactions between the implant and host bone tissue. Lack of proper osteointegration with the host bone tissue is one of the main reasons for aseptic loosening of implants resulting in revision arthroplasties. Recent reports suggest that the total number of hip revision surgery is expected to increase by 200 % by
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2030 [24]. The ever increasing need for implants demand the improvement of current orthopaedic devices to increase its life span and reduce the requirement of a revision surgery [25]. Recent research works in the orthopaedic biomaterials are focusing on generating new orthopaedic implants which can enhance early osteointegration and thereby potentially increase the longevity of implants.

1.2.5 Metallic Biomaterials Employed in THA

The standard requirements for materials used for orthopaedic implants mainly for load bearing applications such as in THA should possess outstanding mechanical as well as biological properties. It should have excellent biocompatibility, superior corrosion resistance in body environment, high specific strength, low elastic modulus, high fatigue and wear resistance, high ductility and high durability [26]. The commonly utilized metals for joint replacement include commercially pure titanium (cpTi), titanium alloy with 6% aluminium and 4% vanadium (Ti-6Al-4V), cobalt alloy with 27-30% chromium and 5 -7% molybdenum (Co-Cr-Mo) and stainless steel grades 316 and 316L [27]. Amongst these materials, cobalt chromium alloys and stainless steel are reported to release chemically active metal ions such as Ni, Cr, Co etc due to the corrosion from the surface. Elevated levels of Co and Cr concentrations were reported in the serum and urine specimens of patients after total knee or hip arthroplasty with porous-coated prostheses fabricated of Co-Cr alloy [28]. Numerous studies have reported the role of nickel, cobalt and chromium in inducing allergic contact dermatitis (ACD) and irritant reactions to the skin. The elevated levels of these metal ions in various anatomic sites such as heart, kidney, liver and lymphatic tissues have also been demonstrated in various studies after joint arthroplasty [29-31]. Several animal studies have proven the carcinogenic effect of nickel containing metal alloys. Local tumours were developed in rats and hamster models after implantation of metal alloys containing a predominance of nickel [32]. In addition to the toxic effects of these metallic biomaterials, both stainless steel and Co-Cr implants have high elastic moduli which can induce a phenomenon known as “Stress Shielding”. Stress shielding occurs when a metal having a high elastic modulus than the native tissue is placed to restore the damaged tissue function, most of the load applied will be taken up by the implant. Since bone being a
highly remodelling tissue, normal stress is required for its remodelling. When the loading on bone decreases, the bone tissue will become less dense and weak leading to bone resorption and loosening of implant [33]. Taken into account the shortcomings of employing SS and Co-Cr metals for joint arthroplasties, Ti implants are getting much attention due to the combination of its outstanding features such as high specific strength, low elastic modulus, corrosion resistance, less allergic problems and suitable biocompatibility. Though Ti and its alloys are basically developed for aerospace applications due to their light weight, its excellent biocompatibility has made it an attractive candidate for biomedical applications [34, 35].

1.2.6 Biomaterial of Choice – Titanium

Titanium is one of the transition elements in group IV with the symbol Ti and atomic number 22. It was first discovered in England in 1791 by William Gregor and named after the Titans of Greek mythology by Martin Heinrich Klaproth. Titanium has a high melting point (1668 °C) and possesses either a hexagonal closely packed (hcc) α phase or the body centered cubic β phase [36]. Commercially pure Ti (cpTi) may be classified as α, near- α, α + β, metastable β, or stable β depending upon the room temperature microstructure. The alloying elements for titanium fall into three categories: (1) α –stabilizers, such as Al, O, N, C; (2) β -stabilizers, such as Mo, V, Nb, Ta (isomorphous), Fe, W, Cr, Si, Co, Mn, H (eutectoid); (3) neutrals, such as Zr. Based on the incorporation of these elements, cpTi is available in four different grades (Grade I-IV) where, each grade has specific physical and mechanical properties. Young’s moduli of α- and (α + β)-type titanium alloys such as Ti and Ti-6Al-4V are higher than those of β-type titanium alloys [37, 38].

The main positive aspect of Ti based metals to be widely utilized as a biomedical prosthesis is its low elastic modulus. Compare to SS (210 GPa) and Co-Cr (240GPa), Ti has a comparatively low modulus of elasticity, thereby resulting in smaller stress shielding. Considering the strength of this material it is almost equivalent to 316 L SS, with almost 50% less dense than SS. Another important factor which makes Ti based metals to be employed as biomaterials for
implants is the presence of a 2-5 nm thick oxide layer. The high corrosion resistance and biocompatibility offered by Ti implants is mainly due to presence of this film which forms spontaneously during the passivation and re-passivation process. Because of this excellent properties Ti and its alloys are used in a wide range of biomedical applications mainly in orthodontic surgeries, joint replacement surgeries and housing devices for artificial heart valves [39- 41]. The elastic moduli of commonly used metals were compared and depicted in Figure 1.4.

![Figure 1.4: Elastic modulus of widely used orthopaedic biomaterials in comparison to cortical bone (Niinomi et al., 2003 [35]).](image)

In hip replacement procedures, Ti and its alloys are widely utilized to make metallic cup and hip stem. Though, Ti based metals are potential candidates for implant applications, some of the fundamental drawbacks of Ti are its poor fretting fatigue resistance and poor tribological properties. Their poor tribological behaviour includes high coefficient of friction, severe wear and low abrasion resistance [42]. Besides these, the bioinert nature of Ti implants tends to develop non-collagenous fibrous tissue between the implant surface and surrounding bone. Hence a good mechanical interlocking with bone tissue is not achieved resulting in loosening of implants (Figure 1.5). Owing to this, 10%-20% of joint prostheses need to undergo a revision surgery. Due to the increasing clinical demands for joint prostheses, significant research is being focused on to improve the performance of Ti based metals. The current research trend is mainly on the
surface modification strategies which have been proposed to improve the bone conductivity or bioactivity of Ti.

![Image of implant failure causes](image)

**Figure 1.5: Schematic representation of various causes of implant failure leading to revision surgery.**

### 1.2.7 Need for Surface Modification of Ti

Surface modification strategies are often performed to improve the bioactivity and bioconductivity of Ti based metals. A wide variety of approaches are currently being employed in order to make Ti biologically bond to bone. Since the material surface plays an essential role in response to the biological environment, most of these approaches are focussed on methods to improve the existing biomaterial surfaces beyond its normal efficiency. The appropriate surface modification strategies not only improve specific surface properties required by different clinical applications but also retain the excellent bulk characteristics such as relatively low modulus, good fatigue strength, formability and machinability. According to Puleo and Nanci [43], these surface modifications can be classified into three main categories- morphological, physico-chemical and biochemical.

The first approach involves altering the surface roughness and topography of implants to elicit favourable tissue response. Several *in vitro* and *in vivo* studies have demonstrated that rougher surfaces promote higher levels of bone
formation and apposition compared to smoother surfaces. Furthermore, the surface roughness approaches also enhanced the surface area and surface energy which in turn enhanced protein adsorption, cell adhesion, proliferation and its subsequent differentiation [44]. The second approach is to alter the composition by incorporating inorganic phases viz; calcium phosphate on to biomaterial surfaces, which due to their chemical resemblance with native bone mineral can support rapid bone formation [45]. In the third approach which is the biochemical modification specific cellular components such as proteins, peptides, growth factors, etc are immobilized onto implant surfaces to elicit specific cell and tissue responses. The goal of this modification is to control the tissue-implant integration with bioactive molecules delivered directly to the interface [46].

Some of the currently employed surface modification strategies are listed as:

(i) Mechanical

(ii) Physical

(iii) Chemical

(iv) Biomimetic approaches

(v) Biomolecular (Biochemical) modifications

(i) Mechanical Methods

Mechanical surface treatments are intended to generate specific surface topographies and roughness, which are favourable for getting improved adhesion in bonding with other substrates. Some of the commonly performed mechanical treatments include machining, polishing, grinding and blasting [47]. These surface treatments enhance the surface area of the substrates and facilitate the biomineralization process to initiate. Surface roughness in the order of 1-2 μm has showed stronger bone response compared to implants with smoother surfaces [48]. Among the mechanical treatments, grit blasting was found to be one of popular methods for getting desirable surface properties. Silicon carbide (SiC), alumina (Al₂O₃), biphasic calcium phosphates (BCP), hydroxyapatite and β-
Tricalcium phosphate have been commonly utilized as the blasting materials [49]. Though these treatments had favoured the osteoblast attachment, proliferation and differentiation, there are also some reports that blasting with SiC and Al₂O₃ may lead to surface contamination and local inflammatory reactions to surrounding tissues due to dissolution of these particles into host bone [50].

(ii) Physical Methods

Physical modification methods involve processes such as thermal spraying, physical vapour deposition (PVD), ion implantation and glow discharge plasma treatment. During these processes chemical reactions do not occur and thin films or coatings formed on Ti and its alloys are attributed to the thermal, kinetic, and electrical energy.

(a) Thermal Spraying

In this process, the materials of coating are thermally melted into liquid droplets and are allowed to condense on Ti surfaces. Thermal spraying techniques often require high temperature flame to operate and can be divided into flame spraying [51], plasma spraying [52], arc spraying [53], detonation gun spraying [54], laser spraying [55] and high velocity oxy-fuel (HVOF) spraying [56]. These methods are usually done to create bioactive coatings on metallic Ti surfaces eg: hydroxyapatite coatings on surfaces.

(b) Physical Vapour Deposition

In PVD the coating materials get evaporated in vacuum to form atoms, ions, molecules etc and get deposited onto the surfaces as a thin uniform film. PVD processes include evaporation [57], sputtering [58], and ion plating [59]. This type of modification is usually done to improve the implant biocompatibility, bioactivity, wear resistance and corrosion resistance [60].

(c) Ion Implantation

Ion implantation is a process in which energetic ions are introduced into a solid substrate surface via bombardment. Ions such as oxygen, carbon, nitrogen,
calcium, magnesium, sodium, helium etc have been implanted onto the surface to improve the biocompatibility of Ti and its alloys. It has been reported that the introduction of these ions improved the surface properties of the metals such as roughness, corrosion and wear resistance, blood compatibility etc [61, 62].

(d) Glow Discharge Plasma Treatment

This is a well established technique for surface cleaning and processing in electronic industry [63]. This process has received much attraction in biomedical arena where it is utilized to modify the surfaces of bulk polymers and production of thin polymer coatings. During glow discharge plasma treatment, the surfaces are usually exposed to plasma treatment and are bombarded with electrons and ions. Plasma treatments have been commonly employed as a method to increase the surface energy and clean the surface of biomaterials before biological evaluation. Aronsson et al [64] had also investigated the effectiveness of glow discharge plasma treatment in the surface cleaning and modification of metallic biomaterials in Ar and O₂ in the pressure range of 10–40 Pa. Glow discharge nitriding, oxynitriding and carbonitriding studies on the surface modification of Ti–1Al–1Mn alloy by Sobiecki et al produced surface layers with a diffusion character exhibiting high hardness, good wear, and corrosion resistance as well as increased fatigue limit [65].

(iii) Chemical Methods

Chemical method of surface modification provides Ti and its alloys with bioactive surfaces. It is regarded as one of the most popular treatment options to impart nanoscale features onto metal surfaces. It includes chemical treatment, sol-gel method, hydrothermal treatment, anodic oxidation and chemical vapour deposition (CVD).

(a) Chemical Treatments

Chemical treatments are mainly based on chemical reactions occurring at interface between titanium and a solution. It mainly involves acid and alkali treatment.
(b) Acid Treatment

It is one of the most popular surface treatment methods to remove oxides and contamination from surface [66]. It usually employs a combination of acids (mainly 10–30 vol% of HNO₃ and 1–3 vol% of HF in distilled water) for pre-treatment. Hydrofluoric acid can quickly attack the native TiO₂ layer and can form soluble titanium fluorides and hydrogen. A group of researchers had investigated the decontamination efficiency to the Ti surface using three acids, Na₂S₂O₈, H₂SO₄, and HCl and they found out that among these HCl was the most effective due to the capability to dissolve titanium salts easily without harming the Ti surfaces [67]. Surface treatments with acids can generate oxide layers of thickness <10 nm, which usually grow slowly in air during a 400-day period. The oxide formed will be predominantly TiO₂, however some residues of etching solution are frequently observed. Wan et al has reported on the effect of a two-step chemical treatments employing HCl and H₂SO₄ and alkaline solution in improving the bioactivity of Ti alloy [68]. A study by Maekawa et al. has demonstrated that polyphosphoric acid treatment of Ti significantly enhanced both the cell attachment and proliferation of hBMSC compared to untreated Ti [69].

(c) Alkali Treatment

This method was first introduced by Kim et al to improve the bioactivity of Ti and its alloys [34]. Nowadays, alkaline treatment is generally combined with subsequent heat treatment to modify the surface properties of Ti. This technique is considered to be one of the widely used method to generate biologically active bone-like apatite layer on the surface of bioactive ceramics, such as Bioglass®, hydroxyapatite and glass– ceramic A/W. In alkali heat treatment, the materials were firstly immersed in a high molar alkaline solution (5–10 M NaOH or KOH solution) for 24 h, followed by rinsing with distilled water and ultrasonic cleaning in acetone for 5 min. The samples were then dried in an oven at 40- 60 °C for 24 h and finally heated to around 600 – 800 °C for 1 h. The heat treatment usually employ at a pressure of 10⁻⁴ to 10⁻⁵Torr. The formed product consists largely of crystalline sodium titanate and a mixture of rutile and anatase phase [70]. A study by Nishio et al. had shown that alkali treatment followed by heat treatment had
significantly improved the apatite formation on Ti surfaces. This bone like apatite formation provided favourable environment for the osteogenic differentiation of rat bone marrow cells cultured on alkali-heat treated Ti compared to pure Ti [71]. Lee et al has also reported on the synergistic effect of a combined alkaline-heat treatment and topographic patterning method in improving the new bone deposition [70].

(d) Sol-Gel Method

A sol is defined as a colloid suspension of solid particles in a liquid system and a gel holds a continuous solid skeleton which encloses a continuous liquid phase [72]. Sol-gel method is considered as one of the widely employed method to deposit thin film coatings mainly ceramic coatings. The deposited films will be usually <10 µm in thickness. The advantage of sol-gel process is that it allows better control of the chemical composition and microstructure of the coating, preparation of homogeneous films, reduction of the densification temperature, and finally simpler equipment and lower cost [73]. It is considered as one of the promising strategies to improve the bioactivity of Ti and Ti based alloys. Usually coatings of TiO₂, Calcium phosphate, TiO₂-CaP were deposited onto Ti to improve the bone bonding ability. It is reported that sol-gel titania coatings can induce calcium phosphate formation and may therefore be able to contribute to enhanced bonding to bone [74-77]. The most commonly utilized sol is prepared by mixing tetraisopropylorthotitanate, ethanol, ethylene glycol monoethyl ether, hydrochloric acid, and water. Titania/hydroxyapatite coating was reported as a very promising method for achieving a good adhesion to the substrate with a very good bioactivity [78]. There was a significant improvement in the adhesion strength (up to 55 MPa) with the introduction of a TiO₂ layer in the HA/TiO₂ coatings.

(e) Hydrothermal Treatment

The term hydrothermal is purely geological in origin. It generally refers to any heterogeneous reaction taking place in presence of aqueous solvents or mineralisers under high vapour pressure and temperature to dissolve and
recrystallise materials that are rather insoluble under normal conditions. Sir Roderick Murchison (1792–1871), the British Geologist, coined the term “Hydrothermal”. He used this term to describe the changes in the earth’s crust due to the action of water at elevated temperature and pressure leading to the formation of various minerals. The first successful commercial application of the hydrothermal method began during the 19th Century. Karl Josef Bayer (1871–1908) employed hydrothermal conditions for obtaining pure aluminum hydroxide with sodium hydroxide as a solvent to leach bauxite. Hydrothermal synthesis includes the various practice of crystallizing substances from high-temperature and high pressure [79]. Hydrothermal method due to its simplicity, considered as one of the promising approaches to improve the bioactivity of Ti implants. Distinctly different TiO$_2$ nanostructures can be developed on Ti surfaces by employing this cost effective technique [80]. The experiment is carried out in a steel autoclave at high temperature and high pressure. Hydrothermally modified Ti surfaces enhanced the attachment, proliferation and maturation of pre-osteoblasts than conventional Ti implants. Due to high surface roughness and surface area imparted by the nanostructures the bone bonding ability or the osteointegration was found to be improved on hydrothermally modified Ti surfaces than the machined or polished Ti. This technique can also be easily translated to other metallic implants such a Co-Cr alloys, stainless steel, NiTi etc for other applications such as cardiovascular stents, urethral stents etc [81].

(f) Anodic Oxidation (Anodization)

Anodic oxidation is commonly used as a surface treatment of Ti and its alloys, to obtain uniform, microporous arrays of oxide nanotubes on the surface of metals. During this process, the metal to be treated works as the anode and oxide layer forms on the surface of the metal [82]. The oxide layers thus formed on the surface imparts the corrosion resistance and wear resistance property to the Ti metal. The formation of nanotubes strictly depends on many factors such as anodic potential, electrolyte composition, temperature and current. This method is often considered as a simple, economical and environmental friendly coating technique because the processing can be carried out at room temperature. Since
the nano TiO$_2$ tubes provide high specific area for reactive nucleation of calcium phosphates, it has been employed to fabricate calcium phosphate coatings for the recent generation of dental implants [83, 84]. Another promising method of producing the porous oxide surface with bioactive composition is anodic spark oxidation, also called micro-arc oxidation (MAO) or plasma electrolytic oxidation. It is one of the novel anodic oxidation strategy to deposit ceramic coatings on the surface of different metals, such as Al, Ti, Mg, Ta, W, Zn, and Zr and their alloys [85, 86]. Nie et al [87] described a hybrid treatment to improve the biocompatibility and durability of Ti–6Al–4V, comprising of micro-arc oxidation and electrophoretic deposition. A phosphate solution was used to produce a relatively thick and hard TiO$_2$ coating and HA was deposited using a combination of plasma electrolysis and electrophoresis oxidation. Anodization is considered as a relatively simple and cost effective method to modify the surface of titanium and its alloys for improving its biocompatibility and bioactivity.

(g) Chemical Vapour Deposition (CVD)

Chemical vapor deposition (CVD) is quite a widely used technique to chemically modify metallic surfaces. In this process, chemical reactions takes place between chemicals in gaseous phase and substrate surface resulting in the deposition of non-volatile compounds. It is different from PVD, which typically employs evaporation and sputtering, while no chemical reactions involved [88]. CVD is mainly used to provide thin organic and inorganic coatings on different metals, semiconductors etc. The different types of CVD so far developed include Atmospheric-pressure chemical vapour deposition (APCVD), Low-pressure chemical vapour deposition (LPCVD), Laser-enhanced chemical vapour deposition (LECVD), Plasma-enhanced chemical vapour deposition (PECVD) and Plasma-assisted chemical vapour deposition (PACVD). The combinations of PVD and CVD processes to improve the bioactivity have also been developed [62]. Over PVD, thin films grown by CVD have a better step coverage. Hence, CVD is considered as a widely adopted technique for the fabrication of microelectronic devices or coating objects with complex geometry. It has also been widely employed to modify the mechanical and biological properties of Ti and its alloys. By CVD method polycrystalline diamond films have been deposited onto Ti to
improve its tribological properties. Tang et al has investigated the effect of CVD diamond on human osteoblasts and found out that there was no adverse effect of diamond on cell growth and its proliferation [89]. Because of its biocompatibility it is frequently used in biomedical implant modification. Diamond like carbon (DLC) coatings have been also been deposited on biomedical implants using various CVD methods [90, 91]. Ianno et al reported on the DLC coatings on Ti–6Al–4V by PECVD method for it to be used in hip and knee prostheses [92].

(iv) Biomimetic Approach- Coating of Ti with Calcium Phosphates

To initiate the de novo bone formation around Ti implants, extensive efforts are being taken by the researchers worldwide. It is well understood that by providing a Ca/P coating the bioactivity of Ti can be improved [93]. Several methods described in literature for the deposition of Ca-P coatings on titanium implants include ion beam deposition plasma spraying, sol-gel methods, laser deposition, radiofrequency sputtering, biomimetic deposition and electrostatic spray deposition [94]. Of these methods, plasma spraying is the most popular method for the deposition of Ca-P coatings on titanium implants. However, the reported drawbacks of this method include potential for coating debris, macrophage infiltration, fibrous tissue encapsulation, and eventual coating failure at the bone-implant interface. Biomimetic deposition of Ca/P is one of the alternative surface modification techniques to improve the bioactivity of Ti implants. This method was originally introduced by Kokubo and his co-workers [95]. This technique allows the deposition of hydroxyapatite and other calcium phosphate crystals from a simulated body fluid (SBF) onto titanium surfaces under physiological temperature (37 °C) and pH (7.4). Usually it involves an immersion period of 14-28 days in SBF to form a uniform Ca/P coating on Ti surfaces. Recently, to fasten the deposition and shorten the incubation period, efforts have been made by a group of researchers. A coating thickness of ~25 µm could be achieved on metallic implants by biomimetic modification [96]. It is well understood that the cell response mainly depends on the composition and topography of the implant surfaces. Bio-mimetically produced apatite surfaces may, therefore, is useful in facilitating early bone ingrowth into porous surfaces. Another advantage of this procedure is that the apatite crystals formed acts as a
tissue-engineering scaffold and different signalling moieties can also be deposited along with this to impart the osteoinductive nature to the Ti implants [97].

(v) Biochemical Surface Modification Approach

Besides the topographical and physicochemical modifications, researchers are actively interested in the biochemical modifications of Ti surfaces wherein different bioactive molecules, peptides, proteins and growth factors are immobilized onto Ti surfaces to elicit specific tissue response [46]. This approach in particular involves the immobilization of biological components such as ECM molecules (Collagen I, RGD peptides, fibronectin), growth factors and peptides onto Ti substrates with the aid of crosslinking agents. As reported by Puleo and Nanci, this alternative biochemical method utilizes the current understanding of the bio-chemistry of cellular functions and differentiation and also on different appropriate surface modification aspects. Numerous methods are reported in literature by which biomolecules of interest (peptides, growth factors, can be immobilized onto implant surfaces [98-102]. These includes physical adsorption, electrostatic interactions, encapsulation, covalent and ionic bonding. ECM serves as a scaffold for cells to interact via the integrin mediated cell signalling receptors [103]. The most explored peptide sequence immobilized onto Ti or polymer surfaces is Arg-Gly-Asp (RGD) derived from fibronectin, which has a high affinity to $\alpha_5\beta_1$, the predominant osteoblast integrin receptor [104]. Ti implants coated with RGD peptide has resulted in increased bone formation in the femur of rats and goats [105]. Another relevant ECM molecule explored for biochemical modification of bone-contacting interfaces is collagen, the major extracellular matrix component of bone. Coating of Ti implants with collagen has shown enhanced bone ingrowths in rabbit femur relative to uncoated Ti implants, suggesting the significance of surface modification using ECM components [106]. The coating of Ti implants with fibronectin has also significantly enhanced the osteointegration in canine models. In general, this appears to be a promising approach to accelerate bone healing and increase new bone formation and bone bonding around orthopaedic implants, hence potentially reducing the problem of implant loosening and infection rates [107]. Another promising approach for improving the osteointegration is the delivery of signalling molecules, particularly
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the osteogenic growth factors. The coating of implants with bone morphogenic proteins (BMPs), to enhance the osteointegration potential has been very well acknowledged in several literatures [108-110]. The osteogenic potential of BMPs around Ti implants have been very well demonstrated in a study using an atelopeptide type-I collagen carrier as a coating [111]. However, an enhancement of bone volume density (BVD) and bone-implant contact (BIC) around coated implants were observed on a study utilizing a collagen/chondroitin sulphate (CS) carrier system on titanium, but they were not able to show any significant difference between bare collagen/CS and BMP integrated coatings [112, 113]. Due to the variation in these findings, the need for an optimal 3D carrier on the implant surface to provide sufficient osteointegration is highly demanding.

Summarizing, the different strategies adopted for surface modification of bare metals, an overview of the history of surface modification techniques practiced over years is presented in Figure 1.6. As evident, the recent trend is towards the use of biomolecular coatings over metallic surfaces to improve the hard-tissue compatibility.

![Image](image.png)

Figure 1.6: An overview of the different modification strategies performed on metals for improving its biofunctionality (adapted from a review article by Hanawa T [114]).

1.2.8 Relevance of Bone ECM Architecture on Implant Modification

Bone tissue implants require not only a material with suitable composition, but also a good structure that mimics the extracellular matrix architecture of native
bone. Bone is a composite material composed of an organic and inorganic part. If the wet weight composition is considered 65% of the weight is contributed by the inorganic part and 20% by the organic components, and the rest by water. Considering the ECM of native bone it is mainly composed of 90% collagenic proteins (type I collagen 97% and type V collagen 3%) and of 10% non-collagenic proteins (NCP) (osteocalcin 20%, osteonectin 20%, bone sialoproteins 12%, proteoglycans 10%, osteopontin, fibronectin, growth factors, bone morphogenetic proteins, etc.). All these proteins play a crucial role in organization of the matrix, the mineralization of the bone, and the behaviour of the bone cells. The inorganic mineral phase of bone serves as an ion repository. Approximately 99% of the body’s Ca²⁺, 85% of P, and 40-60% of Na and Mg²⁺ are associated with the bone mineral crystals, which serve as the major source of these ions. The stiffness and strength of the bone tissue is provided by the calcium-phosphate crystals. The tensile strength of bone is provided by the elastic protein collagen and compressive strength by the hydroxyapatite crystals [115,116].

Figure 1.7: a) Representative image of the microscopic view of bone tissue (picture courtesy http://www.physioweb.org/skeletal/bone_tissue.html), b) SEM image of the collagen scaffolding of ECM (http://collagenguide.net/).
The interaction of osteoblasts with these ECM components is essential for the skeletogenesis and maintenance of osteoblastic phenotype. The structures of ECM encountered by the cells usually are of nanoscale dimensions (Figure 1.7). Type I Col, the most abundant protein fibrils are formed of tropocollagen molecules of ~300 nm long and 1.5 nm diameter polypeptide strands [117]. The hydroxyapatite crystals are embedded in fibrils and have an average size of 50x25x4 nm³ [118]. Numerous research works had already shown the effectiveness of nanoscale features on biomaterials in enhancing cell behaviours including adhesion, ECM production and mineralization, than on conventional materials [119-121]. Divya Rani et al had already reported on the improved osteoblast response on nanomodified titanium implants [80]. Thus, there is a need to produce better implant materials having nanometre scale dimensions. Another important factor required for bone ingrowth into a material is its porosity. Since bone is a highly porous tissue material, introduction of a porous architecture can favour bone ingrowth and provide an adequate biological fixation [122].

1.2.9 Research Strategy Adopted – Biopolymeric Scaffolding Approach

Although literature reports several diverse strategies for generating a surface modified metal, a biopolymeric approach to its surface modification has not been explored yet. The fact that ECM architecture of native bone is microporous and nanofibrous in nature motivated us to adopt a polymeric scaffolding approach for surface modifying metallic Ti. This is hypothesized to promote the proliferation of stem cells and simultaneously aid its differentiation into osteoblasts. The study was proposed to improve the current problems associated with Ti implants such as osteoinduction, osteoconduction and osteointegration. The biopolymers selected for the present work include an endogenous protein fibrin and a natural polymer alginate. Further, the bioengineering of this metallic Ti with osteogenically induced MSCs to generate a surface bony layer construct could further aid in enhanced bone apposition and thereby significantly improve functional implant osteointegration compared to unmodified Ti substrate.
1.2.10 Why the Choice of Biopolymers?

(a) Fibrin

Fibrin is a fibrous non globular protein formed during blood clotting. It is a polymerized product of the action of a proteolytic enzyme thrombin on fibrinogen. Fibrinogen is a 360 kDa glycoprotein produced by the liver having 45 nm in length and made up of three pairs of polypeptide chains, (AαBβγ)$_2$, including the fibrinopeptides A and B, held together by 29 disulphide bonds [123]. The amino termini of all six chains are joined in the central region, which is connected to the end domains by α-helical coiled coils. During fibrin polymerization, thrombin cleaves the fibrinopeptides A and B on fibrinogen producing ‘A’ knobs complementary to ‘a’ holes in the γC-module and B knobs complementary to holes ‘b’ in the βC-module. Interactions with knobs and holes yield fibrin protofibrils which aggregates to form the fibrous fibrin protein (clot). The fibrin clot thus formed is then stabilized by the plasma transglutaminase factor XIIIa [124,125], which gives the clot more mechanical strength and resistance to proteolytic degradation (Figure 1.8).

![Figure 1.8: a) Microscopic image of fibrin clot entrapping RBCs (http://www.marined3.com/nattokinase/), b) SEM image representing the nanofibrous nature of fibrillar fibrin and c) Scheme representing the assembly of fibrin monomers into protofibrils [126]).](image-url)
For commercial purpose fibrin is manufactured from fibrinogen which is extracted from pooled plasma by cryoprecipitation. This cryoprecipitate is rich reservoir of many growth factors such as platelet derived growth factor (PDGF), Transforming growth factor (TGF-β), Fibroblast growth factor (bFGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) [126]. Fibrin is the first nanofibrous scaffold a cell encounters during trauma to tissues. Several studies have used fibrin as a sealant in bone regenerative material due to its adhesive property. Fibrin has two pairs of RGD sites and a pair of AGDV sites through which it can interacts with the integrin receptors present on the cell surfaces. This bioactivity makes fibrin an attractive candidate for stem cell differentiation and tissue engineering. Commercially fibrin is available as a biopolymeric matrix known as fibrin glue which is commonly used for surgical homeostasis and tissue sealing [127]. It has been reported that by modulating the mechanical and chemical properties of a fibrin-based matrix, human mesenchymal stem cells can be successfully differentiated into osteoblasts and mouse embryonic stem cells into neural and astroglial lineages [128,129]. One of the reasons reported for the increased cellular attachment, proliferation and differentiation of stem cells on fibrin matrices is the presence of the binding sites for αvβ3 and αvβ1 integrin receptors which favour cell binding [130]. Fibrin has been utilized in different forms in literature to enhance the osteogenic property of stem cells. To induce the osteoinductive property of different ceramics and silicates, fibrin glue has been mixed along with these materials which resulted in a positive effect on the neo-osteogenesis. Abiraman et al [131] has also demonstrated similar results about the osteoinductive nature of fibrin glue when implanted in the extra skeletal site of Swiss albino mice. It has been reported that human mesenchymal stem cells proliferated well and differentiated into osteogenic lineage when they were entrapped in fibrin gels. However, one of the major limitations encountered with fibrin based scaffolds for hard tissue regeneration is its lack of long term stability and mechanical integrity due to fibrinolysis. Though it can be advantageous in wound healing studies, it becomes a major obstacle to be used as a tissue engineering scaffold. Different fibrinolytic inhibitors such as aprotinin, tranexamic acids and amino caproic acids are added as supplements to slow down the degradation of gels [132]. However, studies show that these inhibitors can
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evoke adverse tissue responses. Krishnan *et al* in their studies showed that these fibrinolytic inhibitors can adversely affect the tissue remodelling during wound healing [133]. Another alternative strategy to overcome the issues associated with mechanical integrity is by combining fibrin with other polymers which can impart mechanical strength to the construct.

(b) Alginate

Alginate otherwise known as alginic acid is a naturally derived polysaccharide distributed widely in the cell walls of brown algae. It is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, which are covalently linked together (Figure 1.9). Sodium salt of alginic acid is soluble in water and forms a viscous solution depending on its molecular composition such as concentration and weight. In the presence of divalent cations such as Ca$^{2+}$, Ba$^{2+}$, Sr$^{2+}$ etc sodium alginate solution is ionically crosslinked between chains to form a hydrogel. The Ca$^{2+}$ ions exchange with Na ions on the G blocks to initiate the gelation of alginate [134,135]. Because of its excellent properties such as non-toxicity, relative low cost, biodegradability and immunologically inert nature, alginate has been widely used as a scaffold material for immobilization of enzymes or cells for bioreactors, and also for tissue engineering [136]. Alginate has been mainly employed in tissue engineering as a cell delivery system [137,138]. It has also been used extensively in the culturing of chondrocytes, hepatocytes and Schwann cells for nerve regeneration [139-141].

Alginate is reported to lose its mechanical stability over time in culture conditions due to the outward flux of divalent cations into the surrounding medium. However, the mechanical strength of alginate based gels can be controlled by optimizing different parameters such as molecular weight, gelation rate, type of crosslink, alginate concentration, crosslink concentration, and mannuronic acid/guluronic acid ratio [142]. Molecular weight is the key factor that influences the degradation as well as mechanical properties of alginate-based biomaterials. Higher molecular weight alginate undergoes slower degradation rates which results in its long term stability [143]. One of the drawbacks reported for alginate based hydrogels is the absence of specific cell adhesion sequences. To
address this, alginate is modified to provide with specific cell adhesion motifs, which thereby enhances its biological performance. The presence of carboxylic acid makes it suitable for appropriate modifications. Reports suggest that cell attachments and its subsequent events got enhanced when they were cultured in peptide modified alginate (RGD functionalized) [144,145]. Also due to the anionic nature associated with alginate, it is reported to inhibit protein adsorption. However, Machida-Sano et al in their studies showed that the critical factors determining protein adsorption not only depends on the surface charge, but also the surface wettability and morphology [146]. Hence efforts are being made to modify alginate matrix to obtain the desirable properties. One such approach to enhance the biological performance of alginate is by combining it with different polymers. Amongst them, chitosan, hydroxyapatite and fibrin were generally used in combination to improve the biological property of alginate [147, 148].

Alginate has been reported to form interpenetrating networks with fibrin. An interpenetrating network (IPN) is a combination of polymers in network form, where at least one polymer is synthesized and/or crosslinked in the presence of the other, either simultaneously or sequentially [149]. The chains of the individual polymers are completely entangled, and there may or may not be chemical bonds between the combined networks. The characteristics of both the polymers will be evident in the IPN network. A study utilizing FA-IPN for ovarian follicle development demonstrated that the construct promoted follicle growth and enhanced the number of meiotically competent oocytes relative to either fibrin or alginate alone. FA-IPNs have got dynamic mechanical properties that can be utilized to enhance the tissue development relative to a single hydrogel [150]. Taking this aspect into consideration, we finally ended up with a combination strategy of mixing fibrin with alginate, for making use of the beneficial effects of both for developing a bioactive and mechanically strong 3D microporous and nanofibrous scaffold over metallic Ti.
Figure 1.9: a) Photograph of seaweed Laminaria digitata (Oarweed-kelp http://www.eastlondonnature.co.uk/2012/04/oarweed-kelp/) (b) Granules of sodium alginate obtained from seaweeds and c) Structural formula of sodium alginate molecule composed of homopolymeric blocks of (1→4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues (www.Intechopen.com).

(c) Dopamine – Biomimetic Anchor

One of the important factors affecting the durability of an implant is the stability of the coating material. Of the known methods available for immobilization (such as mere physical adsorption, electrostatic interactions, encapsulation, covalent and ionic bonding), covalent immobilization is advantageous due to its ability to withstand wear and tear during in vivo conditions [151]. Covalent modification of Ti utilizing biomimetic anchor dopamine is a widely adopted technique due to the ease of processing and cost [152-154]. The non-proteinogenic aminoacid 3, 4 dihydroxyphenylalanine (DOPA) has attracted substantial attention in the context of its binding ability to substrates of different chemistry. The use of this chemical is inspired by nature especially the mussels. Mussels usually attaches to surfaces with different
geometry with the help of proteinaceous glue called Mussel adhesive proteins (MAPs). These proteins consist of tandem repeating sequences of approximately 5-15 amino acids usually consist of 30 mol% of amino acid 3, 4-dihydroxyphenylalanine (DOPA) (Figure 1.10). The high amount of DOPA in the MAPs has been found to contribute to the sticking and cross-linking ability of the marine adhesives.

Figure 1.10: Mussel attached to a rock with the aid of glutinous 3, 4-dihydroxyphenylalanine (Courtesy: Zhao Qin.)

The adhesive property makes it an attractive candidate to use as an anchoring moiety. Messersmith et al has first used DOPA as an anchoring group for the surface immobilization of PEG [155]. This bio-inspired modification technique has been recently employed to modify different materials including metal, metal oxides (e.g. Cu^{2+}, Fe^{3+}, Mn^{2+}, Mn^{3+}, Ti^{3+}, Ti^{4+}, Zn^{2+}, Nb_{2}O_{5}, TiO_{2}) polymer, and ceramic via several proposed mechanisms [152, 156]. Of these, TiO_{2} is drawing worldwide attention as a potential material which has wide applications in implant dentistry, orthopaedics, for making optical sensors, as a photocatalysts, corrosion protective and optical coating agent. Several studies have focused on the study of interactions between catechol and surface of TiO_{2}. Only by a simple immersion of substrate in a dilute aqueous solution of dopamine (of alkaline pH), can easily result in a spontaneous deposition of a thin polydopamine film. Catechols having -OH groups usually adsorbs on the surface either by molecular (hydrogen bonded) adsorption or by dissociative (coordinative) adsorption. The first one involves the formation of Van der Waals complex with hydrogen bonds. In dissociative adsorption the dissociation of catechol –OH bonds take place.
resulting in the formation of either a mono-dendate or a bi-dendate structure. If dissociation of one one -OH group is taking place, it results in the formation of mono-dendate complex. If dissociation of both –OH groups is occurring, bidentate co-ordination complex is taking place [157]. Elimination of H₂O at the defect Ti=O sites leads to the formation of bidentate structure with two chemical bonds from catechol to one surface titanium (Figure 1.11). On Ti, the interaction of dopamine is likely to take the form of a bidentate coordination complex between the catechol oxygen and a Ti atom at the native oxide surface. Due to the presence of free NH₂ group it can easily form amide bonds with other molecules of interest.

![Figure 1.11: Schematic representation of possible Catechol- TiO₂ interactions (adapted from the dissertation works of Barbora Malisova [155]).](image)

DOPA containing peptides are widely used for grafting different polymers (chitosan, HAP) and bioactive molecules (RGD, VEGF, BMP-2etc) onto Ti for inhibiting bacterial adhesion and promoting osteoblast function [158-160,102]. The surface conjugation of polymers over metallic Ti with dopamine was found to be very effective in yielding an adherent polymeric matrix, which in turn promoted osteointegration and inhibited bacterial growth. With this in mind, we have also exploited dopamine, a robust anchor peptide for immobilizing the fibrin alginate scaffolding onto Ti surfaces.

1.2.11 Mesenchymal Stem Cells (MSCs) – Cell of Choice

Different in vitro cell culture models have been applied extensively to determine the biological responses on titanium surface. Bone regeneration around
Ti implants has been regarded as similar to that observed during fracture healing. During healing process a cascade of cellular and molecular events take place. A group of multipotent stem cells residing in the bone marrow microenvironment under specific growth and transcriptional factors differentiate into cells of osteogenic lineage and helps in the new bone formation process. These primitive cells originate from mesenchyme hence called Mesenchymal stem cells (MSCs) [161]. MSCs are widely reported in literature in regard to their multilineage differentiation potential in particular bone and cartilage [162]. These multipotent stem cells can differentiate into a variety of cell types, including: osteoblasts (bone cells), chondrocytes (cartilage cells), adipocytes (fat cells), tendons and ligaments, muscles cells, skin cells, cardiomyocytes and even nerve cells in vitro and in vivo [163]. Friedstein and colleagues (1966) demonstrated that these fibroblastoid like cells could be isolated from single-cell suspensions of rodent bone marrow explants on the basis of their ability to adhere to tissue culture plastics [164]. These mesenchyme originated cells were got their name after the pioneering works by Caplan in early 1990’s [165]. At 2006, Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has proposed a minimal set of four criteria for identification of human mesenchymal stem cells [166]: (1) MSCs have to be plastic-adherent when maintained under standard culture conditions, (2) MSCs should have the ability to differentiate into osteogenic, adipogenic, and chondrogenic lineage (3) MSCs must express positive cluster of differentiation markers such as CD105 (SH-2), CD166 (ALCAM), CD54 (ICAM-1), CD55, CD13 (APN), CD73 (SH-3, SH-4), CD90 (Thy-1) and CD44 and (4) MSCs must lack the expression of hematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD19, CD79 and human leukocyte antigen (HLA)-DR. MSCs can be isolated from different sources such as bone marrow, umbilical cord blood, adipose tissue, adult muscle, corneal stroma, or the dental pulp of deciduous baby teeth. Among these, bone marrow serves as the traditional source of MSCs for basic research and therapeutic use. However, it has been demonstrated that the number and differentiating potential of bone marrow MSCs decrease with age and the fraction of MSCs of the total population of the MSCs present in bone marrow is low (0.001- 0.01% or approximately 1 MSC per 10^5 adherent stromal cells) [167]. Hence, significant
research is carried out in search for an alternative source of MSC. Umbilical cord and blood contains a putative stem cell population which show high morphological and molecular similarities to bone marrow derived MSCs including the lack of hematopoietic surface antigens CD133, CD34 and CD45 [168]. Umbilical cord blood MSCs have more primitive properties than any other adult MSCs. Compared to bone marrow, umbilical cord blood is easy to obtain as it is discarded as a waste after delivery and hence possess no risk of collection. Also, cord blood derived MSCs demonstrate higher proliferation capabilities than bone marrow MSCs [169]. Cord blood MSCs have been successfully differentiated in-vitro into osteogenic, chondrogenic, neural, hepatic and cardiomyogenic lineages [170]. Cord blood derived MSCs are also suitable for allogenic use because of their immunological naivety and weaker response to inflammatory stimuli. Many in vivo studies had also displayed the immunosuppressive capacity of MSCs [171].

Another promising and richest source of MSC is adipose tissue. When compared to bone marrow, there are 500 times more stem cells in 1 gram of fat when compared to 1 gram of aspirated bone marrow. Adipose derived stem cells (ADSCs) are also capable of differentiation into chondrogenic, osteogenic, adipogenic and neuronal lineages [172]. ADSCs can be isolated from fat depots within the body by minimally invasive liposuction and subsequent digestion by collagenase enzyme [173]. A shortage of autologous donor adipose tissue is very unlikely in most individuals. The lipoaspirates are easy to culture and can be differentiated into multiple cell lineages by the appropriate supply of induction agents to the growth medium. Osteogenic supplements such as ascorbic acid, 1, 25-Dihydroxyvitamin D3, β-glycerophosphate and dexamethasone are used to induce osteogenesis in vitro. Dexamethasone stimulates MSC proliferation and supports the osteogenic lineage differentiation by altering the expression levels of many genes such as ALP, COL I, integrins, cadherins and interleukins, while β-glycerophosphate acts as a substrate for ALP and helps in mineralization and modulation of osteoblast activities. Ascorbate and 1, 25-Dihydroxyvitamin D3 are commonly used as supplements for osteogenic induction. It helps in enhancing the expression of alkaline phosphatase and promoting the production of osteocalcin. Furthermore, ascorbate is also involved in the conversion of procollagen to
collagen. Osteogenic induced ADSCs were reported to express high alkaline phosphatase activity and transcription factors c-fos and msx2, both of which are involved in osteoblastic differentiation [174-179].

1.2.12 Animal Model for Assessing Osteointegration Potential

In order to understand the effectiveness of a newly developed implant material, it must be tested both in vitro and in vivo. Often results from the in vitro studies cannot be extrapolated to the in vivo situation always. Appropriate preclinical evaluation is often an essential step in the testing of orthopaedic and dental implants prior to clinical usage [180]. In case of testing orthopaedic and dental implants, it is necessary to use an animal model which is reproducible and in which implant dimensions are comparable to those used in humans. Animal selection depends on many factors such as cost to acquire and care for animals, availability, acceptability to society, tolerance to captivity and ease of housing [181]. For bone implant related studies the most preferred animals are dogs, sheep, goats, pigs and rabbits. The most common implant designs used in animal models are either screw type (threaded) or cylindrical (rod shaped). In order to avoid fracture of test sites, strict guidelines are followed for the dimensions of implants for in vivo studies which is mainly based on the size of animal, bone chosen and on the implant design. ISO has recommended standard sizes for bone implants for testing in animals. For smaller animals like rabbits, cylindrical implants which are to be placed onto tibial and femoral diaphyseal bone, the recommended dimensions should be no larger than 2mm in diameter and 6mm in length. For larger animals such as sheep, goats and dogs the dimensions of cylindrical implants for implantation into the femur and tibia are 4 mm in diameter and 12 mm in length. For orthopaedic bone screw-type implants the dimensions range from 2- 4.5 mm. For larger species such as dog, sheep and pig generally 4.5 mm screws are opted.

For musculoskeletal research studies, rabbit is the most preferred animal model [182] because (1) its small size makes it easy to handle (2) it attains skeletal maturity soon after sexual maturity at around 6 months of age [183]. One of the drawbacks associated with rabbit models is the limitation in assessing
multiple implant materials due to its smaller size. As per ISO standards, a maximum of 6 implants (3 test and 3 control implants) per rabbit (ISO 10993-6:2007) is recommended. Also, if cylindrical implants are to be used it should not be larger than 2 mm in diameter and 6 mm in length. This is half the number recommended for larger animals (International Standard ISO 10993-6:2007). Despite these drawbacks, rabbit remains the popular animal model for bone implant studies.

Several experimental methods have also been proposed in literature for evaluating the bone implant integration. These can be broadly classified into three.

❖ **Push- or Pull-out Test of Transcortically Placed Implants**

Transcortical implantations methods are used extensively for testing orthopaedic implant materials. It represents the simplest *in vivo* model in terms of surgical procedure and mechanical evaluation [184].

❖ **Push- or Pull-out Test of Intramedullary Placed Implants**

Intramedullary implantations are clinically relevant models for joint replacement applications such as the total hip replacement [185]. The ‘push-out’ or ‘pull-out’ test is the most commonly used approach to investigate the healing capabilities at the bone implant interface. In the typical push out or pull-out test, a cylinder-type implant is placed intramedullary in bone and is then removed by applying a force parallel to the long axis of bone. The interfacial strength is calculated by dividing the maximum force by the surface area of implant in contact with the host bone [186, 187]. The push-out and pull-out tests are usually employed for cylinder type non-threaded implants.

❖ **Removal Torque of Rotationally Symmetrical Implants**

Removal torque is usually done to evaluate screw type implants and rely on rotational symmetry of cylindrical, often threaded, implants. Application of a reverse or unscrewing torque is utilized to assess the implant stability [188, 189].
1.3 MAIN OBJECTIVES OF THE STUDY

Having outlined the literatures regarding different modifications for improving the osteointegration, we propose a strategy of bio-polymeric scaffolding approach on metallic Ti, for which the following objectives are outlined.

- Generation of a 3D bio-polymeric scaffolding on metallic Ti using biodegradable polymers such as fibrin and alginate and its physico-chemical characterization.

- A detailed \textit{in vitro} evaluation of the surface modification approach in favouring the adhesion, proliferation and differentiation of human umbilical cord blood derived mesenchymal stem cells into functional osteoblasts, compared to an unmodified Ti surface.

- \textit{In vitro} analysis on the effect of bio-polymeric scaffolding on metallic Ti in inducing hemolysis, platelet adhesion, activation as well as its influence on the clotting kinetics.

- Isolation and characterization of adipose derived stem cells from rabbit adipose tissue and its osteogenic differentiation potential evaluation on Ti rods.

- Evaluation of \textit{in vivo} osteointegration potential of the modified Ti substrates (MTi) and combination of modified Ti and osteogenically induced ADSCs (MTi + cells) with respect to a control polished Ti (CTi) in a rabbit intramedullary model.

1.4 THESIS OUTLINE

\textbf{Chapter 1} includes the general introduction and comprehensive literature review of relevant articles pertaining to the thesis.

\textbf{Chapter 2} includes the materials and experimental methods done to validate the thesis.
Chapter 3 includes the results and discussion of the research studies, represented under four main sections. Each section is addressed with specific research questions and hypotheses.

- Bio-polymeric scaffolding approach on metallic Ti and its physico-chemical characterization.

- *In vitro* biological evaluation of the efficacy of the scaffolding approach in enhancing the proliferation and differentiation of hMSCs into functional osteoblasts.

- *In vitro* blood-material interaction studies.

- *In vivo* osteointegration studies in New Zealand White rabbits.

Chapter 4 summarizes the study and provides its future prospects.

1.5 OVERALL RESEARCH STRATEGY

The research strategy employed in this thesis work is schematically represented here in Figure 1.12.

![Figure 1.12: Schematic representation of the overall thesis outline.](image-url)