IMPLANT MATERIAL FUNCTIONALIZATION TO ENHANCE BIOACTIVITY IN ORTHOPAEDICS

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Summary

Musculoskeletal disease is one of the leading causes of long term physical disability and affects hundreds of millions of people worldwide. One of the key challenges in bone healing and regeneration is the engineering of an implant that incorporates osseointegration to meet the metabolic demands of recovery. Although implants are typically expected to last ten years or more, longevity is still not assured and the lack of integration into the bone for long-term survival often occurs and leads to implant failure.

Revision surgery to address such failure involves increased risks, complications and costs. The main reason for the failure of these implants is due to aseptic loosening which accounts for 60 to 70% of the cases for revision surgery. The success of implants is dependent on firm bonding or fixation of implant biomaterial to bone, for optimal function and lastingness. Thus the aim of this thesis is to develop orthopaedic implant materials with enhanced bioactivity and improved implant-host interactions so as to reduce biological related implant failure.

In this thesis, various approaches of surface functionalization to confer implant materials with bioactivity were developed depending on the materials of interest. At the same time, other important material properties such as surface profile, topography, stability, cytotoxicity and effects on cellular functions were investigated after the functionalization process. The interactions between the fabricated materials and biological systems were evaluated with typical cells involved in osseointegration including human mesenchymal stem cells, osteoblasts and endothelial cells**.** Taken together it is hoped that the work in this thesis will bring continued breakthroughs in

implant technology research which will lead to translational clinical applications for improved implants of the future.

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Chapter 1

Nature and Scope of Thesis

Orthopaedic implants form a diverse group of applications and designs. This is further compounded by the range of biomaterials available with attendant issues affecting success and survivorship. The successful application of an orthopaedic implant depends on the complex interplay of a number of factors. Technology and research in this area thus need to consider all these complex factors in coming up with improved function and outcomes for the future. Within the ambit of this thesis however, we shall be mainly focusing on the implant itself.

The theme of this project is to promote interaction between biological systems and implant materials by using novel applications of functionalization of material surfaces with bioactive factors so as to confer materials with bioactivities that can potentially enhance their results and longevity. The choice of materials selected in this project include metallic alloys and bioactive factors that can improve bone-implant anchorage and osseointegration. The specific aims of the study are:

1) To develop stable and non-cytotoxic strategies to enhance the bioactivity and biointegration of the implant materials.

2) To evaluate the modified implant materials in terms of the stability of the functionalized groups and for cytotoxicity.

3) To assess biological effects of the functionalized implant material.

In Chapter 2, a general overview of the musculoskeletal burden and the different categories of orthopaedic implants and the reasons for implant failure will be discussed.

Joint replacement implants are among the most important orthopaedic devices in use today and accounts for more than half of the orthopaedic implant market [1, 2], therefore current development and evolution in one of the most popular and extensively used joint implant, 'The total hip arthroplasty implant' will be reviewed [3, 4]. In addition tissue-implant response, current strategies and research in enhancing orthopaedic implants will be examined.

In chapter 3, surface immobilization strategies were developed and assessed for the functionalization of Titanium substrates with vascular endothelial growth factor (VEGF). The binding properties, efficiency, release profile and cytotoxicity of each of the various techniques of surface functionalization namely A) physical adsorption, B) cross-linking and C) covalent binding with VEGF were analyzed. From the investigation a viable technique was chosen which would provide us with the most efficient, facile and cost effective method of attaching bioactive molecules to implant materials without the risk of cytotoxicity and undesirable effects.

In chapter 4, we further characterized the effects of Titanium substrates functionalized with VEGF via the chosen technique in terms of surface composition, topography and bioactivity. X-ray photoelectron spectroscopy (XPS) was used to track the surface composition at each stage of surface functionalization. Surface topography was checked with atomic force microscopy (AFM). Cellular functions in terms of cellular adhesion and proliferation were assessed with human dermal microvascular endothelial cells (HDMECs). Biological activity of the immobilized VEGF was evaluated with *in vitro* angiogenesis assay using matrigel and immunofluorescent staining with von Willebrand Factor (vWF) and PECAM1 (CD31). The results of the study indicate new

possibilities for the use of such functionalized surfaces for implanted medical devices. The coating of a biomimetic polymer film onto Titanium substrate followed by VEGF conjugation provides a means for applications where revascularization around implants would be beneficial. This may serve as a model for the immobilization of other bioactive factors onto various different types of metallic substrates.

In chapter 5, using the methodology developed in chapter 4, we investigated if this technique is viable in another metallic alloy with a different bioactive factor. In this instance Cobalt Chromium and bone morphogenetic protein 2 (BMP2) was evaluated. The binding properties, efficiency, release profile and cytotoxicity were analyzed. XPS was used to verify the successful grafting procedures at various stages of surface functionalization. Cellular functions with osteoblastic cells were assessed measuring alkaline phosphatase (ALP) activity and calcium mineral deposition. The results from this study showed that the immobilization of BMP2 on Cobalt Chromium has the ability to confer enhancement of cell-implant interactions, promoted bone matrix formation and bone growth. Thus the immobilization of BMP2 on Cobalt Chromium implants may be beneficial in the establishment of a direct interface between the bone and the implant without intervening fibrous tissue layer which may provide a promising means for enhanced osteogenesis.

In chapter 6, we sourced for an alternative molecule as a viable replacement for BMP2. BMP2 has the shortcomings of needing a huge quantity and a high immobilized density to elicit a response. It is also expensive and has a short shelf life. Hence a synthetic peptide CKIPKASSVPTELSAISTLYL with a cysteine amino acid at the N-terminus was produced. A comparison of the binding efficiency and dosage response of

the peptide versus BMP2 was evaluated. Surface topography was evaluated from the surface profile determined by AFM. Cellular functions, morphology, viability, ALP assay, alizarin red staining and BMP signaling via smad-dependent pathways were assessed. The study showed that the usage of the synthetic peptide in implant functionalization is a cheaper and viable alternative compared to BMP2, especially in instances where costs may be prohibitive.

Chapter 7 gives the overall conclusion of the current work done and Chapter 8 gives some recommendations for possible future work.

Chapter 2

Introduction

2.1 The Musculoskeletal Burden

Musculoskeletal disorder is a major public health concern and accounts for 33% of all cases of disability [5]. Orthopaedic research especially in the fields of biomedical engineering is an important tool for treatment. However long term outcome is not guaranteed and 10% of patients would need to undergo revision surgery at 10 to 15 years and the instances increases to 30% for heavier and younger patients [6]. Revision surgery is a complex procedure, costly to perform and leads to prolonged hospitalization with high health and social costs. In addition the outcome may not be as good as the first replacement due to extensive surgery required for the procedure and higher patient morbidities (longer surgery, more blood loss, etc.). The most common cause for revision surgery is due to aseptic loosening where the bone fail to grow into the surface of the implant [7]. Biological failures continue to prevent true longevity of orthopaedic implants, hence innovation is needed to reduce the necessity for revision surgery surrounding this common orthopaedic procedure.

The orthopaedic implant sector forms a significant portion of the worldwide biomedical industry. In the US alone, the orthopaedic implant market was estimated at over US$14 billion in 2008, and this is projected to rise to US$23 billion by the year 2012 [8]. Within this large and diverse field of orthopaedic surgical practice, there are four major implant applications: reconstructive joint replacements, spinal implants, orthobiologics and trauma implants. Of these joint replacement implants are among the most important orthopaedic devices in use today and accounts for more than 50% of the

orthopaedic implant market [1, 2]. The clinical need is anticipated to continue to grow for the foreseeable future, boosted by local and worldwide ageing populations, as well as increasing prevalence of physically active lifestyles and higher expectations of quality of life in older age groups.

2.2 Orthopaedic Implants

Orthopaedic implants are medical devices used for the treatment of musculoskeletal diseases. They are used for fixation of bones and to replace damaged joints. Generally they are available for the elbow, shoulder, hip and knee. Orthopaedic implants may consist of a single type of biomaterial or comprise a number of different biomaterials working together in modular parts, such as in a total hip replacement system which may contain up to three or more different materials such as polymethylmethacrylate (PMMA or bone cement), Cobalt Chromium and Titanium alloy. Prime examples of widely-used orthopaedic implants would include prosthetic hip and knee replacements for various types of arthritis affecting these joints, spinal fusion instruments for stabilizing degenerate and unstable vertebral segments, and fracture fixation devices of various types such as plates, screws and intramedullary rods. Less common implants in which the technology may still be in varying phases of maturity, as well as those which are in development but may not yet be established in clinical usage, would include other joint replacements such as for shoulder, ankle, elbow and small joints, artificial vertebral disc replacements, and orthobiological implants such as artificial scaffolds for osteochondral defects and knee meniscal implants.

Despite the large number of orthopaedic medical devices in use today, they are predominantly make up of only a few metals and polymers. Metallic alloys such as Cobalt Chromium and Titanium continue to be one of the most important components used in orthopaedic implant devices. They have favorable properties of high corrosion resistance, strength, rigidity and fracture toughness. Cobalt Chromium alloys are used in bearing surfaces due to their hardy surfaces that resists abrasive wear and very high corrosion resistance [2]. However Cobalt Chromium alloy is known to have much less potential for osseointegration. Titanium alloys are used in non-weight-bearing surface components such as femoral necks and stems as they have lower modulus of elasticity, resulting in less stress shielding of bone [9]. The presence of a naturally formed oxide layer on the Titanium surface also increases its biocompatibility and bioactivity [10]. Nonetheless the osseointegrative bioactivity is still often not sufficient to attain true adhesion between the implant and bone, which may ultimately lead to mechanical instability and implant failure [11].

Reasons implant may fail

Orthopaedic implants developed by various manufacturers have different designs. Each manufacturer have their own differing theories on implant designs for specific orthopaedic applications. Generally there are certain guiding principles that will affect the ultimate viability of an implant. The design of the implant has to take into account biomechanical and biological factors that may affect its success. Conformity to native anatomy, material properties and mechanical strength appropriate for the targeted function and environment are some of the considerations that come into play.

Despite the benefits and successes of these medical devices, their use are not without risk of adverse effects. Success in the application of an orthopaedic implant would depend on various factors and implants may fail due to physiologic reasons such as infection, loosening, dislocation and patient-related factors.

Infection

The presence of a large foreign object in the body can serve as a surface for the bacteria to latch onto. Infection is caused by the susceptibility of the implant material to infection by inoculated or circulating bacteria, which can be impossible to eradicate without implant removal. The tissue that has been operated on has an altered blood supply which may be inadequate to combat infection. More importantly a chronic fight against infection would endanger the life of the patient. However with current antibiotic regimens and surgical techniques, the risk of infection is moderately low [12].

Loosening

When implants were placed in they were intended to stay fixed for a long time and bone was expected to grow into the surface of the implant. Unfortunately this does not always happen and leads to micromotion and the generation of wear particles on the surfaces of the implant [13]. Eventually this causes aseptic loosening as the bonds of the implant to the bone are destroyed by the body's attempts to digest the wear particles. When this occurs the prosthesis becomes loose and the patient may experience instability and pain.

Dislocation

Dislocation occurs when an implant become misaligned or is displaced from its normal position. The rate of dislocation is roughly about 10% [14] and may be caused by inadequate soft tissues, incompatibility issues, bony or scar tissue impingement and loosening.

Patient-related factors

Heavier and obese patients have a higher chance of wear and loosening. Young and active patients have a higher incidence of revision [6]. Patients with medical history such as having a previous hip fracture, arthritis and avascular necrosis are also at a higher risk as well.

2.3 Joint Replacement Implants

The total hip arthroplasty which is one of the main joint implants in use has been named the operation of the century [3]. Total hip arthroplasty implants typically consist of a stem, femoral head and an acetabular cup, and are used to replace damaged natural bearing surfaces in patients.

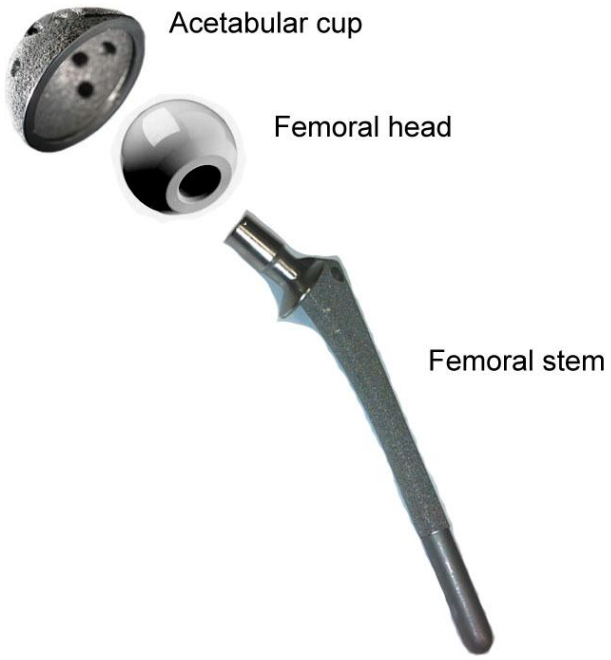


Figure 2.1 Diagram of a typical hip implant. Acetabular cup and femoral head is usually made of Cobalt Chromium for the bearing strength, while the femoral stem is usually composed of Titanium for the biocompatibility.

Developments and evolution in hip implants

Throughout their evolution, total hip arthroplasty implants have incorporated metal-on-metal, ceramic-on-ceramic and metal-on polyethylene designs, each with its own distinct advantages and unique drawbacks. New combinations and improvements in

materials are being developed, however at present there is still no clear winner in the search for the perfect bearing surface material combination.

Metal-on-polyethylene implants

From the early to mid 20th century, various different combinations of materials were being explored as candidate bearing surfaces for total hip arthroplasty. High-density polyethylene was first used clinically by Sir John Charnley and this bearing material dominated the total hip replacement implants in the 1970s. First-generation results of metal-on-polyethylene hip arthroplasty showed impressive long-term results of 77% to 81% survivorship at 25 year follow up [15, 16]. However a new complication was arising, presenting as insidious hip pain and appearing on radiographs as massive bone lysis resembling metastatic malignancy [17]. An outbreak of periprosthetic loosening took the orthopaedic world by surprise. Tissue examinations revealed an inflammatory response around the implant interface with macrophages displaying minute particles embedded in them [18]. In the beginning these particles were thought to be bone cement, leading to the erroneous term "bone cement disease" being coined in 1987. Eventually it was ascertained that the problem was due to polyethylene wear particles stimulating a macrophage response, but not before the perceived problem with bone cement had given impetus to a new direction of development in hip arthroplasty: that of uncemented hip designs relying on biological fixation. The identification of polyethylene wear particles as the main cause in periprosthetic osteolysis in turn led to a resurgence in interest in alternate bearing combinations. Another impending issue is that younger, active patients as opposed to their older and more sedentary counterparts inflict as much as 40 fold

greater wear on their hip joint bearing surfaces [19]. Therefore alternative bearing surfaces such as ceramic-on-ceramic or metal-on-metal (i.e. hard on hard) as compared to metal on polyethylene (hard on soft) are being developed to address these issues.

Ceramic-on-ceramic implants

In 1970s, the first ceramic-on-ceramic total hip replacement was introduced by Pierre Boutin [19]. However during then there were problems as the material quality was not as good as it is today and strengths were much lower. Recent improvements over the years have now created ceramic powders with inherently higher strength. The ceramic-on-ceramic hip replacement implants are made from aluminum oxide ceramic. Ceramic components are extremely wear resistant and have much smaller debris particles compared to that of metal-on-polyethylene and metal-on-metal bearing surfaces. Ultra-smooth surfaces, hardness and very low wear rates have popularized ceramic as a choice for younger patients so as to delay or prevent the need for revision surgery. However the use of ceramics too, have risks. They are more fragile than metals or polymers and as such are more vulnerable to fracture and consequently an implant failure of catastrophic proportions [19].

Metal-on-metal implants

Metal-on-metal total hip replacements were first used in the 1930s, and further developed by pioneering surgeons like McKee and Ring in the 1950s and 1960s [20]. The early generations suffered from inferior material quality, poor fixation and generally had high device failure rates. As a result the better performing metal-on-polyethylene

prostheses supplanted metal-on-metal designs as the preferred bearing surface for several decades. In the past decade, improved industrial fabrication and manufacturing processes have incorporated stronger, more wear resistant metallic alloys such as Cobalt Chromium and Titanium into hip implant designs which have spurred a renewed interest in the metal-on-metal hip prostheses. Use of metal-on-metal hip implants has been further bolstered by their more-recent success in hip resurfacing applications. Hip resurfacing was developed as an alternative to total hip replacement. Hip resurfacing entails reshaping the patient's natural femoral head and then capping it with a matching metal cup rather than replacing the entire joint. Long-term follow-up of implants using metal-on-metal bearings showed good survival and wear resistance without the problems associated with polyethylene bearing surfaces [21]. Polyethylene bearing surfaces have high wear rates in the region of 0.1 mm per year compared in contrast to 0.004mm per year for metal surfaces. Unlike ceramics, metal is also ductile, not brittle, hence, implant sizes can be kept thinner without risk of implant fracture. Thus, for a given acetabular shell size, a large head diameter can be used, which provides enhanced joint stability and a large range of movement before the neck impinges on the socket. Another advantage of metal-on-metal bearings is that they are also self-polishing, allowing for self-healing of surface scratches [20].

2.4 Tissue-Implant Response

All implant materials will elicit some response to the host and none are truly considered inert [22]. The response generally occur at the bone-implant interface and is dependent on various factors. The most important factors in implant studies would include the surface topography, intrinsic properties of the implant and any implant-mediated biological reactions [23].

Generally there are 2 types of implant-tissue responses [24-26]. The first type is the response of the hosts' tissues to the toxicity of the implanted material. Implanted material may be toxic or releases chemicals that damage the surrounding tissues. The second response which is also the commonest is the formation of a nonadherent fibrous capsule between the implant and the hosts' tissues. This is a natural response to protect the body from a foreign object which may eventually lead to complete fibrous encapsulation [27]. Metallic alloys and polymers when implanted into bone may be surrounded by both bone and fibrous tissue. Under ideal conditions osseointegration would be desired, however the instances of the development of a fibrous capsule is high and will eventually lead to implant loosening and extrusion.

2.5 Current Research in Enhancing Orthopaedic Implants

Two types of fixation are usually used to hold the implants in place. Cemented fixation would use PMMA to anchor the implant in place while cementless fixation (direct biological fixation) would rely on bone growing into the surface of the implant achieving solid fixation. Patients will not be able to feel the difference between the two types of fixation, and the fixation technique chosen would be based on the bone stock quality, age and the demands of the patient. Controversy still exists regarding the optimal method of fixation, although traditionally the method of fixation of an implant to bone has relied on the use of bone cement. However due to the problems of implant loosening and loss of bone stock observed especially in younger and more active patients there is a shift from cemented fixation to direct biological fixation. The idea of implanting prostheses without bone cement and eventually becoming a part of the body with complete incorporation is becoming more popular. Nonetheless the comparative outcomes, advantages, disadvantages and preferred indications of cemented versus direct biological fixation remain unresolved. The superiority of either fixation technique is still not established.

Bone-implant interface

So far most research efforts have been concentrated on improving the bone-implant interface, with the aim of enhancing bone healing and implant integration via either physical or chemical approaches [28]. The physical approach is focused on the modification of the implant surface morphology and topography using mechanical methods such as machining, acid-etching, plasma spraying, grit-blasting and anodization

to improve the microtopography of the surface. The rationale behind this is that an increase in surface roughness of the implant material would provide a higher level of surface energy which would improve bone anchorage, matrix protein adsorption, osteoblasts functions and ultimately osseointegration [29].

The chemical approach is towards the creation of a bioactive implant surface via application of coatings onto the implant layer by biochemical and physicochemical techniques. In biochemical techniques, organic molecules such as growth factors, peptides or enzymes are incorporated to the implant layer to affect specific cellular responses [30]. While in physicochemical techniques, the incorporation is achieved with inorganic phases such as calcium phosphate which may increase the biochemical interlocking between bone matrix proteins and surface materials thereby enhancing bone-bonding [29]. Many implant modifications may combine both physical and chemical engineering methods, in the following sections we will discuss some of the more popular strategies used to enhance implant integration and bone-bonding.

Inorganic coatings

Calcium phosphate coating has been widely used in the orthopaedic field due to their similarity with the mineral phase of bone [31] and are known for their bioactive properties which are beneficial in bone-bonding [32]. As calcium phosphate generally lack the mechanical strength for use as bulk materials under loaded conditions, they are often coated onto the surface of metallic implants. There are several studies published which have shown the favorable use of calcium phosphate coatings in increasing bone-implant interface, implant anchorage and integration [33]. The calcium phosphate layer

functions as a physiological transition between the implant surface and the hosts' tissues which guides bone formation along the implant surface and the surrounding tissues. One of the most successful method for the application of calcium phosphate coatings is via the plasma-spraying method due to its advantage of extensive coating capability and high deposition rate. However despite numerous findings [34] that report the beneficial osteoinductive properties of plasma-sprayed calcium phosphate coatings, there are still some concerns regarding its use. Plasma-sprayed coatings are not uniform and there is poor control over thickness and surface topography, which may result in implant inflammation when particles are released from these heterogeneous coatings. To overcome these drawbacks, various other deposition strategies have been developed and employed such as biomimetic deposition, electrophoretic deposition and electrospray deposition etc. However care should be taken when comparing the efficacy of each of these methods which would require a comprehensive evaluation of both biological response and clinical performance. Although calcium phosphate coatings have been shown to be beneficial in enhancing bone-bonding, there is still no general consensus on the use of calcium phosphate coating systems. The main problems include large variation in the quality of calcium phosphate coatings, even between different batches and market forces which offer other cheaper alternatives [35].

Organic coatings

Surface modification of implant materials with growth factors and peptides is gaining popularity in the recent years [36, 37]. Various therapeutic biomolecules of interest can be immobilized onto implant surfaces to enhance the bone-implant interface interactions. Currently more popular approaches would include the immobilization of

bone growth factors such as bone morphogenetic proteins (BMPs) to enhance osteogenesis and the deposition of peptide sequences to induce specific cellular functions. Growth factors immobilized on orthopaedic devices have been reported to enhance osteoblastic activity and favor implant integration [38]. The most commonly used growth factors in orthopaedics are members of the transforming growth factor beta (TGF-β) superfamily including the BMP family, especially BMP2 and BMP7. Growth factors may be physically adsorbed or covalently grafted onto the implant surface and various studies have shown that the loading of implant with these factors can enhance interactions at the bone-implant interface and aid the remodeling process ultimately improving implant integration [39-41]. However critical factors in the successful use of growth factors in orthopaedic devices are the optimum dosage, exposure period and release kinetics, all have to be considered carefully to avoid the detrimental effects associated with growth factor use such as high initial burst rate, ectopic bone formation and short half-life. More recently, peptide sequences with the ability to target specific osteogenic cellular functions of differentiation and mineralization have been developed [42, 43]. These short functional fragment derived from the original protein have increased shelf life, can be synthetically produced and are more resistant to denaturizing effects. Their usage would provide significant clinical benefits over the use of conventional proteins. They can be linked to the implant surface to provide biological cues for bone formation. Additionally other peptide sequences in use include the RGD, YIGSR, IKVAV and KRSR which have been used to improve cellular adhesion and bone matrix formation [44-46].

Organic–inorganic composite coatings

Research in the recent years have concentrated on the development of bioactive composite coatings which mimics the structure of the bone tissue. These composite coatings would combine calcium phosphate with growth factors, peptides, antibodies etc. to enhance interactions at the bone-implant interface. However due to the fact that often high temperatures or non-physiological conditions are needed in the preparation of calcium phosphate coatings, only physical adsorption is employed in deposition of the biomolecules on the implant surface [47, 48]. However with physical adsorption techniques, initial high burst rate is often observed, which is not desired [49]. Therefore coating techniques that create a gentle sustained release kinetics are preferred. A recently published paper have shown that calcium phosphate coating combining slow release of antibiotics, aids in early success at recruitment of bone cells [50]. Many other studies have shown that depositing BMP2 and TGF-β onto the implant surface would greatly enhance bone-bonding at the bone-implant interface [38, 47]. The biological efficacy of orthopaedic implants can be improved greatly by both physical and chemical modifications. The use of a wide multitude of engineering techniques in the manipulation of surface topography, morphology and incorporating the use of various inorganic and organic components would directly influence the response in the local bone-implant interface and the apposition of new bone. With the development of new techniques and strategies on composite coatings to better mimic the human bone structure this would result in a new generation of orthopaedic implants with improved implant integration and bone healing.

Summary

Despite the successes of orthopaedic implants, their usage are not without risks and the most common cause of implant failure is due to aseptic loosening. Aseptic loosening alone causes about 60 to 70% of the cases for revision surgery and the reason is due to sub-optimal osseointegration [51, 52]. Strategies to enhance osseointegration include the use of surface modification techniques to enhance implant integration with bone and to induce acceleration of the bone healing phenomena. Many attempts to activate the surface have been made based on the control of surface topography, surface energy and biological cues [53]. Therefore to increase the success of implant integration and to reduce revision surgery, our study will investigate surface modification of metallic implant materials with biomimetic coatings immobilized with therapeutic biomolecules of interest to enhance bone-implant interface interactions. The specific metallic implant materials and bioactive factors investigated are as follows: 1) Metallic alloys (Titanium and Cobalt Chromium) which will provide the structural support for mechanical function, and delivery of growth factors. 2) Bioactive factors (VEGF, BMP2 and BMP Peptide) that will facilitate early angiogenesis and induce osteogenesis.

Chapter 3

3.1 Novel Strategies for Conferring Bioactivity to Implant Material

Despite the advances in current implant technology, there are still problems associated with their usage including loosening and tissue rejection. A variety of implant materials have been developed for orthopaedic applications however the results have not been fully satisfactory. The clinical strategies to manage musculoskeletal defects would center around three components: cells, structure and growth factors. For the design of implant materials, cells and proteins at the implant interface plays a critical role [54]. The utilization of biosignal proteins such as growth factors for development of bioactive implant materials holds great potential. Especially due to the scarcity of stem cells in the body, materials which regulates cellular functions such as adhesion, growth and differentiation are desired.

One promising way to incorporate growth factors usage with implant materials would be by surface immobilization of growth factors. Soluble growth factors work by binding with cognate receptors on cells to form complexes which would result in autophosphorylation of the cytoplasmic domains of the receptors and this phosphorylation activates intracellular signal transduction. The formed complexes are then aggregated and internalized into the cells by both clathrin-dependent and clathrin-independent mechanisms which leads to the recycling of the receptors for degradatory down-regulation [55]. Similarly immobilized growth factors work by forming complexes with the cell surface receptors, however the signal transduction is expected to last longer than soluble growth factors due to the inhibition of the internalization process. Multivalency is another important phenomenon responsible for this prolonged enhanced

mitogenic effect. Multivalent ligands interact and bind avidly to multiple surface cell receptors through several binding modes. This enhances the formation of ligand-receptor complexes which are critical for signal transduction and the multivalent ligands are able to stabilize and prevent lateral diffusion of the formed complexes leading to the prolonged effect. Figure 3.1 shows the interactions of cells with the different forms of growth factor and the cross relationship between dosage and mitogenic effects.

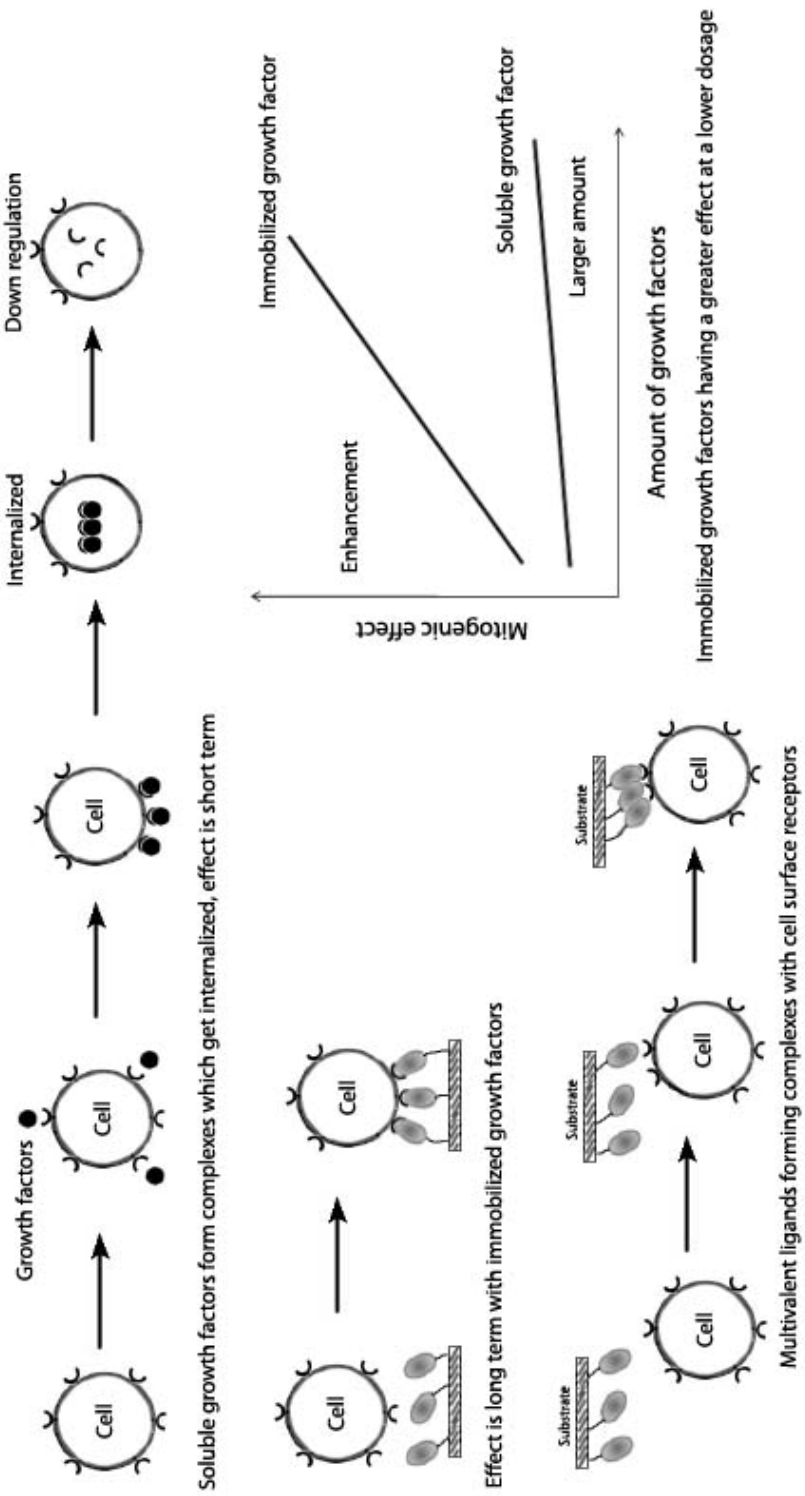


Figure 3.1 Effects of soluble growth factors compared to immobilized growth factors

In order to effectively derive the effect from immobilized growth factors, strategies have to be developed that can optimize the structure to elicit the desired biological response. One of the problems encountered with implant materials for surface functionalizaton is the lack of suitable chemical groups on the surface. For more versatility and applicability, the concentrations of the OH group and other reactive groups such as amino or carboxyl groups have to be increased. The initial organic layer immobilized on the implant materials can then be used as a tether for biomolecular components used to mediate cell attachment. Another issue which merits investigation is the control of the retention and/or release of the biomolecules from the implant surface. The easiest and most common method employed for delivery of biomolecules is physical adsorption, which unfortunately provides little control over the delivery and orientation of the biomolecules. Bonding of the biomolecules and use of coatings incorporating them would be alternative methods of delivery to the bone-implant interface. Regardless, the preferred and chosen immobilization technique would depend on the specific working mechanism of the biomolecules. Given the above scenario, chemical modification (functionalization) of biomaterials in order to enhance biocompatibility and promote osseointegration has great potential in addressing the problems of prosthetic joint implant longevity and survival.

Immobilization techniques

Immobilization techniques are broadly classified into four categories, namely a) physical adsorption (via van der Waals or electrostatic interactions), b) physical entrapment (use of barrier systems), c) cross-linking and d) covalent binding. The choice

of the technique would depend on the nature of the bioactive factors, substrates and its application. It will not be possible to have a universal means of immobilization, however developing a viable methodology which can provide for a facile, secure immobilization with good interactions for orthopaedic implants is vital.

Physical adsorption

This is the simplest of all the techniques available and does not alter the activity of the bioactive factors. Physical adsorption techniques are mainly based on ionic and hydrophobic interactions. If the bioactive factors are immobilized via ionic interactions, adsorption and desorption of the factors will depend on the basicity of the ion exchanger. A reversible dynamic equilibrium is achieved between the adsorbed factors and substrates which is affected by the pH as well as ionic strength of the surrounding medium. Hydrophobic interactions offer slightly higher stability with less loss of the factors from the surface of the substrates. Although physical adsorption systems are simple to perform and do not require extensive treatment to the bioactive factors and substrates used however there are certain drawbacks. These systems suffer from low surface loading and biomolecules may desorbed from the surface in an uncontrolled manner.

Physical entrapment

This method is employed with barriers including natural polymers like gelatin, agar and alginate entrapment systems. Other synthetic polymers employed include resins, polyurethane prepolymers etc. Some of the major limitations of the entrapment system is

the diffusional problem where there is possible slow leakage during continuous use due to the small molecular size of bioactive factors, and steric hindrance which may affect the reactivity of the factors. Recent development of hydrogels and water soluble polymers attempt to overcome these drawbacks and have attracted much attention from the biomedical field.

Cross-linking

Bioactive factors can also be immobilized through chemical cross-linking via homo- as well as heterobifunctional cross-linking agents. Among these glutaraldehyde cross-linking are the most popular due to its low cost, high efficiency and stability [56-58]. Glutaraldehyde is often used as an amine reactive homobifunctional crosslinker for biochemistry applications..

Covalent binding

Covalent binding is another technique used for the immobilization of bioactive molecules. The functional groups investigated are usually the carboxyl, amino and phenolic group of tyrosine. Bioactive factors are covalently linked through functional groups in the factors not essential for the bioactivity. The covalent binding should be optimized so as to protect the active site and not alter its conformational flexibility.

Therefore based on the above scenario, the objective in this part of the study was to devise a suitable methodology to immobilize VEGF onto the surface of Titanium alloy substrates (the reason for the choice of the growth factor and substrate will be explained in the following chapter). As physical entrapment is not suitable in this case of improving

the bone-implant interface via the surface of the implant material, therefore this system is not investigated. The binding properties, efficiency and cytotoxicity of each of the various functionalization procedures with VEGF were analyzed. We aim to use the above scheme as a means to develop an efficient and effective modification strategy to promote osseointegration and implant integration thereby reducing the need for revision surgery.

3.2 Materials and Methods

Materials

Ti-6Al-4V (denoted as Ti in the subsequent discussion) foils were purchased from Goodfellow Inc. of Cambridge, UK. Recombinant human VEGF was obtained from R&D Systems, US. The viscosity-average molecular weight was approximately 2.2 X 10^5 as determined by the viscometric method. The degree of deacetylation was 84% as determined by elemental analysis using the Perkin-Elmer Model 2400 elemental analyzer [10]. 3,4-dihydroxyphenylalanine (dopamine) and glutaraldehyde were obtained from Sigma-Aldrich Chemical Co. Ultrapure water (>18.2 MG cm, Millipore Milli-Q system) was used in the experiments. The CellQuanti-MTT^{TM} cell viability assay kit was purchased from BioAssay Systems. Endothelial Cell Growth Medium MV Bulletkit CC-3125 were purchased from Lonza Walkersville, Inc.

Preparation of substrates

Ti foils (0.52 mm thick) were cut to a size of 1 cm × 1 cm. The substrates were polished using 600 and 1200 grid sandpaper and then sonicated for 10 min in water. The carbide deposited during polishing were removed by sonicating the substrates in Kroll's reagent (4.0% HF, 7.2% HNO_3, 88.8% water) for 10 min [59]. The reaction was terminated by the addition of 1 N sodium hydroxide. The substrates were then cleaned ultrasonically for 10 min each in dichloromethane, acetone, water and placed in 40%

HNO_3 for 40 min for surface passivation. The acid-treated substrates were then rinsed thoroughly with water.

Figure 3.2 Substrates preparation to 1 cm by 1 cm and placing them in 24 well plates for subsequent experiments.

Energy-dispersive X-ray spectroscopy (EDX)

Unmodified Ti substrates were coated with platinum and the surfaces of the substrates were analyzed using a scanning electron microscope equipped with energy-dispersive X-ray spectroscopy (SEM-EDX) (JEOL, model 5600LV).

Cell culture

HDMECs (Lonza Walkersville, Inc) were cultured in endothelial cell growth medium MV and passage 5 HDMECs were used for the analysis of cell viability assay. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 with the growth medium changed every 2-3 days.

Physical Adsorption

Ti substrates were coated with VEGF in 1 μg/ml concentration dissolved in a mixture comprising 0.1% gelatin and 1% low-melting agarose in the ratio 1:1, and allowed to dry at room temperature overnight. The substrates were then gently rinsed with PBS and allowed to dry. A second thin coating of the gelatin/agarose mixture was then layered over each substrate. The substrates were rinsed with PBS and allowed to dry before use. The substrates are denoted as Ti-VEGF in the subsequent discussions.

Cross-linking

Dopamine was anchored to the surface of the Ti substrates by immersing in a 1mg/ml aqueous solution of dopamine overnight in the dark. The substrates were then thoroughly rinsed with ultrapure water to remove the unattached dopamine and dried under nitrogen flow. Subsequently, the substrates were immersed in a stirred 3% aqueous solution of glutaraldehyde (pH=8.0) at room temperature overnight. Glutaraldehyde acts as a cross-linking agent by providing the reactive aldehyde groups for bonding with dopamine and the free amino groups of VEGF [60, 61]. The substrates were rinsed with water to remove unbound glutaraldehyde. The glutaraldehyde-treated substrates were

then coated with VEGF in 1 μg/ml concentration and allowed to air dry in a sterile environment. Following which, the substrates were rinsed 3 times with sterile PBS to remove unattached VEGF and left to air dry in a sterile environment before use. The substrates are denoted as Ti-GLU-VEGF in the subsequent discussions.

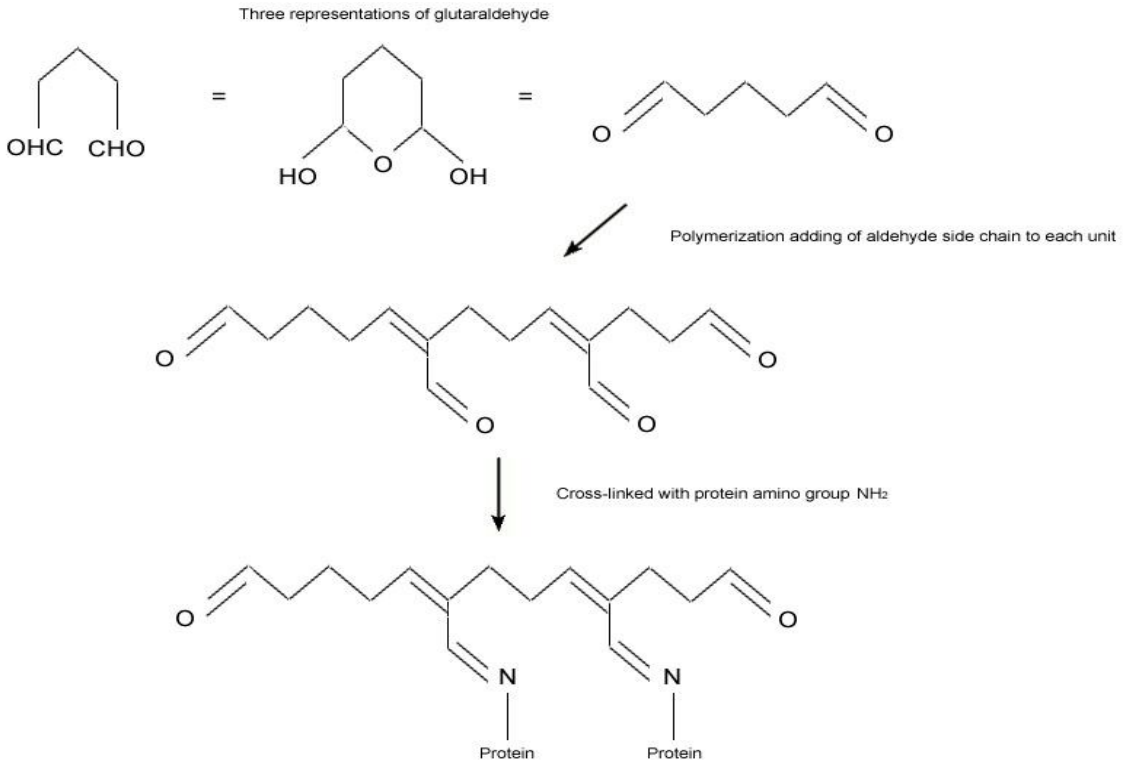


Figure 3.3 Schematic diagram showing glutaraldehyde cross-linking with proteins.

Covalent binding

Polydopamine was anchored to the surface of the Ti substrates via immersion in a 2 mg/ml solution of dopamine (10mM Tris buffer, pH=8.5) overnight in the dark. Under alkaline conditions, dopamine self-polymerize to form a thin layer on the surface of the Titanium substrates via strong intermolecular interactions [62, 63]. The substrates were then rinsed with copious ultrapure water to remove the unattached dopamine and dried

under nitrogen flow. The polydopamine-grafted Ti substrates were then coated with VEGF in 1 μg/ml concentration dissolved in deoxygenated 10mM Tris buffer, pH=8.5 and incubated overnight in a humid atmosphere at room temperature. Polydopamine coating is a very complex surface having its own pH-dependent behaviour and its precise chemical composition is still unknown [62, 63]. Two functional groups catechol and quinone are present on the polydopamine coating, and under alkaline conditions, latent reactivity is shifted towards the quinone groups [62] which is expected to react with the amine groups on the VEGF molecule. The substrates were then washed three times with sterile PBS to remove unattached VEGF and left to air dry in a sterile environment before use. The substrates are denoted as Ti-PDOP-VEGF in the subsequent discussions.

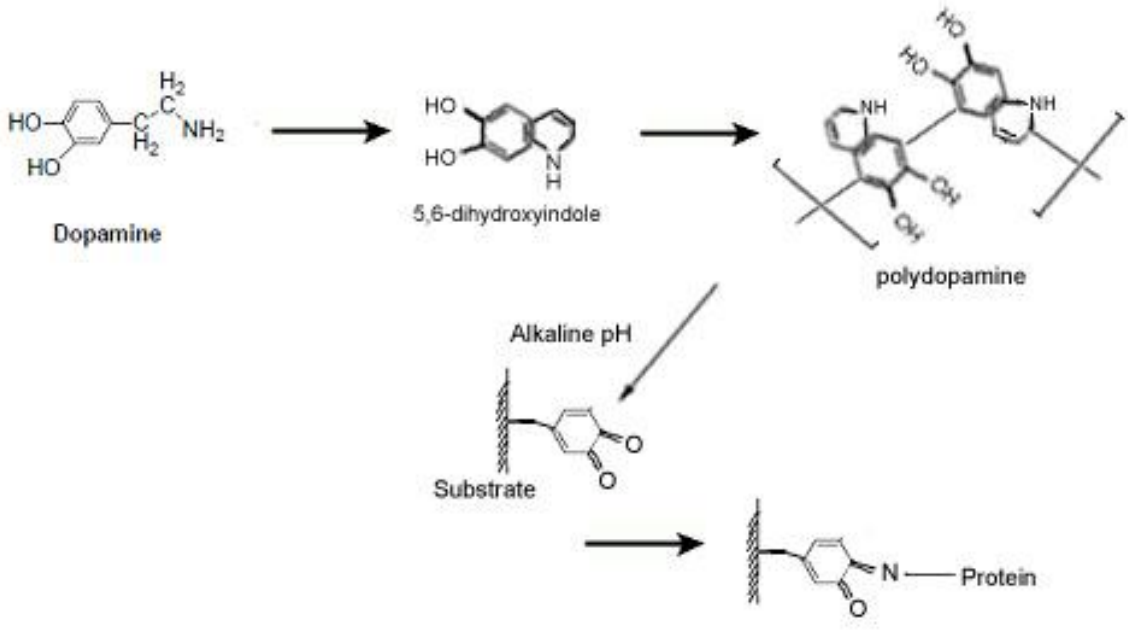


Figure 3.4 Schematic diagram showing polymerization of dopamine under alkaline pH and the equilibrium shift towards the quinone groups for reactivity with amine groups of the proteins.

Characterization

The chemical composition of the surfaces was analyzed by X-ray photoelectron spectroscopy (XPS) on an AXIS HSi spectrometer (Kratos Analytical Ltd, UK) with an AlKα X-ray source (1486.6 eV photons). All binding energies (BEs) were referenced to the C 1s hydrocarbon peak at 284.6 eV.

Binding efficiency

To determine the binding efficiency of the surface functionalization procedures, the quantity of VEGF in the loading solution and the combined washing solution was performed using an enzyme-linked immunosorbent assay kit (VEGF ELISA, R&D System, Minneapolis, MN) according to the manufacturer's instructions. The surface density of bound VEGF was calculated from the difference between the initial and remaining VEGF in the washing buffer.

Cytotoxicity assay

After 1 week of culture, the viability of HDMECs on the Titanium substrates was assessed with MTT assay. MTT assay is based on the conversion of yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan by metabolically active cells. The insoluble purple formazan product is then dissolved in a solubilization reagent into a coloured solution. The absorbance of the resulting coloured solution was quantified by measuring at a wavelength of 570 nm by a spectrophotometer. Briefly, a suitable quantity of MTT reagent was added to the cells at day 7 of culture on the different substrates and then incubated for 4 hours at 37 ^{o}C. A suitable amount of

solubilization solution was added to respective cultures and then gently mixed on an orbital shaker for one hour at room temperature. The intensity of the colour in each well was measured at 570nm on an absorbance plate reader.

VEGF release overtime

To measure the amount of VEGF being released into the culture medium, Ti-VEGF, Ti-GLU-VEGF and Ti-PDOP-VEGF substrates were subjected to soaking in PBS for 30 days at 37 °C and 7 time points were measured.

Statistical analysis

At least three samples per time point for each experimental condition were used. The data were tested for normal distribution by Shapiro-Wilk test. One-way analysis of variance (ANOVA) *post-hoc* Tukey test was used to assess the normally distributed data and the results are reported as mean ± SD. Statistical significance was accepted at $P < 0.05$.

3.3 Results

SEM-EDX characterization

SEM-EDX was used to characterize the Ti substrates obtained from the manufacturer to check for the uniformity and quality of the alloy obtained. From the results (Fig. 3.5) more than 90% of the substrates consist of the element Ti and is uniformly distributed which conforms to the requirements in our studies.

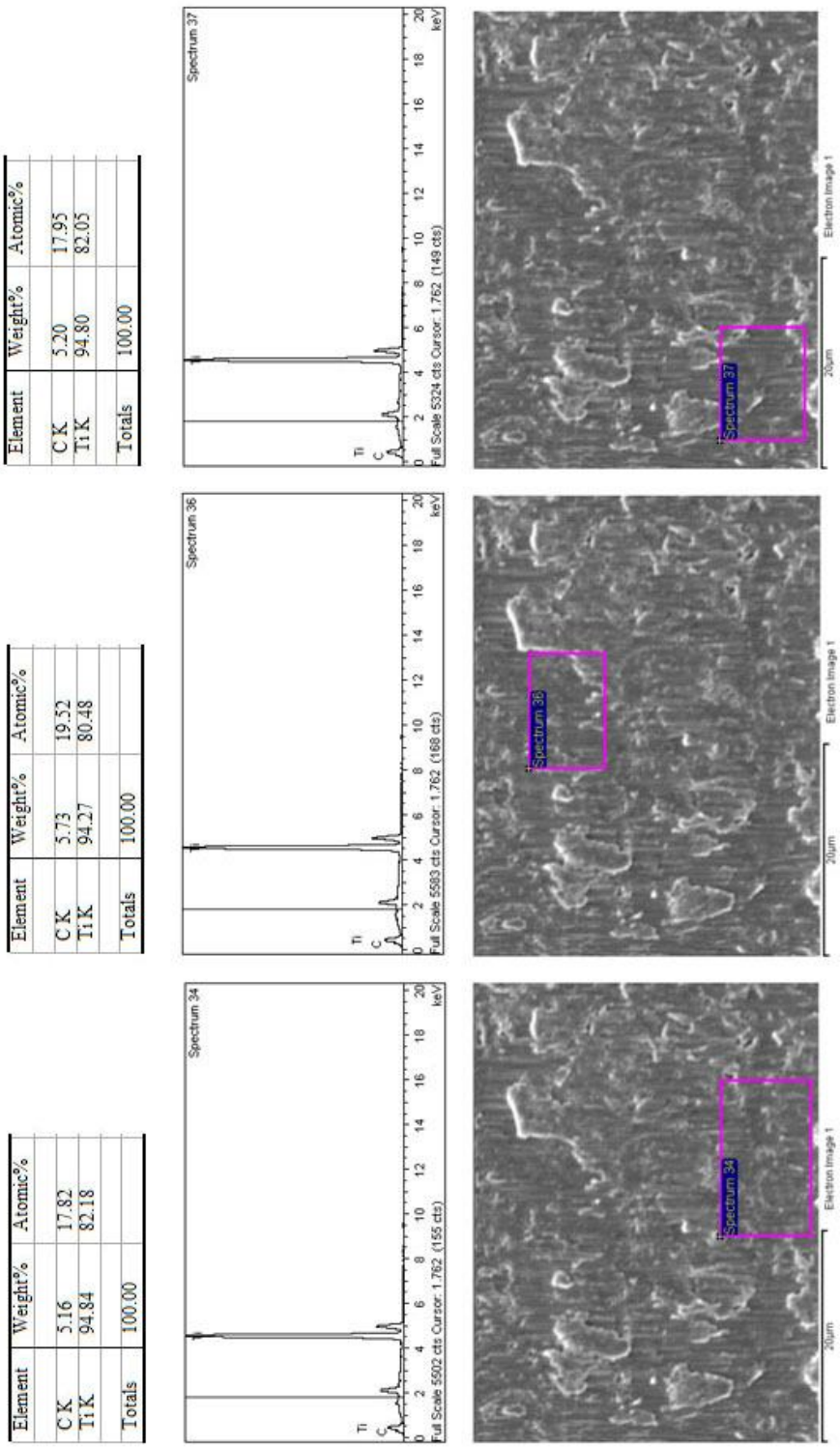


Figure 3.5 Elemental analysis of unmodified Ti substrates from three different regions.

Surface characterization

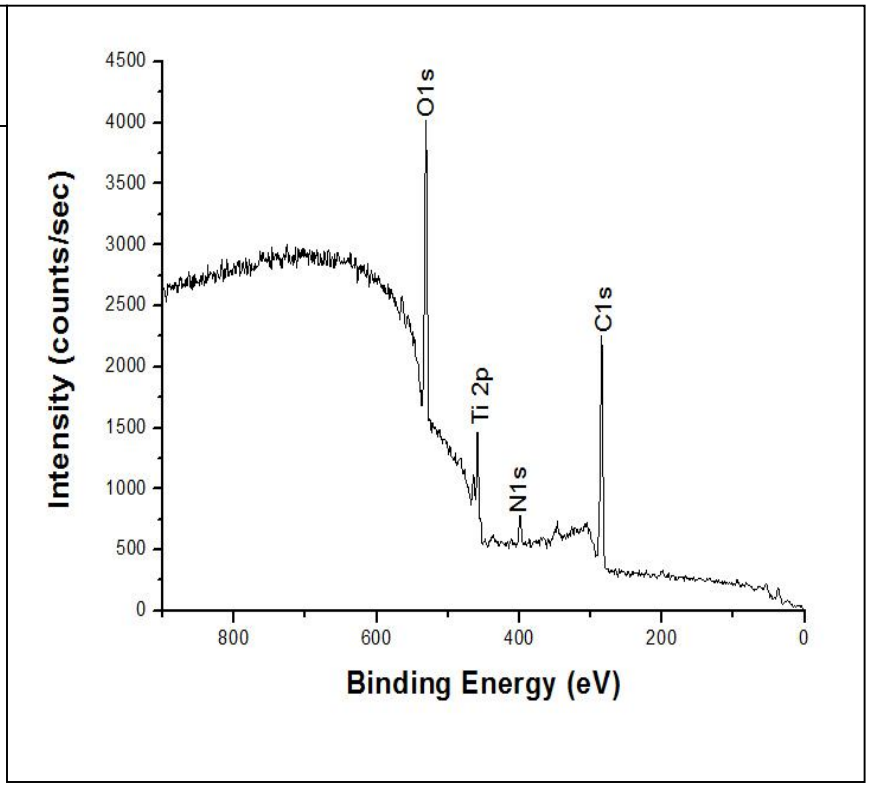
The XPS wide scan spectra of the (A) pristine Ti, (B) Ti-VEGF, (C) Ti-GLU-VEGF and (D) Ti-PDOP-VEGF and their corresponding surface elemental compositions are shown in Fig. 3.5. and Table 3.1. In the wide scan spectrum of the pristine Ti (Fig. 3.6(A)), the predominant components are C 1s (285 eV), Ti 2p (460 eV) and O 1s (530 eV). Successful anchoring of dopamine and glutaraldehyde was indicated by a increase in the C 1s and N 1s peak intensities and a concomitant decrease in the Ti 2p peak intensity (Figure 3.6(C,D)), while successful deposition of the growth factor on the Ti substrate was indicated by an increase in the N contents. The changes in the surface atomic ratio of the functionalized substrates are quantified and summarized in Table 3.1.

Substrate	C%	N%	O%	Ti%
Pristine Ti	35.3	1.7	49.6	13.4
Ti-VEGF	32.3	8.0	48.2	11.5
Ti-GLU-VEGF	68.7	12.0	19.3	-
Ti-PDOP-VEGF	69.1	11.1	19.8	-

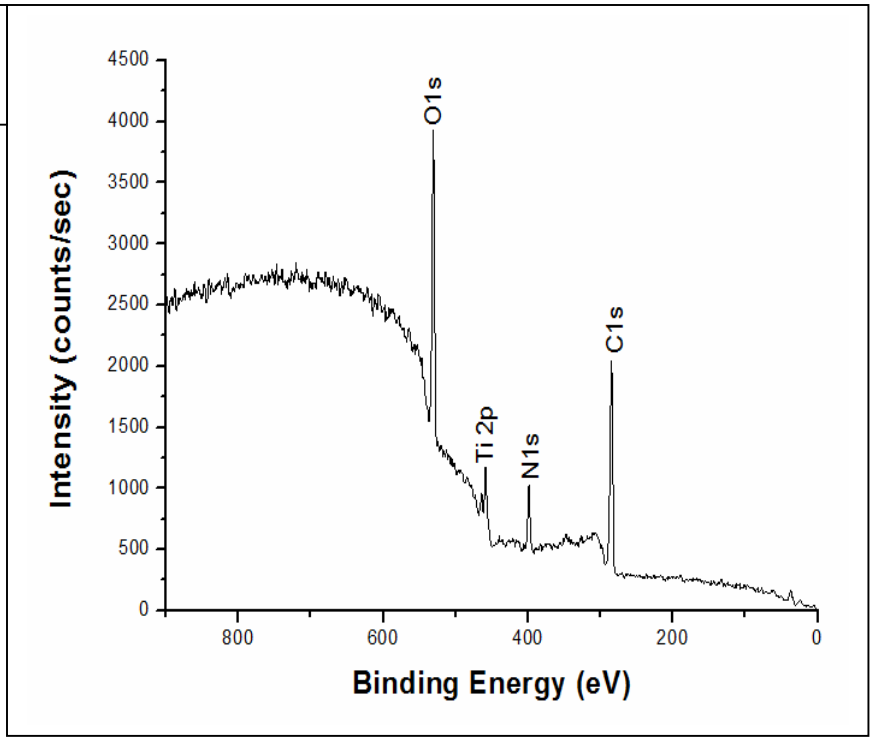
Table 3.1 Elemental Composition* at the Surface of Pristine, Ti-VEGF, Ti-GLU-VEGF and Ti-PDOP-VEGF substrates as Determined by XPS.

*Percentages computed based on the C, N, O and Ti contents only.

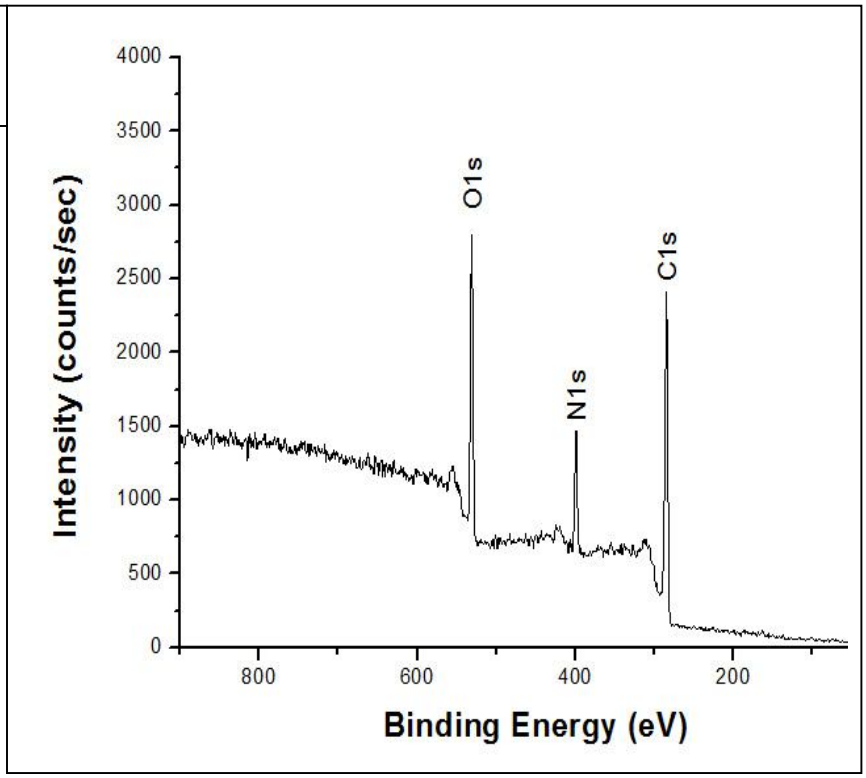
(A) Pristine Ti



(B) Ti-VEGF



(C) Ti-GLU-VEGF



(D) Ti-PDOP-VEGF

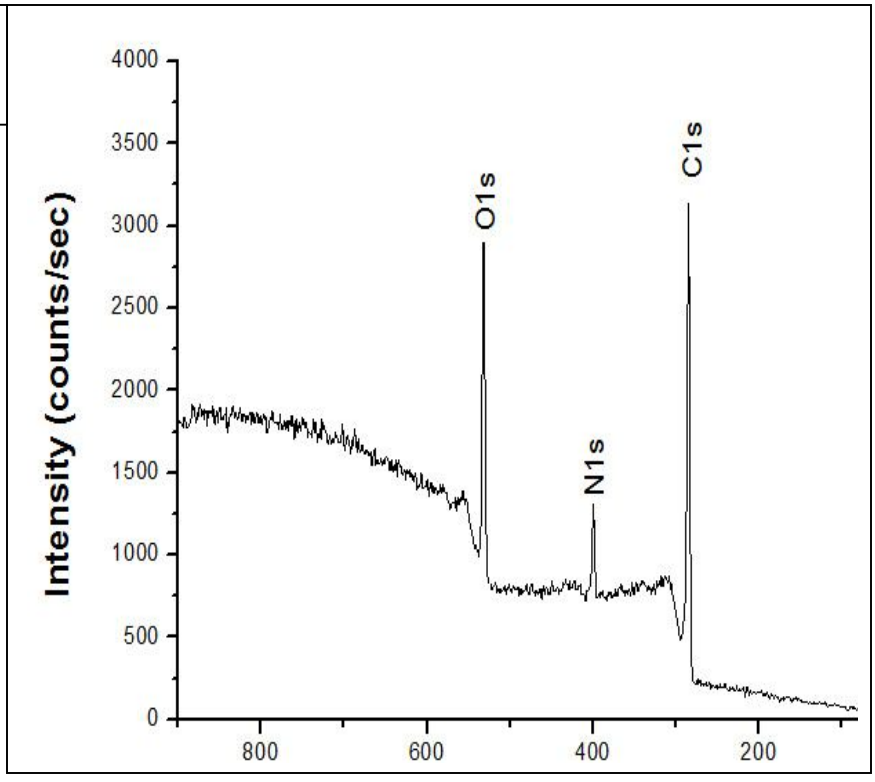


Figure 3.6 XPS wide-scan spectra of (A) pristine Ti, (B) Ti-VEGF, (C) Ti-GLU-VEGF and (D) Ti-PDOP-VEGF to determine the chemical composition of the surfaces at each stage of modification.

Determination of bioconjugation

The amount of VEGF bound to the coated substrates was determined by ELISA kit to be 43 ± 5.0 ng/cm^2 (86%), 28 ± 2.5 ng/cm^2 (56%), and 26 ± 2.5 ng/cm^2 (52%) for Ti-VEGF, Ti-GLU-VEGF and Ti-PDOP-VEGF substrates respectively.

Cytotoxicity assay

Cell viability on the control, pristine Ti, Ti-VEGF, Ti-GLU-VEGF and Ti-PDOP-VEGF substrates were assessed using the MTT assay, to give an indication of the effect of the substrates on the survival and proliferation of the attached cells after 1 week of culture (Fig. 3.7)

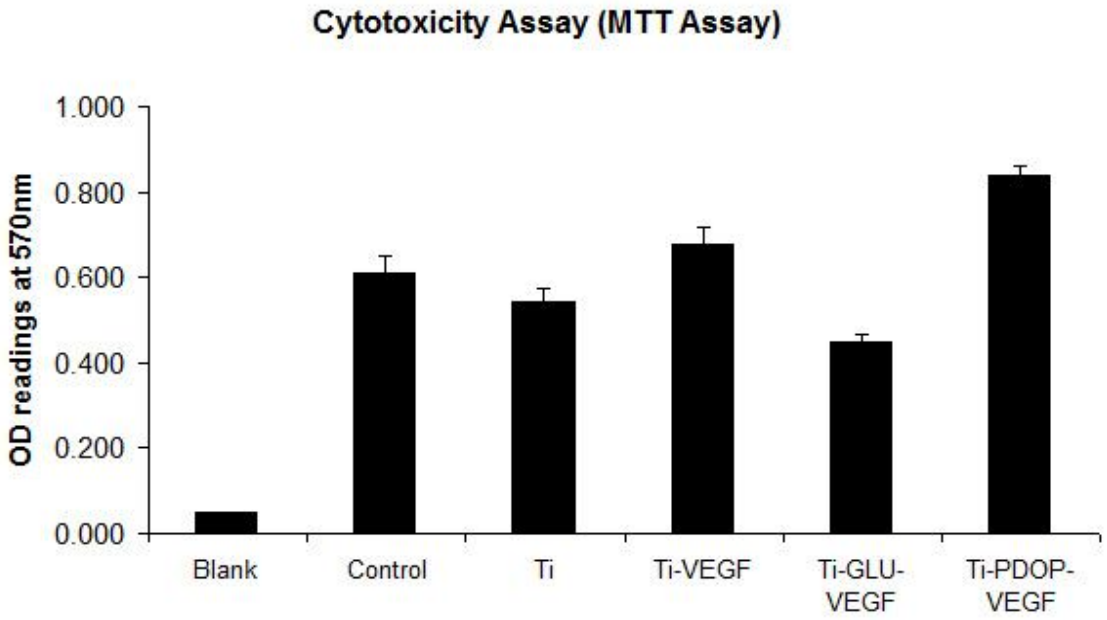


Figure 3.7 Cell viability as measured by MTT assay on the control, pristine Ti, Ti-VEGF, Ti-GLU-VEGF and Ti-PDOP-VEGF substrates after 1 week (*n*=3). The assay showed that the Ti-VEGF and Ti-PDOP-VEGF substrates do not affect cell viability while Ti-GLU-VEGF substrates may be toxic to cells.

VEGF release overtime

There is no release of VEGF from the Ti-GLU-VEGF and Ti-PDOP-VEGF substrates, while VEGF is progressively released in the Ti-VEGF substrates and its concentration in solution reaches a peak at about day 15 (Fig.3.8).

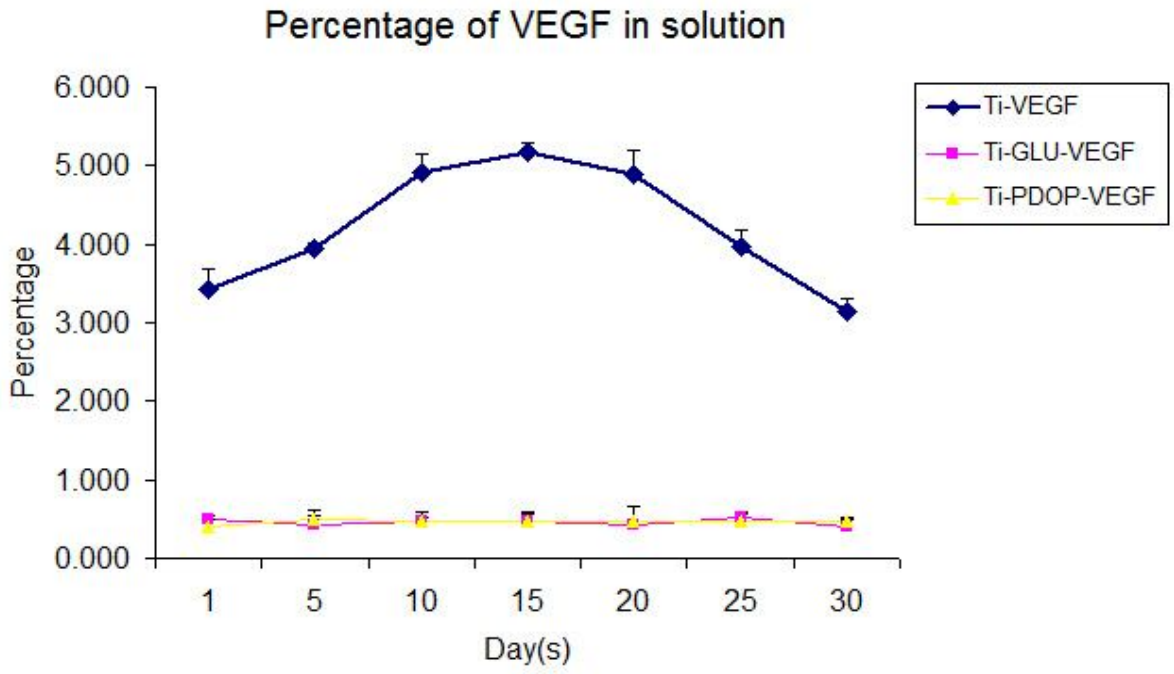


Figure 3.8 Concentration of VEGF in PBS, expressed as percentage of the initial coating concentration of VEGF on Ti-VEGF, Ti-GLU-VEGF and Ti-PDOP-VEGF substrates (*n*=3). Error bars represent standard deviations

3.4 Discussion

Several methods of immobilizing angiogenic growth factors onto substrates have been studied and reported [64-69]. In our study here we investigated the efficacy of immobilization via various modes of functionalization including physical adsorption, cross-linking and covalent binding. One of the main problem faced with surface functionalization is that complicated procedures and extensive modifications are required to both the substrates and the growth factors. Pretreatment of the surfaces is usually needed before producing the required surface functionalization effect, however surface pretreatment may alter and affect the integrity of a substrate [70]. Similarly most growth factors require certain chemical modification before they can be conjugated onto the implant material [66, 67]. For example, VEGF is oxidized with periodate before grafting onto dihydrazide modified PLGA [67]. However the bioactivity of growth factors may be compromised after chemical modification which could adversely affect their ability to bind to their respective cell surface receptors and hence disrupt their biological functions [71]. The two oxidized human VEGF isoforms, $VEGF_{165}$ and $VEGF_{121}$ have been reported to lose their binding properties to VEGF cell surface receptors [71]. Hence facile and effective functionalization procedures without altering the beneficial effects of the implant materials and growth factors should be used whenever possible. With each additional modification, the risk of compromising the integrity of the implant and bioactivity of the growth factors increases. To validitate the various surface modification procedures and integrity of the substrates, the chemical composition of the surfaces was checked by XPS. The increase in the C 1s and N 1 s and a concomitant decrease in the Ti 2p peak intensity (Fig.3.6(C,D)) showed the successful deposition of the dopamine and

glutaraldehyde layers. Successful anchoring of the growth factor on the Ti substrate was indicated by an increase in the N content as shown in Table 3.1, due to the abundance of NH_2 groups in VEGF.

	Binding Efficiency (50ng loading)	Cytotoxicity	VEGF release overtime	Active VEGF form	Number of steps required for fabrication
Physical adsorption	86%	0.677	> 30% after 1 month	Soluble	Single step
Cross-linking	56%	0.449	Nil	Immobilized	Three steps
Covalent Binding	52%	0.841	Nil	Immobilized	Two steps

Table 3.2 Summary of the various parameters of the functionalization process for the bioconjugation of VEGF with Titanium.

A summary of the binding efficiency, cytotoxicity, VEGF release profile and number of steps required for the fabrication of the substrates is listed in Table 3.2. As shown in Table 3.2 although physical adsorption had the highest rate of binding however there was also uncontrolled release of the factors from the substrate which may be undesirable [72-74]. A measurement of the percentage of VEGF released into the solution over a 30 day period showed that more than 30% of the factors were released. A number of studies have examined simple coating or loading of VEGF onto implants [72-78] in order to provide local and sustained delivery of VEGF after implantation. However with this strategy some studies showed an uncontrolled initial burst in the release kinetics of VEGF from such implants [72-74]. High levels of VEGF in the local

microenvironments of these implants may be detrimental to healing and may promote the formation of malformed vessels [79]. To avoid the deleterious effects, secure immobilization strategy to promote angiogenesis would be preferred [64, 67-69]. Immobilization of growth factors on implants have been shown to promote desirable cell substrate interactions and enhance cell functions [65, 66]. Furthermore it has been demonstrated that immobilized VEGF is more effective in promoting proliferation of endothelial cells compared to soluble VEGF [68]. Both immobilized and soluble VEGF bind to receptors on cells, however they have differing effects due to the fact that soluble VEGF is internalized and subsequently degraded, while immobilized VEGF inhibits internalization and prevents down regulation [67, 80], thereby enabling the VEGF to stimulate proliferation for an extended period of time. A comparison of cross-linking and covalent binding shows that they come quite close in terms of binding efficiency and there is no release of growth factors into the solution which is the preferred methodology. However from our cytotoxicity tests (Table 3.2) we found that there is a lower cell viability with glutaraldehyde cross-linking compared to the other groups. This may be due to the fact that glutaraldehyde is known to be toxic and is able to kill cells quickly by cross-linking with their proteins. There have also been reports of its toxicity implicated in poor cell growth, attachment and apoptosis [56-58] by other groups. Although glutaraldehyde cross-linking effectively anchors a high density of VEGF onto the Ti-GLU substrate surface and the molecules are also more firmly attached than those which are physically adsorbed however the associated toxicity has made it unsuitable for our study. The use of covalent immobilization with polydopamine looks promising. Polydopamine has been found to be able to form thin adherent films onto a wide variety

of metallic substrates via covalent bonds and various strong intermolecular interactions including metal chelation, hydrogen bonding and π-π interactions [62] which cannot be disrupted by normal mechanical forces. The use of this bioreactive layer for covalent bioconjugation with bioactive factors for orthopaedic applications holds great potential. However research on the effect of polydopamine in this area is still very limited, hence we will go on to further evaluate the efficacy of its use.

3.5 Conclusion

Therefore because of the concerns of glutaraldehyde cross-linking which may have associated cytotoxicity and an additional step is required, we found that surface functionalization using covalent immobilization with polydopamine would be a more suitable methodology for our study. This would provide us with a facile and efficient method of attaching bioactive molecules to implant materials without the risk of uncontrolled adverse effects of unwanted ectopic bone formation, undesirable effects at locations beyond the implant site in the body and associated cytotoxicity. In the next chapter we will go on to investigate in greater detail the effects of such functionalized substrates on cellular functions in terms of the topography and bioactivity.

Chapter 4

4.1 Effects of Surface Functionalized Titanium on Revascularization

Using the methodology developed in the previous chapter, we went on to characterize the physical structure and to investigate the effects of such functionalized substrates in greater detail.

Osseointegration

One of the most important process in determining the success of an orthopaedic implant is osseointegration. Osseointegration is defined as the formation of a direct structural and functional connection between the living bone and the surface of a implant [81, 82]. An implant is considered osseointegrated if there is no progressive relative movement between the implant and the bone it has direct contact with [82]. Under ideal conditions, implants could permanently become incorporated within the bone and persist under all normal conditions of loading, that is the two could not be separated without fracture. Vascularization which is the provision of blood supply is a critical component for the process of osseointegration. The differentiation of osteogenic cells is highly dependent on tissue vascularity and ossification is closely linked to the vascularization of differentiating tissue [82]. Therefore the success of tissue healing, regeneration and integration lies in the key process of revascularization which is crucial in improving the successful integration of implants [83, 84].

Vascular endothelial growth factor (VEGF)

Bone healing around implants involves a cascade of cellular and biological events that take place at the bone-implant interface until finally the entire surface of the implant is covered by newly formed bone. This cascade of biological events is regulated by differentiation of cells stimulated by growth factors secreted at the bone-implant interface [82]. There has been considerable interest in modifying implant surfaces with growth factors to improve their cell functions and tissue integration capacity at the bone-implant interface. Enhanced cell functions and cell substrate interactions have been demonstrated with growth factors immobilized onto bioimplants [39-41]. One of the more important growth factors for stimulating neovascularization (i.e. formation of new blood vessels) in target areas [85] would be angiogenic growth factors, crucial in improving the successful integration of implants both *in vitro* and *in vivo* [83, 84]. Of these angiogenic factors, VEGF is the most potent and widely used key regulator of neovascularization [85, 86]. [85, 86]. VEGF is a crucial factor in not only angiogenesis regulation but also in osteoblast [87] and osteoclast function [88-90] during bone repair. VEGF acts directly on osteoblasts, promoting cell functions such as proliferation, migration and differentiation [91, 92]. In addition, VEGF also indirectly affect osteoblasts via its influences on endothelial cells [93, 94]. VEGF is known to induce endothelial cells in surrounding tissues to migrate, proliferate and form tubular structures [95] and is an essential survival factor for endothelial cells [93] and new vessel formation [96]. Endothelial cells are needed to provide complex interactive communication networks in bone for gap junction communication with osteoblasts crucial to their formation from osteoprogenitors [97]. Furthermore VEGF stimulates endothelial cells in the production of beneficial bone

forming factors acting on osteoblasts [92]. In all, the effects of VEGF on osteoblasts, osteoclasts and endothelial cells may synergistically act to enhance bone formation.

Titanium alloy

Titanium alloy was the choice of substrate for this study as it is one of the most extensively used metal in orthopaedic implants especially in non-weight-bearing surface components such as femoral necks and stems [9]. Titanium has good mechanical properties, chemical stability and a naturally formed oxide layer on its surface which further increases its biocompatibility [10]. Titanium has inherent bioactivity to osseointegrate [10], however this is still not sufficient to truly and reliably incorporate into living bone [11]. Therefore enhancing vascularity in Titanium implants would be of great benefit in improving bone-implant anchorage and clinical results.

Clinical need

There are clinical situations where compromised vascularity in the immediate vicinity of an implant leads to impaired fracture healing, which can result in implant failure. One commonly encountered example of such a problem is in high grade long bone fractures associated with significant surrounding soft tissue damage, where the blood supply to the fracture site can be greatly affected. This often leads to either delayed or non-union of the fracture, which can persist despite fracture fixation with orthopaedic devices. In time the non-healing fracture places high stresses on the implant and this can result in mechanical implant failure. Another clinical situation in which bone quality can be compromised leading to implant loosening is in hip resurfacing of a femoral head. Hip

resurfacing is a relatively new surgical technique that is an alternative to hip replacement in selected patients; its advantages are that it involves much less resection of the patient's native hip bone, and the preserved bone makes subsequent revision surgery less difficult. However hip resurfacing depends on the integrity of the underlying femoral head bone stock for implant support and success, and this can be affected by compromised vascularity of the femoral neck and head, either from the surgical procedure or from pre-existing conditions such as avascular necrosis. In both of these cases, any advance in technology that can enhance revascularization in the salient anatomical regions would be of great benefit in improving clinical results and reducing complications and patient morbidity.

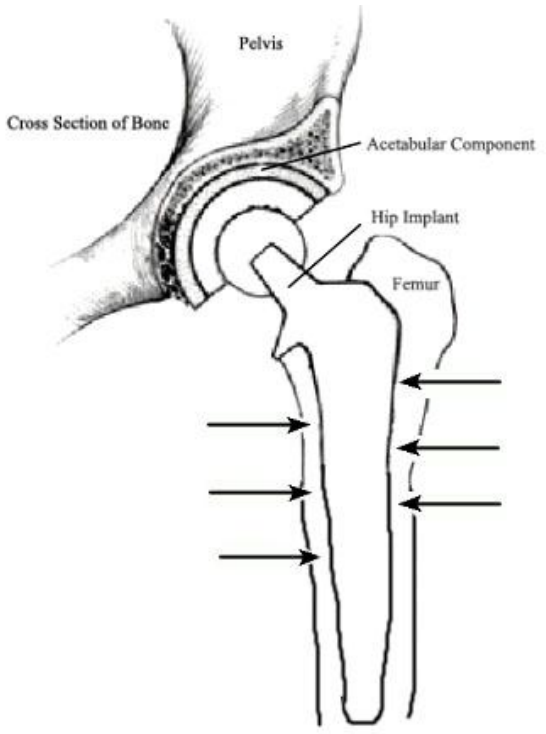


Figure 4.1 Schematic figure of a hip resurfacing implant: hemispherical cobalt chrome alloy component capping onto the femoral head of the hip joint, with Titanium guide stem inserted in femoral neck region. The femoral neck is the region at risk of compromised vascularity. Arrows indicate area of compromised vascularity.

Therefore in this chapter we assessed and evaluated the physical properties and the bioactivity of the VEGF functionalized substrates. We aim to use the scheme developed in chapter 3 as a means to promote angiogenesis in the host tissues surrounding the functionalized implant, thereby enhancing healing and integration of healthy bone tissue around the implant. The functionalization of implant materials in order to enhance biocompatibility and promote revascularization has great potential in addressing the problems of implant longevity and survival.

4.2 Materials and Methods

Materials

Materials and methods specific to this chapter are described below. All other materials and methods used in this chapter are described in Chapter 3 (Materials and methods section).

FITC-conjugated polyclonal goat anti-mouse IgG antibody was purchased from AbD Serotec (UK). Unconjugated monoclonal mouse antihuman von Willebrand Factor (vWF) antibody and PECAM1 (CD31) were purchased from Abcam (UK). Mesenchymal Stem Cell Growth Medium Bulletkit PT-3001 were purchased from Lonza Walkersville, Inc.

Preparation of substrates & covalent immobilization

The substrates were prepared as described in chapter 3. The substrates are denoted Ti (pristine Titanium), Ti-PDOP (polydopamine-grafted Ti) and Ti-PDOP-VEGF (VEGF-coated, polydopamine-grafted Ti) in subsequent discussions.

Binding efficiency

Binding optimization was carried out as described in Chapter 3 (Materials and methods section).

Characterization

Unmodified and modified Ti substrates were coated with platinum and the surfaces of the substrates were imaged using a scanning electron microscope (SEM) (JEOL, model 5600LV). Surface roughness (*Ra*) was calculated from the roughness profile determined by atomic force microscopy (AFM) (Nanoscope III, Digital Instruments, Santa Barbara, CA). These analyses were performed in the tapping mode with a scan rate of 0.5-1 Hz.

Cell culture

HDMECs (Lonza Walkersville, Inc) were cultured in endothelial cell growth medium MV and passage 5 HDMECs were used for the analysis of attachment and proliferation studies. hMSCs (Lonza Walkersville, Inc) were cultured in mesenchymal stem cell growth medium and passage 2 hMSCs were used for the *in vitro* analysis of the biological activity of the immobilized VEGF. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 with the growth medium changed every 2-3 days. Attached cells were detached by trypsinization and resuspended in fresh culture medium for subsequent experiments described below.

Cytotoxicity assay

Cytotoxicity analysis was carried out as described in Chapter 3 (Materials and methods section).

Cellular functions

Cell attachment on the various Titanium substrates was evaluated by counting the number of attached cells 12 hours after cell seeding. The substrates were placed into a 24-microwell plate (Nalge, Nunc International) and seeded with HDMECs at a density of 5 000 cells/cm^2. The number of attached cells on Ti, Ti-PDOP, Ti-PDOP-VEGF and the bottom of a culture well (control) was evaluated. At the time of cell counting, unattached cells were rinsed off with PBS. Adherent cells were then detached by trypsinization and counted using a haemocytometer. Cell proliferation on the substrates was evaluated by counting the number of attached cells on days 1, 7, and 14. The number of attached cells on the bottom of a similar culture well without any Titanium substrate was counted at the respective points in time and was used as a control. At each designated point in time, the unattached cells were rinsed off with PBS and the attached cells were trypsinized and counted using a haemocytometer. The number of attached cells is reported as number of cells/cm^2.

Biological activity of VEGF

In vitro angiogenesis assay was performed using 50 µl of BD Matrigel in each well of a 96-microwell plate (Nalge, Nunc International) at 4^oC [98-100]. The matrigel was left at room temperature for 1 hr to allow it to solidify before use. hMSCs grown on the substrates for 2 weeks were detached with trypsin and the trypsin was removed by centrifugation. The cells were resuspended in normal cell culture medium and 2,000 cells in 50 µl of medium were plated onto the matrigel. The cells were then incubated for 3 hrs

and the capillary-like tube formation was observed under a light microscope (Olympus IX71).

Immunofluorescent (IF) microscopy

hMSCs grown on the substrates for 2 weeks were detached and plated on cover slips overnight. Cells plated on the cover slips were fixed in methanol at -20^{o}C for 10 min and then rinsed with PBS, following which the cells were incubated with vWF or CD31 staining (1:300) for 1 h, rinsed in PBS and further stained with FITC-conjugated goat anti-mouse secondary antibody (1:300) for 1 hr. The cells were then viewed using a fluorescent microscope (Olympus IX71).

Statistical analysis

At least three samples per time point for each experimental condition were used. The data were tested for normal distribution by Shapiro-Wilk test. One-way analysis of variance (ANOVA) *post-hoc* Tukey test was used to assess the normally distributed data and the results are reported as mean ± SD. Statistical significance was accepted at $P < 0.05$.

4.3 Results

Determination of bioconjugation

The amount of VEGF bound to the coated substrates was determined by ELISA kit to be 8.2 ± 2.0 ng/cm^2 (66%) at a loading of 12.5ng, 14.3 ± 2.0 ng/cm^2 (57%) at a loading of 25ng, 26.7 ± 3.0 ng/cm^2 (53%) at a loading of 50ng, 39.1 ± 4.0 ng/cm^2 (39%) at a loading of 100ng and 63.6 ± 5.0 ng/cm^2 (25%) at a loading of 250ng. The results is summarized in table 4.1. Loading at 1μg/ml would provide the optimal binding rate, therefore this concentration was used for the fabrication of the Ti-PDOP-VEGF substrates.

Absolute loading quantity (ng)	Concentration (μg/ml)	Binding as determined by ELISA(ng/cm^2)	Percentage bound over absolute quantity loaded
12.5	0.25	8.2 ± 2.0	66%
25	0.5	14.3 ± 2.0	57%
50	**1**	**26.7 ± 3.0**	**53%**
100	2	39.1 ± 4.0	39%
250	5	63.6± 5.0	25%

Table 4.1 Binding optimization of Titanium with VEGF. Loading at a concentration of 1 μg/ml would provide the optimal binding concentration without the loss of too much proteins.

Surface characterization

The XPS wide scan spectra of the pristine Ti, Ti-PDOP, and Ti-PDOP-VEGF and their corresponding surface elemental compositions are shown in Table 4.2 and Fig. 4.2 respectively. In the wide scan spectrum of the pristine Ti (Fig. 4.2(a)), the predominant components are C 1s (285 eV), Ti 2p (460 eV) and O 1s (530 eV). Successful deposition of polydopamine on the Ti substrate was indicated by an increase in the N and C contents as shown in Table 4.2. Nitrogen-to-carbon (N/C) ratios is 0.126 on Ti-PDOP which is similar to the theoretical N/C of 0.125 for dopamine. Furthermore, complete suppression of photoelectron peaks unique to Ti 2p (about 780 eV) confirms formation of the polydopamine thin film (Fig. 4.2(b)), while successful deposition of the growth factor on the Ti substrate was indicated by an increase in the N content. The changes in the surface atomic ratio of the functionalized substrates are quantified and summarized in Table 4.2.

Substrate	C%	N%	O%	Ti%
Ti	36.6	1.3	49.7	12.4
Ti-PDOP	72.0	9.1	18.9	-
Ti-PDOP-VEGF	68.6	13.5	17.8	-

Table 4.2 Elemental Composition* at the Surface of Pristine and Ti -PDOP and Ti-PDOP-VEGF substrates as determined by XPS.

*Percentages computed based on the C, N, O and Ti contents only.

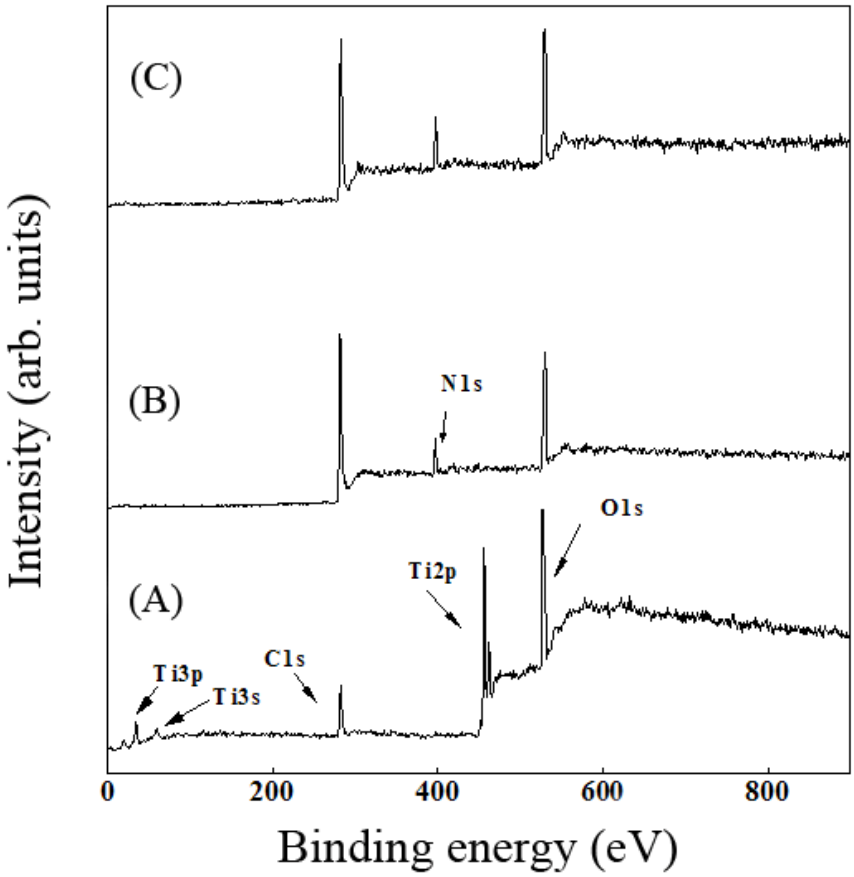


Figure 4.2 XPS wide-scan spectra of (A) pristine Ti, (B) Ti-PDOP and (C) Ti-PDOP-VEGF to determine the chemical composition of the surfaces at each stage of modification.

Surface structure by SEM

SEM investigations revealed the differences in the structure between the unmodified pristine Ti and the modified Ti-PDOP-VEGF substrates (Fig. 4.3).

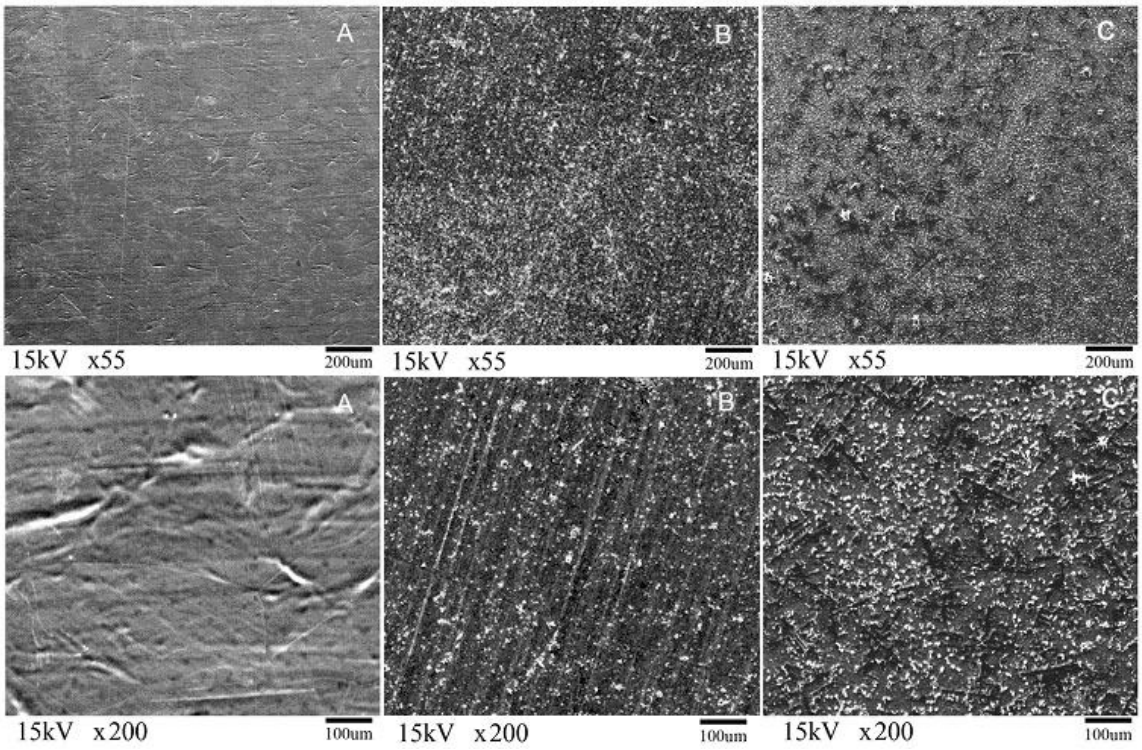


Figure 4.3 SEM images of (A) pristine Ti, (B) Ti-PDOP and (C) Ti-PDOP-VEGF substrates with different surface properties.

Surface texture by AFM

Surface roughness as determined by AFM (Fig. 4.4) was 147 ± 25 nm, 349 ± 48nm and 367 ± 53 nm for pristine Ti, Ti-PDOP, and Ti-PDOP-VEGF substrates respectively.

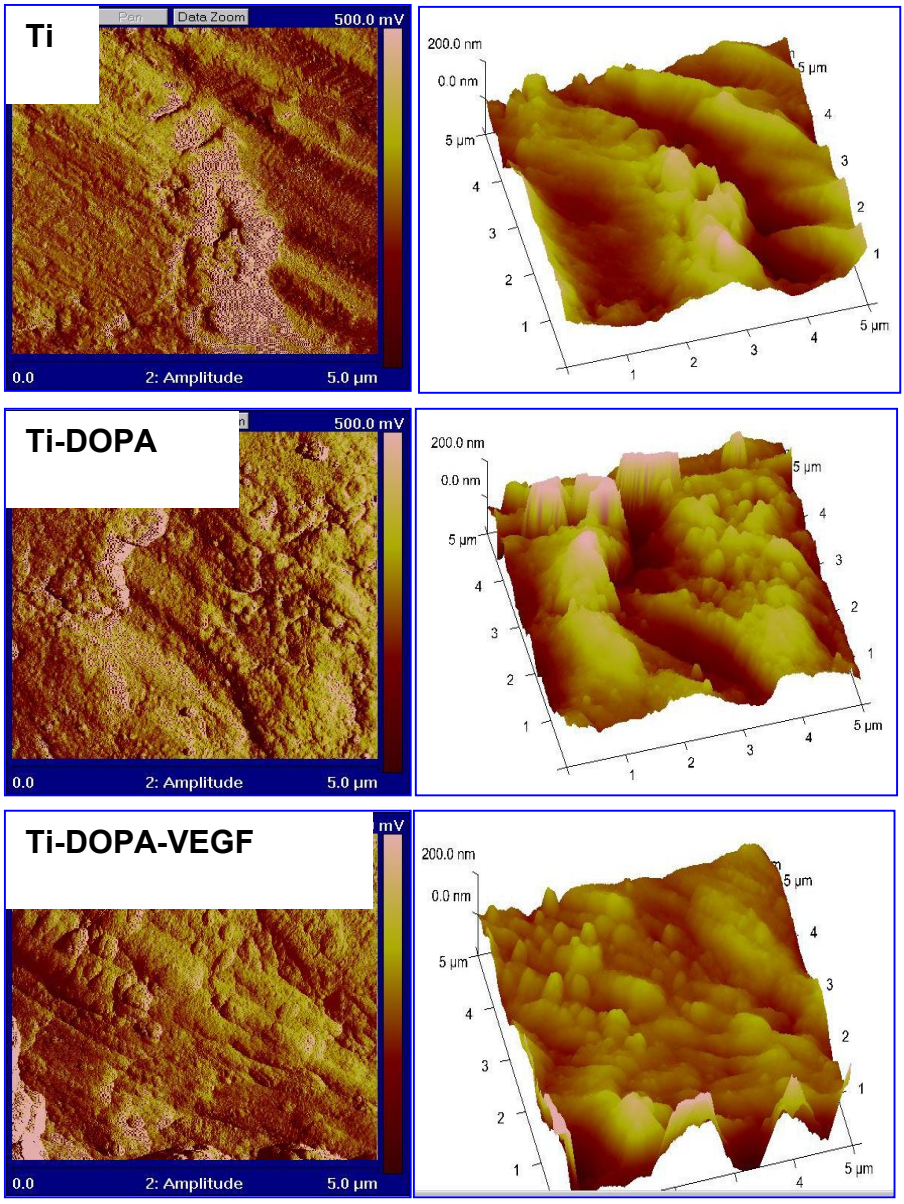


Figure 4.4 AFM images of (A) pristine Ti, (B) Ti-PDOP, (C) Ti-PDOP-VEGF substrates. There is a general increase in surface roughness of the substrates after each step of modification.

Cellular functions

From the results (Fig. 4.5) approximately 63% of the cells seeded were attached to the pristine Ti. In contrast on Ti-PDOP-VEGF substrates approximately 83% of seeded

cells were attached, indicating an increase of about 20% over the pristine substrates. Cell proliferation progressed steadily over 14 days of culture on the controls, pristine Ti, Ti-PDOP and the Ti-PDOP-VEGF substrates as shown in Fig. 4.6.

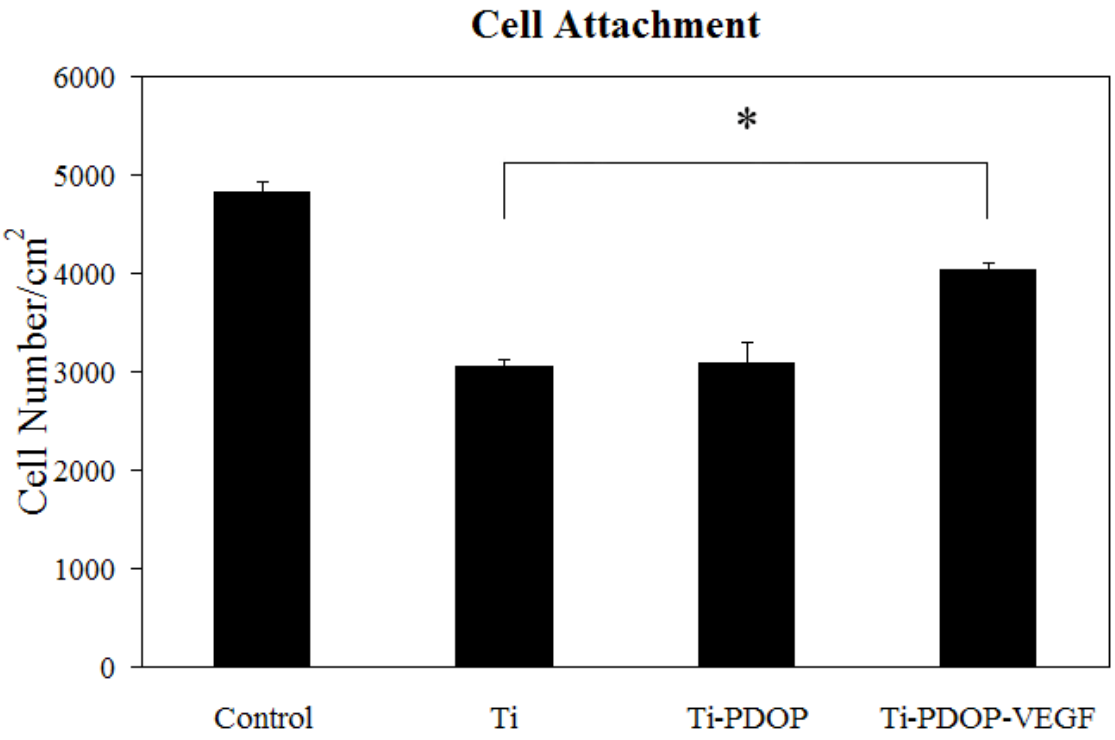


Figure 4.5 Number of adherent HDMECs per cm^2 on surfaces of control, pristine Ti, Ti-PDOP and Ti-PDOP-VEGF substrates (n=5). There is about a 20% increase in HDMEC attachment on the Ti-PDOP-VEGF over the pristine Ti. (*) denote significant differences ($P < 0.05$) compared with the pristine Ti.

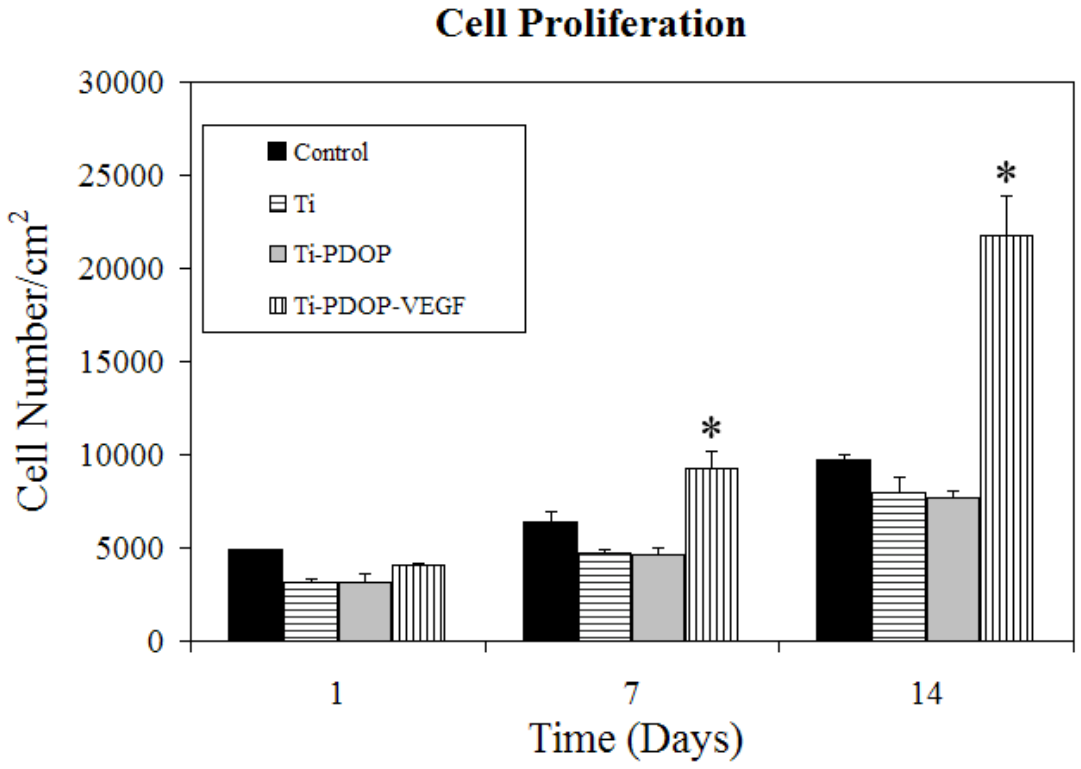


Figure 4.6 Comparison of HDMEC proliferation over 2 weeks on surfaces of control, pristine Ti, Ti-PDOP and Ti-PDOP-VEGF substrates by counting the number of attached HDMEC on each type of substrate on days 1, 7 and 14 (n=5). There was approximately a 2 fold increase in cell number on the Ti-PDOP-VEGF substrates compared to the pristine Ti. (*) denote significant differences ($P < 0.05$) compared with the pristine Ti.

Cytotoxicity assay

As shown in Fig. 4.7, significantly higher viability of HDMECs was observed on the Ti-PDOP-VEGF substrates compared to both the pristine Ti and culture wells controls.

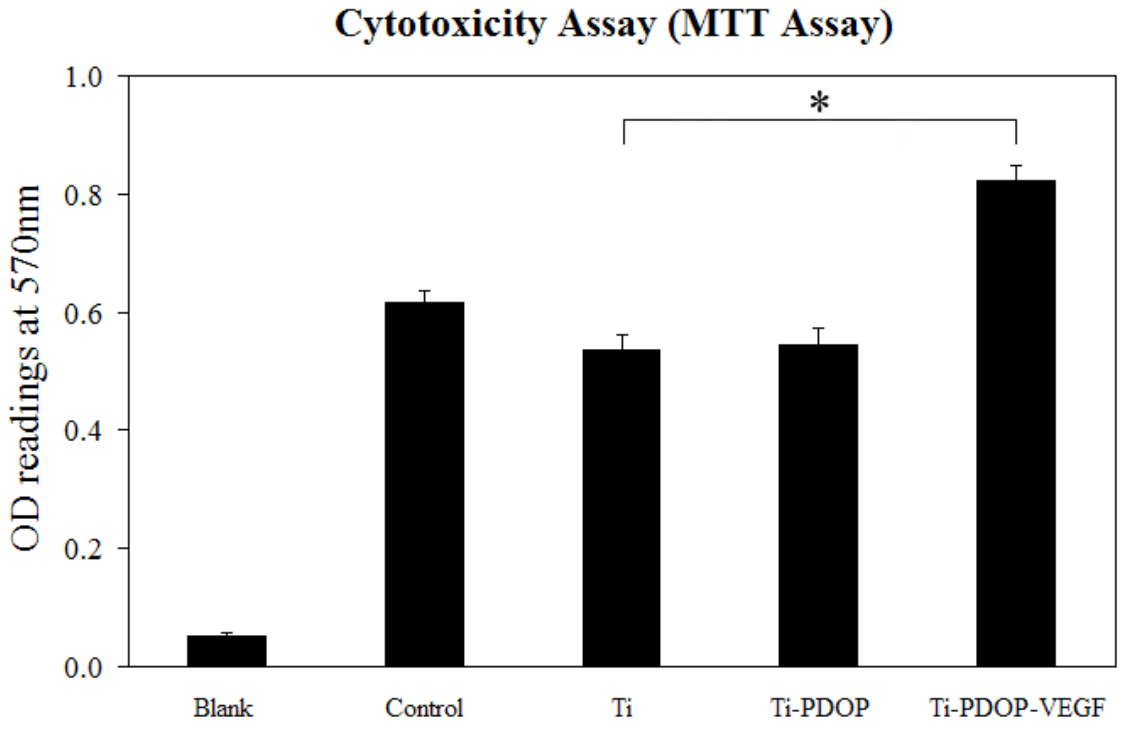


Figure 4.7 Cell viability measured by MTT assay on the control, pristine Ti, Ti-PDOP and Ti-PDOP-VEGF substrates after 1 week (n=5). The assay shows that the substrates do not affect cell viability and there is significantly higher viability of HDMEC observed in the Ti-PDOP-VEGF compared to the pristine Ti. (*) denote significant differences ($P < 0.05$) compared with the pristine Ti.

In vitro analysis of the biological activity of VEGF

Ti and Ti-PDOP-VEGF substrates for 2 weeks were plated onto a Matrigel surface. Capillary networks were observed on the hMSCs grown on Ti-PDOP-VEGF substrates (Fig. 4.8a) while hMSCs grown on pristine Ti remained as isolated, single cells on the Matrigel (Fig. 4.8b). The differentiated cells expressed strong vWF and weak CD31 after 2 weeks of VEGF mediated differentiation.

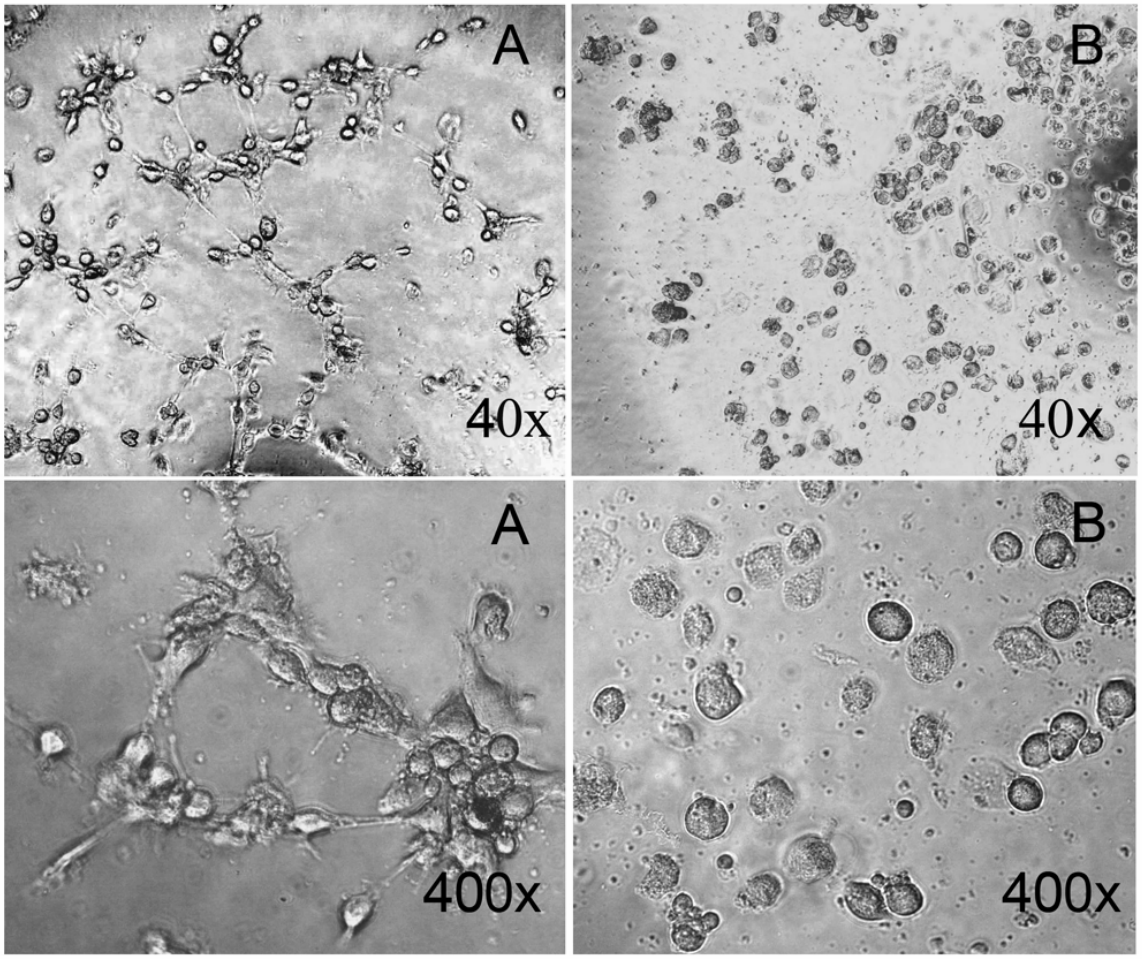


Figure 4.8 hMSCs grown on Ti-PDOP-VEGF substrates display a capillary network formation on Matrigel (A). hMSCs grown on pristine Ti substrates remain as isolated, single cells on the Matrigel surface (B).

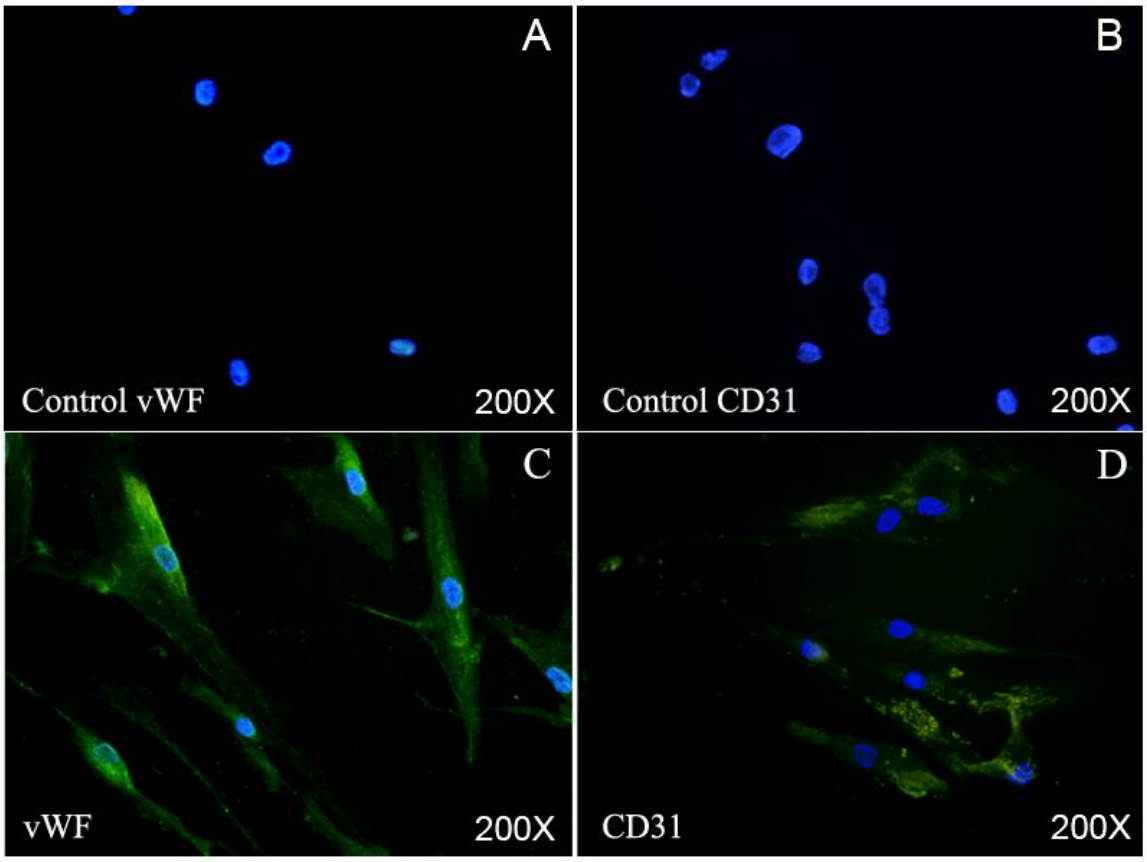


Figure 4.9 hMSCs immunofluorescent staining reveals that the hMSCs derived cells show expression of endothelial cell markers, vWF (C) and CD31 (D) after 2 weeks differentiation.

4.4 Discussion

The fixation of prosthetic components to the bone can be done with or without bone cement. In the cemented technique PMMA is used to "glue" the metal to the bone. In direct biological fixation, precise bone cuts are required to achieve maximum contact between metal and bone. The advantage of cement fixation is that the prosthetic components are instantly fixed, allowing movement immediately after surgery. However in the instances where revision surgery is required, it is extremely difficult to chip out all the cement during implant replacement. Cement fixation is usually employed on elderly patients over sixty-five where their bone stock is more osteoporotic with less likelihood of growing into the prosthesis and chances of revision is lower due to less demands on the implant and shorter remaining life expectancy compared to younger patients. Direct biological fixation is generally used for young patients due to better bone stock and ingrowth potential. The disadvantage of biological fixation is that it can take weeks or months to be fully complete during which weight bearing activity is restricted. However the final fixation achieved is more natural with complete incorporation of implant within the bone in ideal situations. Furthermore in young patients the chances for future revision surgery is higher and it would be easier to revise a cementless prosthesis without the need for cement removal. Another problem perceived was that cementless Titanium stems have been reported to be more resistant to osteolysis and mechanical failure compared to similar cemented Titanium stems [101]. The features of Titanium that are detrimental to the cement environment seems to have no effects in the cementless environment and may in fact be beneficial leading to different in performances of the two techniques. Therefore the enhancement of the bone implant interface especially in direct biological fixation with

Titanium implants would be extremely useful. This would greatly reduce the lag period in which osseointegration occurs between the prosthesis and the patient's bone.

To achieve this, VEGF was immobilized onto the surface of Titanium substrates. The biological activity of VEGF plays a dominant role during neovascularization of an implant *in vivo* and endothelial cells will come into direct contact with the implant surface [75]. Therefore HDMEC was used for the investigation because of their dominant role in revascularization and they are found predominantly in the area surrounding the implant [75, 102]. As cell attachment is the single most important factor during cell-implant interactions [103], and is critical in determining the initial success of a bioengineered implant, therefore we investigated the effects of the functionalized substrates on cell adhesion. An increase of about 20% cell adhesion was observed with the Ti-PDOP-VEGF substrates (Fig. 4.5) compared to the pristine Ti. This suggests that the surface-immobilized VEGF is able to enhance cell attachment and may increase the affinity for endothelial cells to form a protective monolayer layer on the surface, where further cellular developments can occur. This observation is consistent with other reported studies where substrates immobilized with VEGF [68, 104] were shown to result in higher cell density.

The availability of the immobilized VEGF on the substrate surface to stimulate appropriate cellular responses is of crucial importance. Therefore, the growth of HDMECs was accessed at various time points over 2 weeks. The cell proliferation rates were not significantly different on the pristine Ti, Ti-PDOP substrates and in the controls on culture wells. However on the Ti-PDOP-VEGF substrates cell proliferation rate was markedly increased from day 7 to 14 and we can see significantly higher proliferation

rates and cell numbers compared to the pristine Ti and control culture wells. The cell number on day 14 was approximately 2-fold higher on the Ti-PDOP-VEGF substrates in comparison to the pristine Ti and control culture wells. This finding indicates that the Ti-PDOP-VEGF substrate immobilized with VEGF promotes greater proliferation. The effect of proliferation observed in the Ti-PDOP-VEGF substrates was likely due to the immobilized VEGF providing a controlled and sustainable effect on the endothelial cell functions. Immobilized growth factors can regulate and guide local cell functions, simulating a local microenvironment *in vivo* [85, 105]. The continuous stimulated growth of the endothelial cells by the immobilized VEGF over 14 days is in line with the observations by other groups that immobilized VEGF can provide extended signaling to the endothelial cells and is able to continuously stimulate their growth without down-regulation by receptor/ligand complex internalization [106-109].

Cell viability on the pristine Ti, Ti-PDOP and Ti-PDOP-VEGF substrates was assessed using the MTT assay, to give an indication of the effect of the substrates on the survival and proliferation of the attached cells after 1 week of culture. As shown in Fig. 4.6, significantly higher viability of HDMECs was observed on the Ti-PDOP-VEGF substrates compared to both the pristine Ti and culture wells controls. The results suggest that Ti-PDOP-VEGF substrates are not cytotoxic to the cells, and the immobilized VEGF on its surface can promote the survival and proliferation of HDMECs. These observations can be explained by similar findings seen by other groups where immobilized VEGF enhanced the survival of endothelial cells through interactions with integrin receptors [110] and signal transduction pathways [93]. To ensure that the survival and proliferative effects of VEGF observed was not because of the immobilized VEGF being released into

the culture medium, HDMECs was cultured on a permeable support (0.4 μm pore size, Costar®, Corning) in transwells with or without substrates immobilized with VEGF placed at the bottom of the wells for 2 weeks. No significant difference was observed in cell attachment, cell proliferation and cell viability tests (data not shown) between the two groups. Hence it can be concluded that the enhancement in endothelial cell functions observed with the Ti-PDOP-VEGF substrates is due to the immobilized VEGF and not VEGF released into the culture medium.

Polydopamine conjugation of VEGF may affect its biological functions, therefore to show that the immobilized VEGF retained its bioactivity, we did a further test to confirm this. VEGF is known to cause the *in vitro* differentiation of mesenchymal stem cells into endothelial cells [111-113]. Based on this we carried out an *in vitro* model of angiogenesis to test the functional features of the differentiated endothelial cells. A hallmark of endothelial cells is their ability to undergo morphogenic changes to become tubular structures [100]. *In vitro* angiogenesis studies have shown that endothelial cells form a network of tubular structures when incubated on extracellular matrices such as matrigel [98-100]. The hMSCs purchased from Lonza, US, were positive for the markers CD 166, CD29 and CD44 and negative for typical endothelial markers, hence the possibility that the differentiated cells may have originated from a small population of endothelial progenitors is excluded. hMSCs grown on the pristine Ti and Ti-PDOP-VEGF substrates for 2 weeks were plated onto a Matrigel surface. The formation of a capillary network on the hMSCs grown on Ti-PDOP-VEGF substrates was observed 3 h later (Fig. 4.8a). hMSCs grown on pristine Ti did not form any capillary network and remained as isolated, single cells on the Matrigel (Fig. 4.8b). To show that the

differentiated cells were indeed endothelial cells, endothelial cell markers vWF and CD31 [114, 115] were used to confirm this. As shown in Fig. 4.9 the differentiated cells expressed strong vWF and weak CD31 after 2 weeks of VEGF mediated differentiation. Thus, the results further confirmed that the VEGF immobilized on the polydopamine coated substrates retained its biological activity and may mediate endothelial cell differentiation.

4.5 Conclusion

In summary, we have shown that immobilization of angiogenic growth factors onto the surface of metal substrates may be a viable approach in promoting revascularization and enhanced implant integration in a controllable manner. By immobilizing VEGF onto a metal substrate using biomimetic polymer film, we demonstrated that the modified substrate promotes the survival and proliferation of endothelial cells and is able to induce the differentiation of hMSCs into endothelial cells. This enhanced recruitment and differentiation of cells and their progenitors to the target implant is beneficial in accelerating vasculature formation and new bone tissue formation. Thus, the coating of a biomimetic polymer film onto a metal substrate followed by VEGF conjugation provides a means for applications where revascularization around implants would be beneficial in tissue and bio-engineering applications. The method reported here of immobilizing VEGF onto metal substrates has the advantage of efficiency, ease of fabrication and possibly of usage on various types of materials without extensive surface preparation which reduces overall risk associated with the implant use *in vivo*. This may serve as a model for the immobilization of other growth factors onto various different types of metal substrates.

Chapter 5

5.1 Effects of Surface Functionalized Cobalt Chromium on Osteogenesis

Having determined a suitable model for the immobilization of VEGF onto Titanium alloy substrates in the previous chapter, we went on to investigate if this model is viable in another metallic alloy and with a different growth factor.

Cobalt Chromium

Most metallic orthopaedic implants are currently made from a mixture of alloys [116]. Cobalt Chromium alloy (CoCr) is one of the other most used metal besides Titanium alloy due to its toughness, hardness (HV=350), strength, high wear and corrosion resistance [117]. However CoCr does not osseointegrate well, there is a tendency for the formation of an intervening fibrous layer between the bone-implant interface. In clinical situations this compromised bone growth in the immediate vicinity of an implant leads to weakened bone quality and can result in implant failure. One encountered example of such a problem is in the acetabular component (typically made of CoCr) of a hip implant (Fig. 5.0), where the patient loses bone in the pelvis area. In this instance, enhancing bone growth would be of great benefit in improving clinical results and reducing complications and patient morbidity.

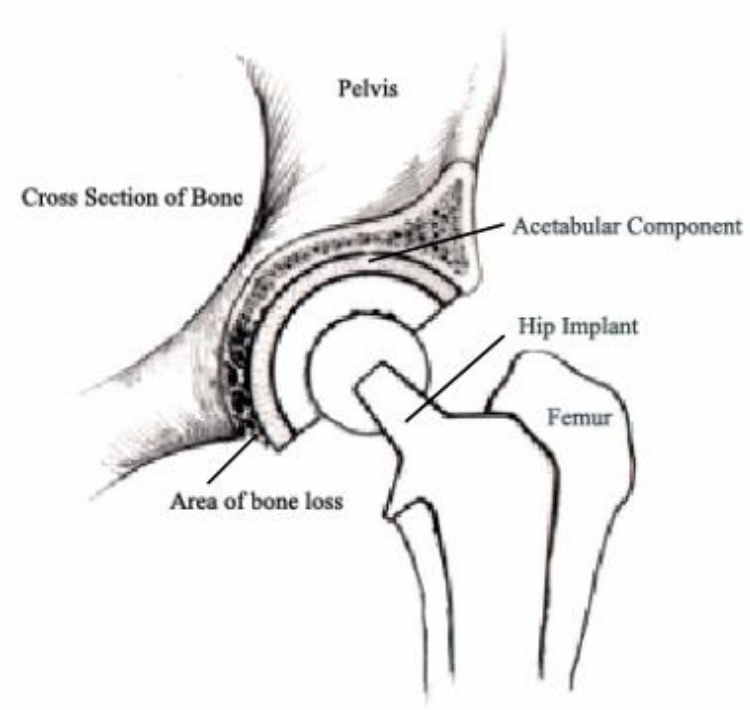


Figure 5.1 Bone loss in acetabular component (made of CoCr) of a hip implant

Bone morphogenic proteins

Coating implants with growth factors such as bone-morphogenetic proteins (BMPs) may be a solution to enhance and accelerate the quality of osseointegration [118, 119]. Bone-morphogenetic protein 2 (BMP2) has been shown to be particularly effective in enhancing bone formation [120, 121]. BMP2 plays a major role during skeleton and cartilage formation and the maintenance of homeostasis during bone remodeling. BMP2 is also known to be involved in the processes of differentiation, calcification and binds to BMP2 receptors on osteoblastic cells stimulating ALP activity [42]. To date only BMP2 and bone-morphogenetic protein 7 (BMP7) have been approved for use by the Food and Drug Administration (FDA) in specific orthopaedic applications [122].

Therefore in this study we aim to functionalize CoCr with BMP2 using the methodology developed in chapter 3. An aqueous dopamine solution at pH 8.5 was used to create a thin layer of adherent polydopamine surface on the CoCr substrates via autopolymerization [123]. This reactive layer contains catechol and quinone functional groups which can be used for covalent coupling to nucleophiles [62]. The effects of the functionalized substrates on the enhancement of bone growth were then investigated. The biological activities of the immobilized BMP2 were analyzed with osteoblastic cells (MC3T3-E1). MC3T3-E1 was used here as osteoblastic cells are usually found in the peri-implant area and these cells migrate to the implant interface, adhere, proliferate and eventually differentiate into osteoblasts to form high quality bone matrix [124].

5.2 Materials and Methods

Materials

Cobalt Chromium alloy foils (denoted as CoCr in the subsequent discussion) were purchased from Goodfellow Inc. of Cambridge, UK. Osteoblastic cells (MC3T3-E1 subclone 14) were obtained from American Type Culture Collection. The QuantiChrom^{TM} alkaline phosphatase (ALP) assay kit and QuantiChrom^{TM} Calcium Assay Kit were purchased from BioAssay Systems.

Preparation of substrates

CoCr foils were cut to a size of 10 mm × 10 mm. The substrates were polished using 600 and 1200 grid sandpaper and then sonicated for 10 min in water. The carbide deposited during polishing were removed by sonicating the substrates in Kroll's reagent (4.0% HF, 7.2% HNO_3, 88.8% water) for 10 min. The reaction was terminated by the addition of 1 N sodium hydroxide. The substrates were then cleaned ultrasonically for 10 min each in dichloromethane, acetone, and water and placed in 40% HNO_3 for 40 min for surface passivation. The acid-treated substrates were then rinsed thoroughly with water. Polydopamine was anchored to the surface of the CoCr substrates, via immersion of the substrates in a 2 mg/ml solution of dopamine (10 mM Tris buffer, pH=8.5) overnight in the dark.[62, 123] The substrates were then rinsed with copious ultrapure water to remove the unattached dopamine and dried under nitrogen flow. The polydopamine-grafted CoCr substrates were then coated with BMP2 at concentration of 1 μg/ml and 10μg/ml and incubated overnight in a humid atmosphere at room temperature [62, 63].

The substrates were then washed three times with sterile PBS to remove unattached BMP2 and left to air dry in a sterile environment before use. The substrates are denoted as CoCr (pristine cobalt chrome), CoCr-PDOP (polydopamine-grafted CoCr) and CoCr-PDOP-BMP2 (BMP2 coated, polydopamine-grafted CoCr) in subsequent discussions.

Binding efficiency

To determine the binding efficiency of the surface functionalization procedures, the quantity of BMP2 in the loading solution and the combined washing solution was performed using an enzyme-linked immunosorbent assay kit (BMP2 ELISA, R&D System, Minneapolis, MN) according to the manufacturer's instructions. The surface density of bound BMP2 was calculated from the difference between the initial and remaining BMP2 in the washing buffer.

Characterization

XPS and SEM-EDX analysis was carried out as described in Chapter 3 (Materials and methods section).

Cytotoxicity assay

Cytotoxicity analysis was carried out as described in Chapter 3 (Materials and methods section).

Cell culture

MC3T3-E1 were cultured in alpha minimum essential medium (Invitrogen, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Passage 5 MC3T3-E1 was used for the studies. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 with the growth medium changed every 2-3 days. Attached cells were detached by trypsinization and resuspended in fresh culture medium for subsequent experiments described below.

Cell adhesion

Cell attachment on the various CoCr substrates was evaluated by measuring the number of attached cells with Vialight Cell Proliferation Assay Kit (Lonza Walkersville, Inc) 12 hrs after cell seeding. The substrates were seeded with MC3T3-E1 at a density of 3 000 cells/cm^2. At the time of cell counting, unattached cells were rinsed off with PBS. ATP monitoring reagent in Tris Acetate buffer was prepared according to the manufacturer's instructions and added to each sample and incubated for 5 min at room temperature protected from light. The emitted luminescence was then measured by using a luminometer (GloMax-96 Microplate Luminometer). A reference standard curve was created for converting the observed luminescence values into cell numbers.

Alkaline phosphatase (ALP) activity assay

ALP activity was assessed using QuantiChrom^{TM} alkaline phosphatase assay kit according to manufacturer's instructions. Cells were seeded onto the substrates at a density of 3000 cells/cm^2 in a growth medium supplemented with 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate. At specific time intervals during cultivation, the

cell layers were washed with PBS and scraped off from the surfaces in cell lysis buffer and sonicated to disrupt the cell membranes. After sonication, cellular debri was removed by centrifugation and aliquots of the cell lysates were collected for analysis of ALP activity and quantification of total protein level. A 10 µl aliquot of the sonicated cell lysate was added to 190 µl of reagent solution containing 10 mM p-nitrophenyl phosphate and 5 mM magnesium acetate and the color intensity of the reaction mixture was measured at 405 nm at time zero and again after 4 min on an absorbance plate reader. The ALP activity of each sample was calculated according to a formula provided in the kit. ALP activity was normalized with respect to the total protein content obtained from the same cell lysate, and expressed as number of IU of p-nitrophenol formation per minute per gram of total proteins (IU min^{-1} g^{-1} protein).

Calcium deposition

The amount of calcium deposited on the substrates by the cells after 3 weeks of culture was measured using QuantiChrom^{TM} Calcium Assay Kit according to manufacturer's instructions. Briefly, the substrates were washed twice with PBS and soaked in 6 N hydrochloric acid overnight with shaking to dissolve the calcium content. The supernatants were then collected and 5 µl aliquot was added to 200 µl of working solution of the kit. After incubation for 3min, the absorbance was measured at 612nm on an absorbance plate reader. A Ca^{2+} standard curve calibrated using known Ca^{2+} concentrations were used to correlate the measured intensities.

Statistical analysis

At least three samples per time point for each experimental condition were used. The results are reported as mean ± SD and one-way analysis of variance (ANOVA) with Tukey-Kramer *post-hoc* test was used to assess the normally distributed data. Statistical significance was accepted at $P < 0.05$.

5.3 Results

Determination of bioconjugation

The amount of BMP2 bound to the coated substrates was determined by ELISA kit to be 7.7 ± 2.0 ng/cm^2 (62%) at a loading of 12.5ng, 13.6 ± 2.0 ng/cm^2 (54%) at a loading of 25ng, 23.0 ± 3.0 ng/cm^2 (52%) at a loading of 50ng, 37.3 ± 4.0 ng/cm^2 (37%) at a loading of 100ng, 59.2 ± 5.0 ng/cm^2 (24%) at a loading of 250ng and 108.5 ± 12.0 ng/cm^2 (22%) at a loading of 500ng. Loading at 1µg/ml would provide the optimal binding rate, however because high density BMP2 is needed for osteogenesis, therefore another concentration 10µg/ml was used for the fabrication of the CoCr-PDOP-BMP2 substrates for comparison. The results is summarized in table 5.2.

Absolute loading quantity (ng)	Concentration (µg/ml)	Binding as determined by ELISA(ng/cm^2)	Percentage bound over absolute quantity loaded
12.5	0.25	7.7 ± 2.0	62%
25	0.5	13.6 ± 2.0	54%
50	**1**	**23.0 ± 3.0**	**52%**
100	2	37.3 ± 4.0	37%
250	5	59.2± 5.0	24%
500	**10**	**108.5±12.0**	**22%**

Table 5.2 Binding optimization of Cobalt Chromium with BMP2.

SEM-EDX characterization

SEM-EDX was used to characterize the CoCr substrates obtained from the manufacturer to check for the uniformity and quality of the alloy obtained. From the results (Fig. 5.1a) approximately 70% of the substrates consist of the element Co and 30% consist of the element Cr, uniformly distributed which conforms to the requirements in our studies.

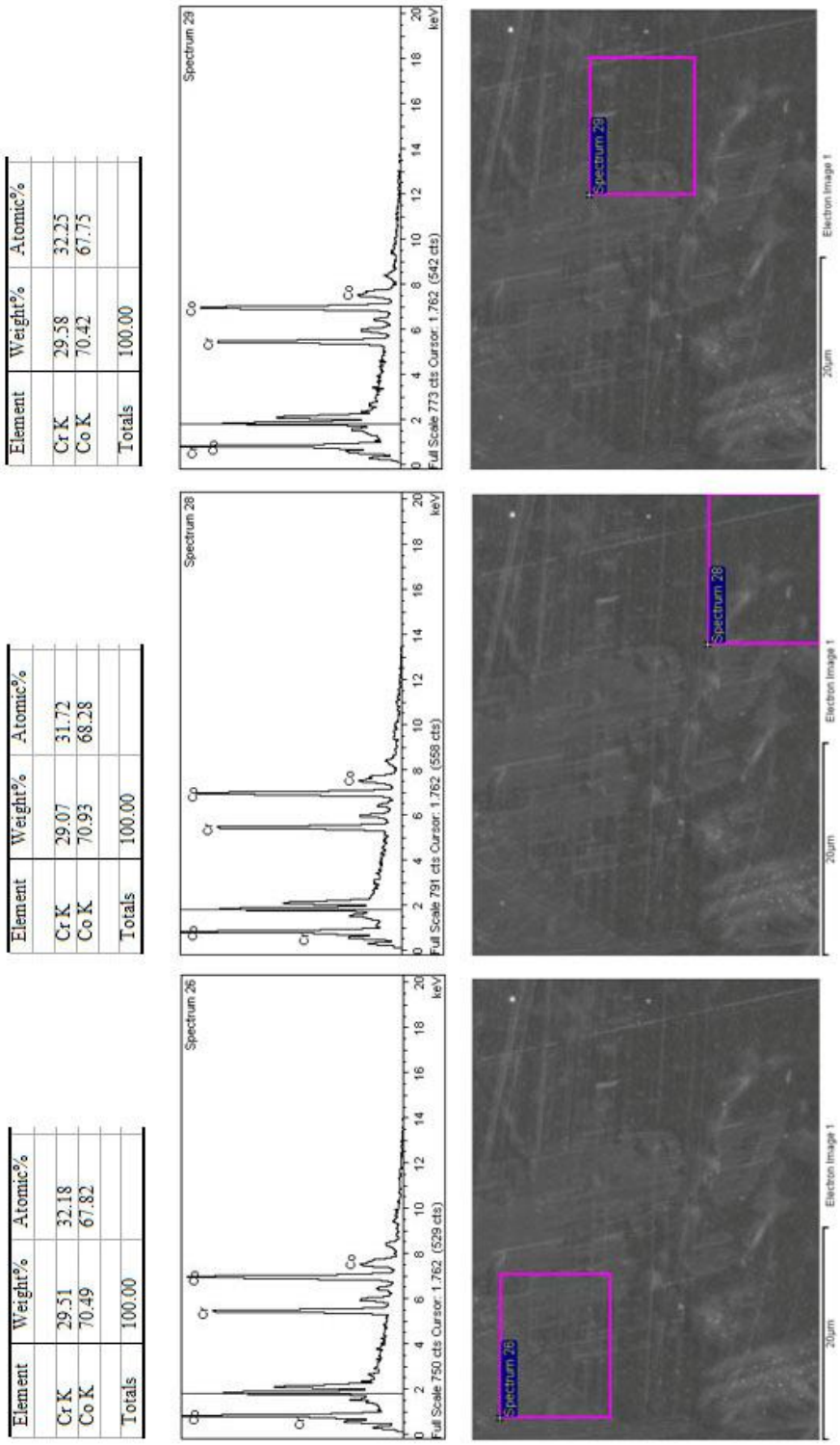


Figure 5.2 Elemental analysis of unmodified CoCr substrates from three different regions.

Surface characterization

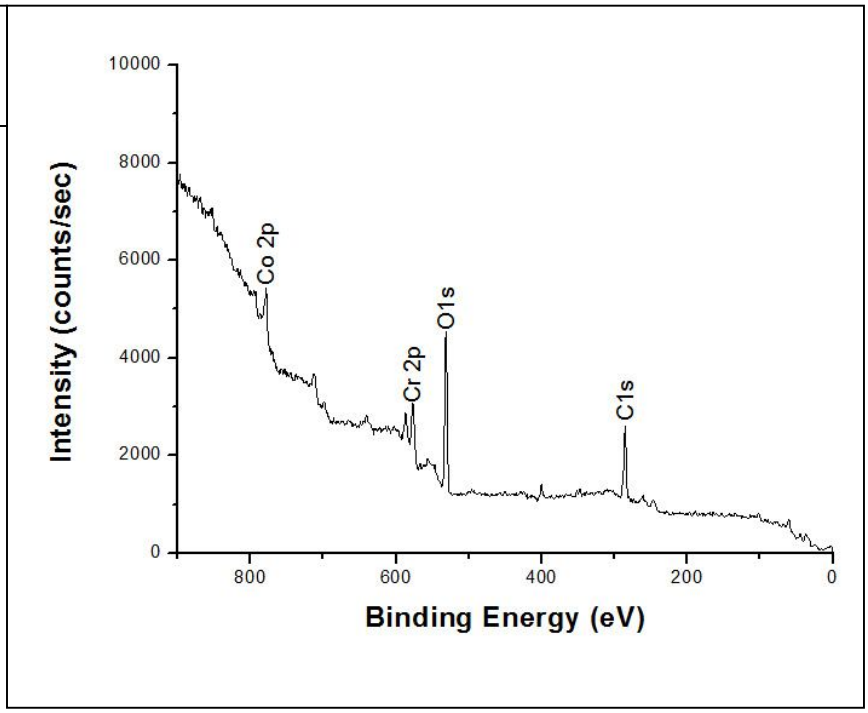
The XPS wide scan spectra of the pristine CoCr, CoCr-PDOP and CoCr-PDOP-BMP2 and their corresponding surface elemental compositions are shown in Table 5.1 and Fig. 5.3 respectively. In the wide scan spectrum of the pristine CoCr (Fig. 5.3(A)), the predominant components are C 1s (285 eV), O 1s (530 eV) and Co 2p (780 eV). Successful deposition of polydopamine on the CoCr substrate was indicated by an increase in the N and C contents while successful deposition of the growth factor on the CoCr substrate was indicated by a further increase in the N content. The changes in the surface atomic ratio of the functionalized substrates are quantified and summarized in Table 5.1.

Substrate	C%	N%	O%	Co%
CoCr	36.7	0.9	45.2	17.2
CoCr-PDOP	71.8	8.9	19.3	-
CoCr-PDOP-BMP2(1µg/ml)	71.2	9.5	19.3	-
CoCr-PDOP-BMP2(10µg/ml)	69.1	11.7	19.2	-

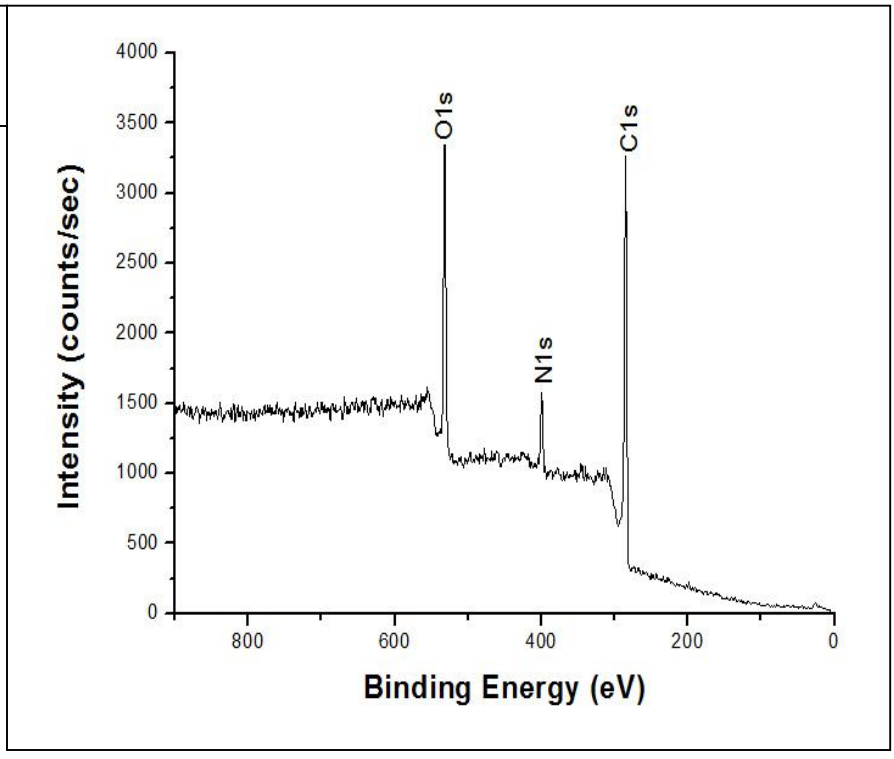
Table 5.1 Elemental Composition* at the Surface of Pristine and CoCr -PDOP and CoCr-PDOP-BMP2 substrates as determined by XPS

*Percentages computed based on the C, N, O and Co contents only.

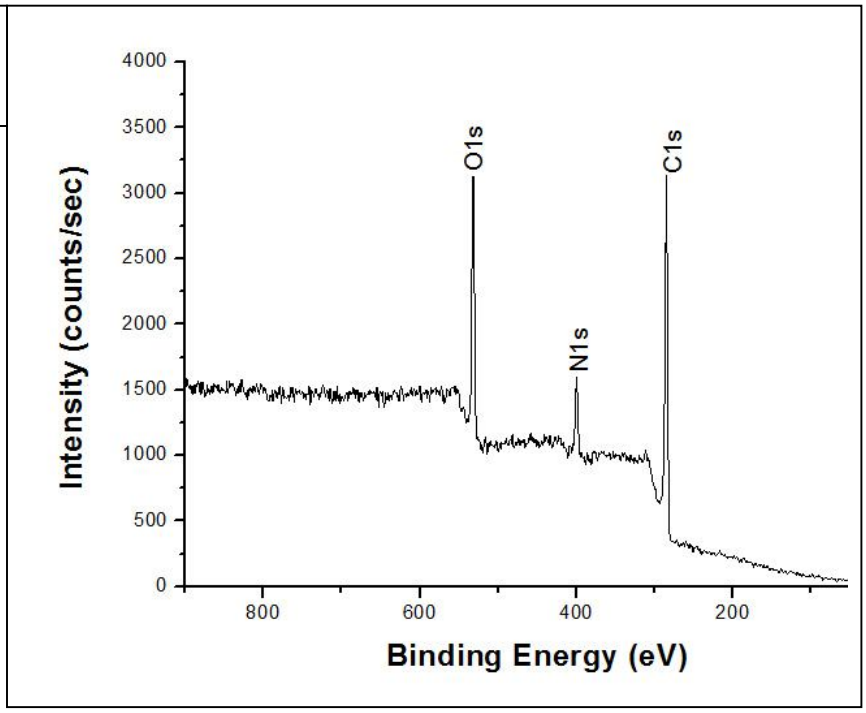
(A) Pristine CoCr



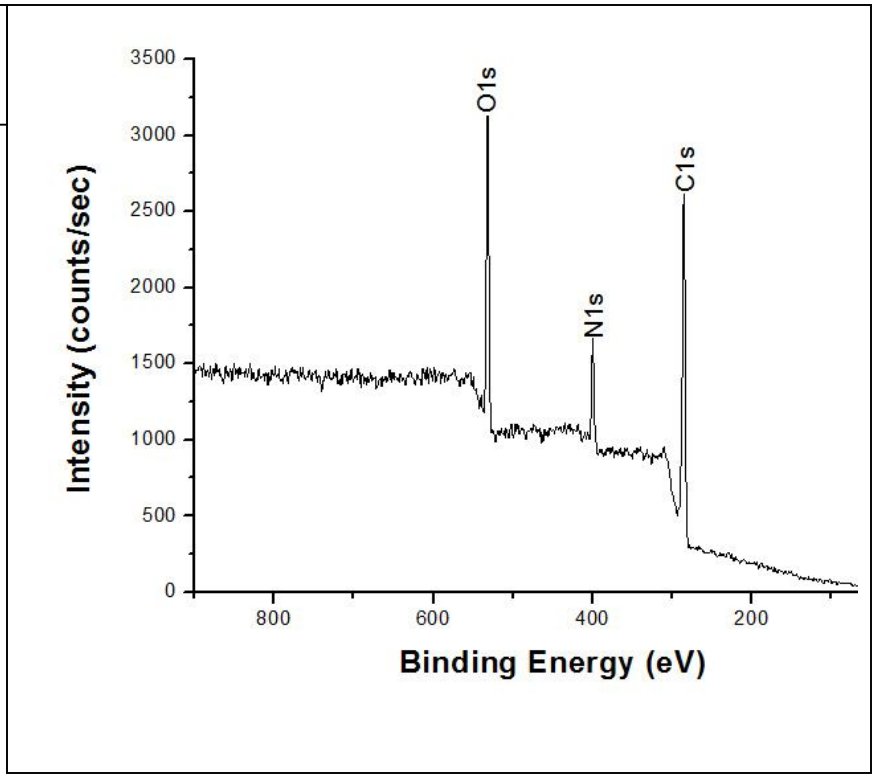
(B) CoCr-PDOP



(C) CoCr-PDOP-BMP2 (1µg/ml)



(D) CoCr-PDOP-BMP2 (10µg/ml)



Cell adhesion

From the results (Fig. 5.4) cellular adhesion on the CoCr-PDOP-BMP2 (10 µg/ml) substrates was significantly higher than that on the pristine CoCr. There is approximately 2-fold increase in the number of cells attached to the CoCr-PDOP-BMP2 (10 µg/ml) substrates compared to the pristine CoCr.

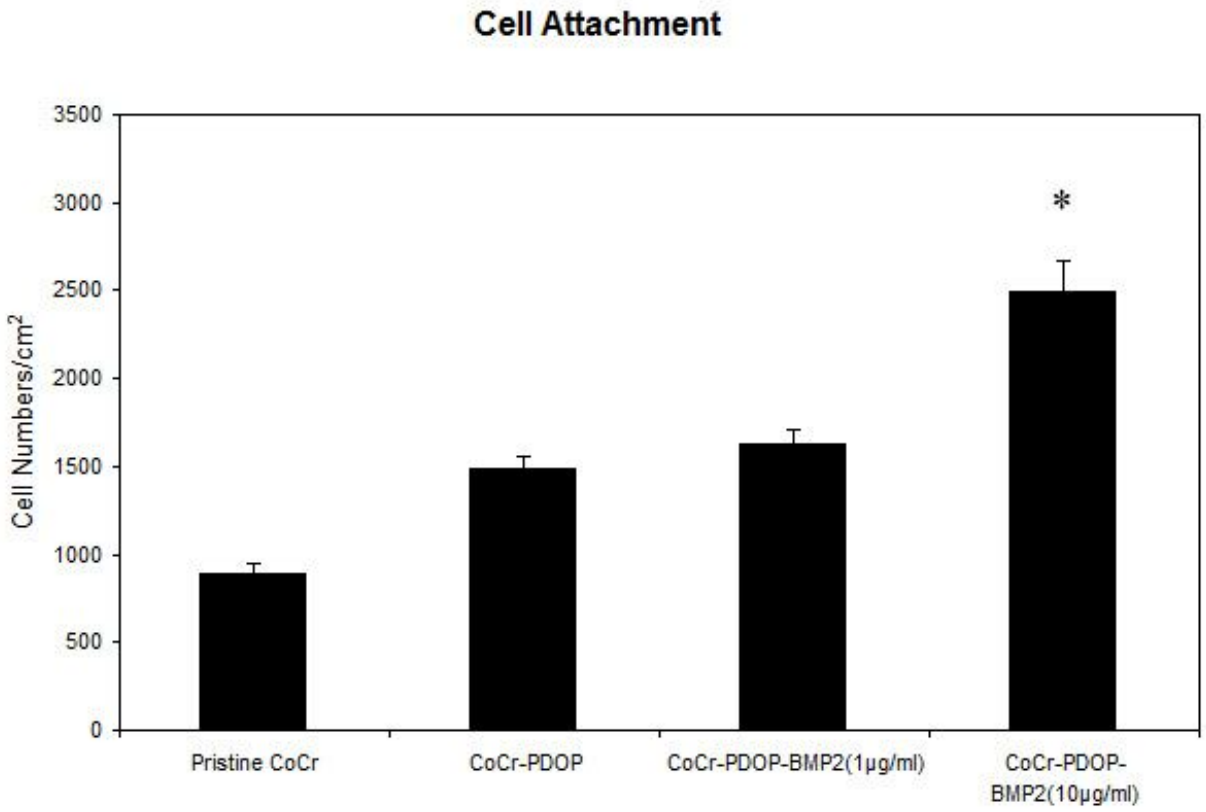


Figure 5.4 Number of adherent MC3T3-E1 per cm^2 on surfaces of pristine CoCr, CoCr-PDOP, CoCr-PDOP- BMP2 (1 µg/ml) and CoCr-PDOP-BMP2 (10 µg/ml) substrates (n=3). (*) denote significant differences ($P < 0.05$) compared with the pristine CoCr.

Cytotoxicity

Cell viability was assessed using MTT assay to investigate the cytotoxic profile of the different substrates. As shown in Fig. 5.5, MC3T3-E1 seeded on the CoCr-PDOP-

BMP2 (10 µg/ml).substrates showed significantly higher viability compared to both the pristine CoCr and CoCr-PDOP substrates.

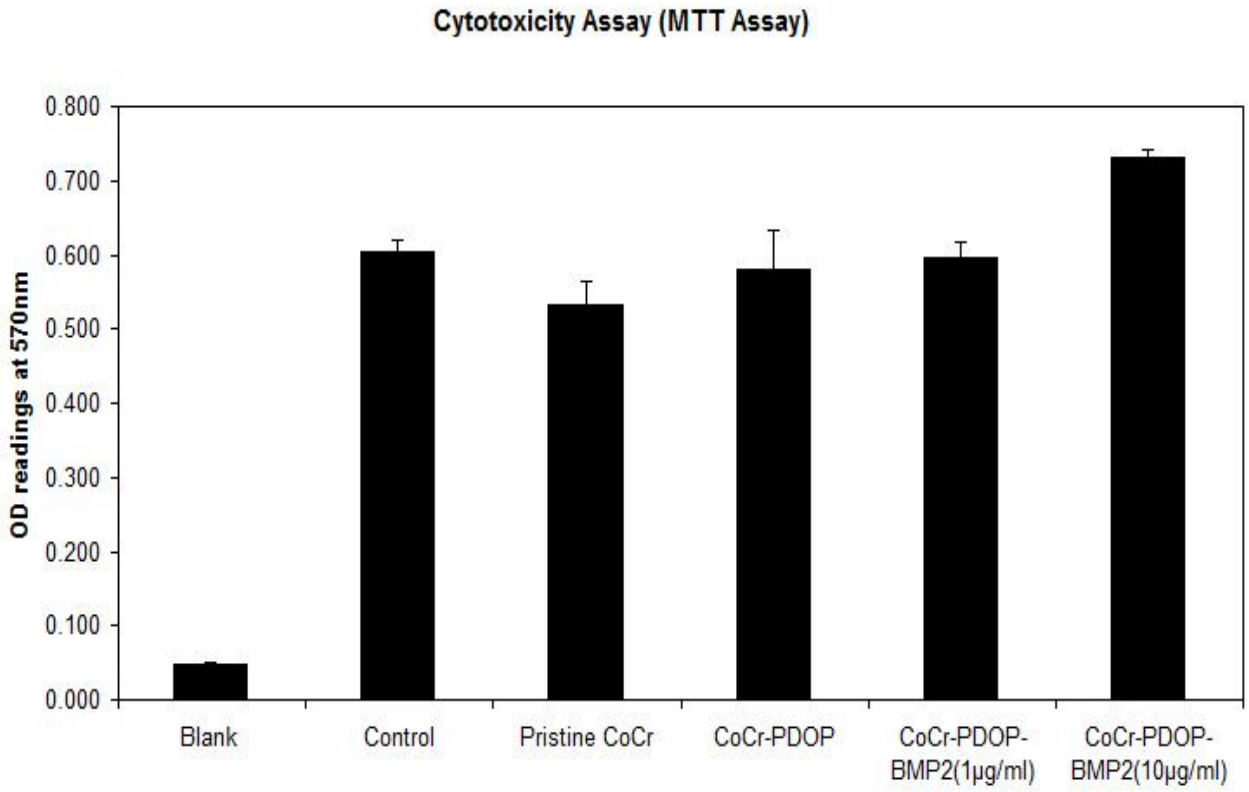


Figure 5.5 Cell viability measured by MTT assay on the control, pristine CoCr, CoCr-PDOP, CoCr-PDOP- BMP2 (1 µg/ml) and CoCr-PDOP-BMP2 (10 µg/ml).substrates after 1 week (*n*=3).

ALP assay & calcium deposition assay

ALP activity was measured after the cells were cultured for 1 and 2 weeks on the substrates. Calcium deposition was measured after the cells were cultured for 3 weeks on the substrates. The choice of the different time points is due to the fact that ALP usually peaks before mineralization actually begins [125]. From Fig. 5.6 & Fig. 5.7, it can be seen that ALP activity and calcium deposition of the CoCr-PDOP-BMP2 (10 µg/ml). substrates were significantly higher than those on the pristine CoCr, CoCr-PDOP and

CoCr-PDOP-BMP2 (1 µg/ml) substrates. There is roughly a 2-fold increase in ALP activity after 2 weeks of incubation and a 3-fold increase in calcium content after 3 weeks of incubation in CoCr-PDOP-BMP2(10 µg/ml) substrates compared to the pristine ones.

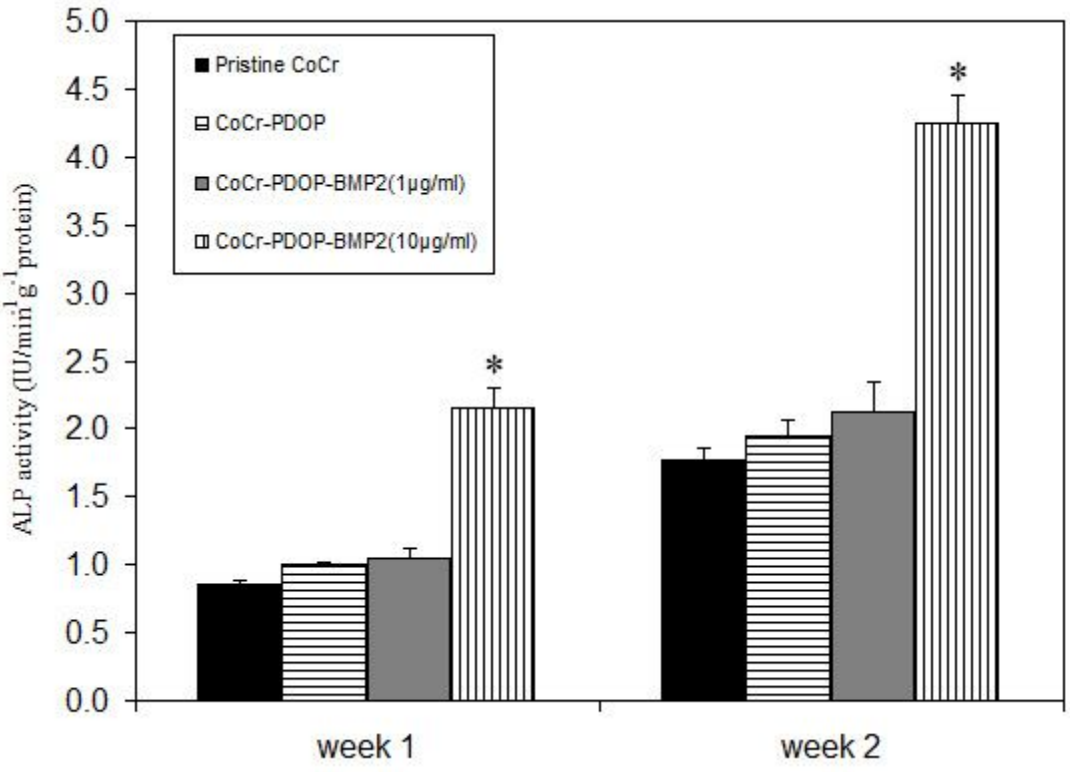


Figure 5.6 ALP activity of MC3T3-E1 seeded on pristine CoCr, CoCr-PDOP, CoCr-PDOP- BMP2 (1 µg/ml) and CoCr-PDOP-BMP2 (10 µg/ml)substrates on week 1 and 2 (n=3). (*) denote significant differences ($P < 0.05$) compared with the pristine CoCr.

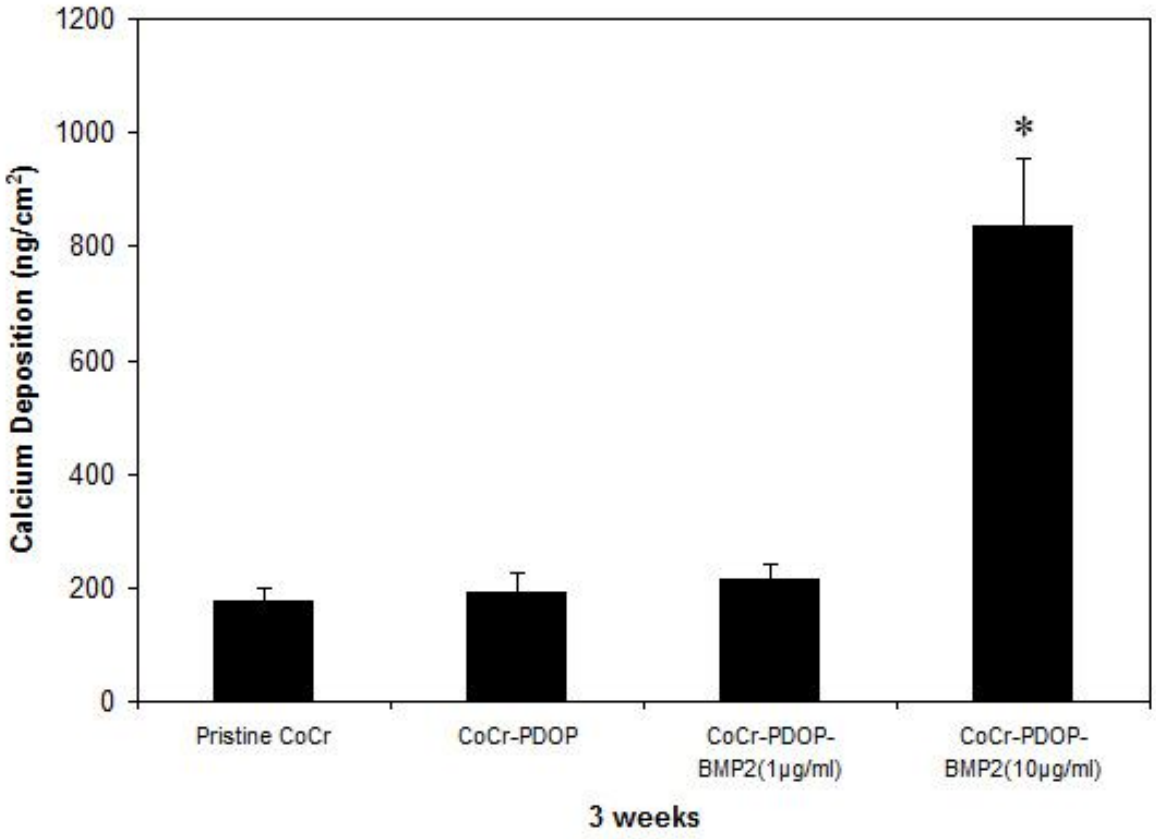


Figure 5.7 Calcium deposition of MC3T3-E1 seeded on pristine CoCr, CoCr-PDOP, CoCr-PDOP-BMP2 (1 µg/ml) and CoCr-PDOP-BMP2 (10 µg/ml) substrates on week 3 (n=3). (*) denote significant differences ($P < 0.05$) compared with the pristine CoCr.

5.4 Discussion

Cobalt Chromium alloys are currently one of the most popular implant materials used in orthopaedics [126]. However due to its lack of osseointegrative properties, implant improvements conferring enhanced bone growth would drastically improve clinical results and reduce peri and post-operative complications and patient morbidity. BMP2 is known to induce bone and cartilage formation and plays a key role in osteoblast differentiation. Therefore in this study we determined the effects of CoCr functionalized with BMP2 on osteoblastic cells. To validitate the functionalization procedures, the chemical composition of the surfaces at various stages of surface modification was determined by XPS. Successful deposition of polydopamine on the CoCr substrate was indicated by an increase in the N and C contents as shown in Table 5.1. Nitrogen-to-carbon (N/C) ratios is 0.124 on CoCr-PDOP which is similar to the theoretical N/C of 0.125 for dopamine. Complete suppression of photoelectron peaks unique to Co 2p (about 780 eV) confirms formation of the polydopamine thin film (Fig. 5.3(B)). Successful deposition of the growth factor on the CoCr substrate was indicated by a further increase in the N content. Two concentrations of BMP2 was used for the investigation as during the initial stages of the experiments, it was found that loading at a concentration of 1 μg/ml was not sufficient to elicit any significant responses, therefore an additional concentration at 10 μg/ml was used.

Attachment of cells onto the implant surface is a prerequisite for successful osseointegration and cell attachment assays (Fig. 5.2) showed that the CoCr-PDOP-BMP2 (10 μg/ml) substrates had a significantly higher number of cells compared to the other groups. This suggests that immobilizing a high concentration of BMP2 was able to

enhance the initial cell attachment. This is in accord with other studies where immobilized BMP2 was able to increase the number of attached osteoblasts [127, 128]. Cytotoxicity assays did not show any significant differences among all the experimental substrate groups. Besides the initial cellular adhesion and material cytotoxicity, subsequent ALP activity and calcium deposition are important considerations for osseointegration. ALP activity is used as a marker for early differentiation of osteoblastic cells [129, 130] and was measured after the cells were cultured for 1 and 2 weeks on the various substrates. From Fig. 5.4 it can be observed that ALP activity of osteoblastic cells seeded on CoCr-PDOP and CoCr-PDOP-BMP2 (1 μg/ml) is at a similar level as those found on pristine CoCr throughout the 2 weeks period. On the other hand, osteoblastic cells cultivated on the CoCr-PDOP-BMP2 (10 μg/ml) substrates have a significantly higher ALP activity. A high immobilized BMP2 density is able to increase the ALP activity of osteoblasts significantly which in turn indicates enhanced bone cell functions and matrix production. Calcium mineral deposition is a late bone differentiation marker for bone matrix formation [131]. The amount of calcium deposited after the cells were cultured for 3 weeks on the various substrates are shown in Fig. 5.7. As corroborated by the ALP activity, there is no significant difference in calcium deposition between pristine CoCr, CoCr-PDOP and CoCr-PDOP-BMP2 (1 μg/ml). However in the CoCr-PDOP-BMP2 (10 μg/ml) substrates the calcium deposition was increased to about 3 fold, indications of a stimulating effect on matrix formation. *In vitro* work with BMP2 in soluble, free form have shown that it can stimulate early differentiation and matrix mineralization of osteoblastic cells [38, 41, 47, 132, 133]. However such an approach is not appropriate for implant applications, as there are concerns about the long-term effects

of released soluble growth factor on the fracture site and their non-specific distribution throughout the body via the bloodstream [134, 135]. The use of covalent immobilization would circumvent these issues.

5.5 Conclusion

This study has shown that the immobilization of BMP2 on Cobalt Chromium has the ability to confer enhancement of cell-implant interactions between the originally inert substrate and osteoblastic cells, without demonstrable cell toxicity, resulting in enhanced bone matrix formation and bone growth. However the cost would be prohibitive for most patients, as huge quantities and a high immobilized BMP2 density would be needed and the problem is further aggravated by the short shelf lives of the growth factor. All these would pose significant problems for the usage of BMP2 in clinical applications, therefore in the next chapter we sourced for a viable alternative to replace BMP2.

Chapter 6

6.1 Growth Factor Versus Peptide

In the previous chapter the surface functionalization of BMP2 on CoCr substrates was achieved. However there are certain issues associated with the use of BMP2 including the huge quantity of BMP2 needed to stimulate bone growth, it is expensive and has a short shelf life. Therefore we sourced for an alternative molecule as a viable replacement for BMP2.

BMP Peptide

BMP2 plays integral roles in bone and cartilage formation and have been demonstrated to be promising in modulating bioimplant-cell interactions. However current BMP2 applications are limited in its usage, such as in cell culture supplements, with bone matrix materials such as collagen for enhanced osteoinduction, in recalcitrant non-union fractures and spinal fusion, albeit at hefty costs, to the tune of US$3500 onwards [120, 136, 137]. Recent studies have reported the utility of BMP Peptides on effective induction of bone growth and accelerated bone healing [42, 43, 127]. BMP Peptides possess various advantages compared to BMP2, such as increased stability, incorporation at higher concentrations, and more significantly, lower costs [43]. Shelf lives of BMP Peptides are also significantly longer, as they can be stored in freeze-dried conditions for over 2 years, whereas BMP2 can only be stored for a short time, e.g. 6 months at $4^{o}C$ [43].

Therefore the objective of this study is to investigate the effects of CoCr substrates immobilized with BMP peptide on osteogenesis. Dopa polymerization was

used to create a thin reactive layer for covalent coupling with the BMP Peptide. The BMP Peptide is derived from residues 73-92 of BMP2 that is known to induce differentiation and mineralization of bone-marrow stromal cells [42]. The peptide was synthesized with a cysteine amino acid at the N-terminus using miniPEG (Fmoc-8-amino-3,6-dioxooctanoic acid) as the linker to produce a cys-tag functionalized BMP Peptide for site specific conjugation via thiol directed chemistry [109, 138]. Several approaches are available for the site-specific labeling and we have chosen the insertion of a cys-tag at the N-terminus to keep interference on the functional activities of the peptide to a minimum [138-140].

The effects of the functionalized substrates on the enhancement of osteogenesis were then investigated. The binding properties and efficiency of the biomimetic coating with BMP Peptide were evaluated and the biological activities of the immobilized BMP Peptide were analyzed with osteoblastic cells (MC3T3-E1). CoCr substrates immobilized with BMP2 were used as a basis of comparison here. We aim to use the above approach to provide an alternative viable cost effective solution compared to conventional BMP2 for the development of orthopaedic implant devices for use in clinical applications.

6.2 Materials and Methods

Materials

Materials and methods specific to this chapter are described below. All other materials and methods used in this chapter are described in Chapter 3 and 5 (Materials and methods section).

O-Phthaldialdehyde (OPA), 2-mercaptoethanol and Alizarin Red S were purchased from Sigma, USA. Rabbit polyclonal anti-phospho Smad1/5/8 were purchased from Millipore, USA. Goat anti-rabbit Alexfluor 488 and Phalloidin-Alexfluor 566 was purchased from Invitrogen, USA. DAPI containing mounting medium was purchased from Vectorlab, Canada.

BMP Peptide

The cys-tag functionalized BMP Peptide, CKIPKASSVPTELSAISTLYL was obtained from 1st BASE Pte. Ltd. (Sg). The purity was 96.5%, as determined by reversed-phase high-performance liquid chromatography (HPLC) on a Venusil XBP-C18 column with a 10-70% acetonitrile gradient in 0.1% trifluoroacetic acid in water, at a flow rate of 1.0ml/min, with detection at 220 nm. Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis was employed and a miniPEG (Fmoc-8-amino-3,6-dioxooctanoic acid) was used as the linker for the cysteine amino acid at the N-terminus.

Details

Sequence :Cys-miniPEG-KIPKASSVPTELSAISTLYL

Length :20AA

Purity :96.5%

HPLC Report:

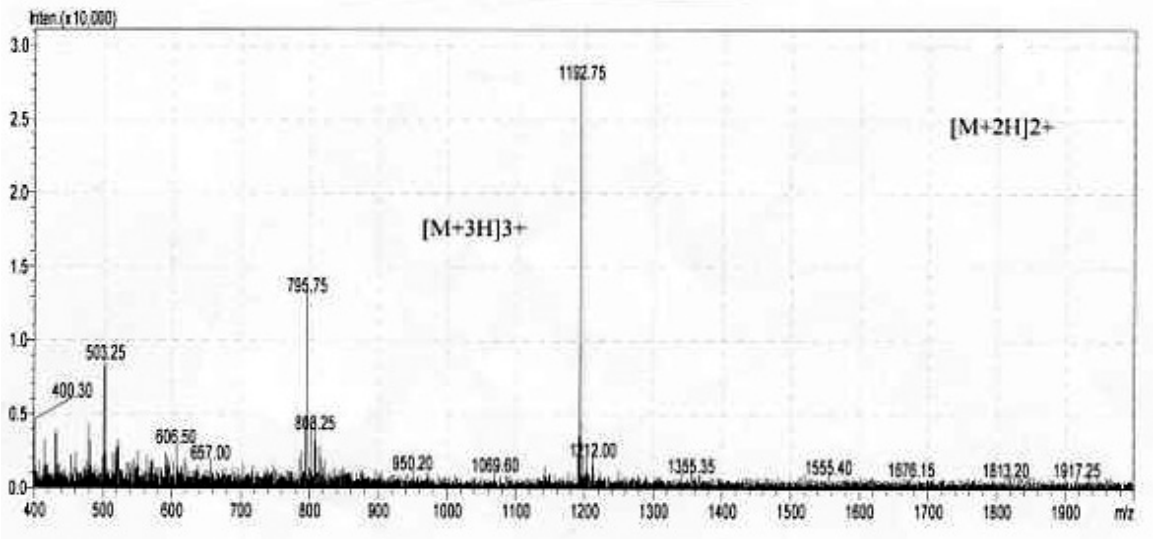
Column : 4.6mm*250mm, Venusil XBP-C18
Mobile phase :A=0.1% TFA/Acetonitrile,
:B=0.1%TFA/water,

Gradient :	A	B
0.01min	23%	77%
25min	48%	52%
25.1min	100%	0%
30.0min	Stop	

Flow rate :1.0ml/min
Wavelength :220nm
Volume :5ul

Mass Spectrometry Report

Item	Parameter	Item	Parameter
Probe:	ESI	Probe bias:	+4.5kv
Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
CDL:	-20.0v	T.Flow:	0.2ml/min
CDL Temp:	250°C	B.conc	50%H_2O50%ACN
Block Temp:	200°C		



Preparation of substrates

CoCr foils were prepared as described in chapter 5 (Materials and methods section). The polydopamine-grafted CoCr substrates were then coated with BMP2 (10 μg/ml) or BMP Peptide (1000 μg/ml) dissolved in 10mM Tris buffer, pH=8.5 and incubated overnight in a humid atmosphere at room temperature [62, 63]. The substrates were then washed three times with sterile PBS to remove unattached BMP2 or BMP Peptide and left to air dry in a sterile environment before use. The substrates are denoted CoCr (pristine cobalt chrome), CoCr-PDOP (polydopamine-grafted CoCr), CoCr-PDOP-BMP2 (10 μg/ml) (BMP2 coated, polydopamine-grafted CoCr) and CoCr-PDOP-BMP Peptide (1000 μg/ml) (BMP Peptide coated, polydopamine-grafted CoCr) in subsequent discussions.

Determination of bioconjugation

O-Phthaldialdehyde (OPA) assay was used to verify the bioconjugation efficiency of BMP Peptide. OPA reacts with amino groups in the presence of thiol-containing

molecules such as 2-mercaptoethanol, generating a fluorescence product. Detection limits for proteins in liquid are in the μg/cm^3 range [141]. A solution of 1ml borate buffer (50mM PH=9.2), 250 μl o-Phthaldialdehyde (OPA) (Sigma, USA, 20mg/ml) and 250μl 2-mercaptoethanol (Sigma, USA) were added to the amino-group containing carrier and reacted for one hour at room temperature. After washing three times with 75% ethanol, the fluorescence marking by OPA were observed under a fluorescence microscope (Axioplan 4.4, Zeiss) using excitation wavelength of 360nm and emission wavelength of 436 nm.

Characterization

XPS, SEM and AFM analysis was carried out as described in Chapter 3 & 4 (Materials and methods section).

Cell culture

Cell culture were performed as described in Chapter 5 (Materials and methods section).

Cellular adhesion

Cellular adhesion assays were performed as described in Chapter 5 (Materials and methods section).

Cell morphology

The cell morphology of MC3T3-E1 cells were examined by detecting filamentous actin of the cytoskeleton by immunofluorescence. After seeding on the substrate for 48 hours, MC3T3-E1 was washed with PBS three times and fixed with 4% paraformaldehyde. After permeabilizing with 0.2% Triton X-100 in PBS for 5 min the cells were incubated with phalloidin-rodamine (Invitrogen, CA) for 20 min and the nucleus counterstained with DAPI. The cells were then viewed using a fluorescent microscope (Olympus IX71).

Cytotoxicity assay

Cytotoxicity analysis was carried out as described in Chapter 3 (Materials and methods section).

Immunostaining

Immunostaining with anti-phospho Smad1/5/8 was performed to evaluate BMP signaling via Smad-dependent pathways. Cells were seeded on the substrates for 24 hours before fixation with 4% paraformaldehyde. The primary antibody for immunostaining was rabbit polyclonal anti-phospho Smad1/5/8 (1:200 dilution; Millipore, USA). Secondary antibody was goat anti-rabbit Alexfluor 488 (Invitrogen, USA). Phalloidin-Alexfluor 566 (Invitrogen, USA) was incubated with specimens for cytoplasm counterstaining. Cell nucleus was counterstained with DAPI containing mounting medium (Vectorlab, Canada).

Alkaline phosphatase (ALP) activity assay & calcium deposition

ALP activity and calcium deposition were assessed as described in Chapter 5 (Materials and methods section).

Alizarin red staining

Cells grown on the various substrates were stained with 1% alizarin red for 2 minutes and then washed with PBS. The stained cells were then observed under a light microscope.

Statistical analysis

At least three samples per time point for each experimental condition were used. The results are reported as mean ± SD and one-way analysis of variance (ANOVA) with Tukey-Kramer *post-hoc* test was used to assess the normally distributed data. Statistical significance was accepted at $P < 0.05$.

6.3 Results

Determination of equivalent functional dosages of BMP Peptide to BMP2

Various concentrations of BMP Peptide and BMP2 was functionalized onto the CoCr substrates and a comparison of MC3T3-E1 proliferation over 7 days was tabulated. From the results (Fig. 6.1), immobilization of BMP Peptide at 1000 µg/ml would provide equivalent functional dosages of BMP2 at 10 µg/ml.

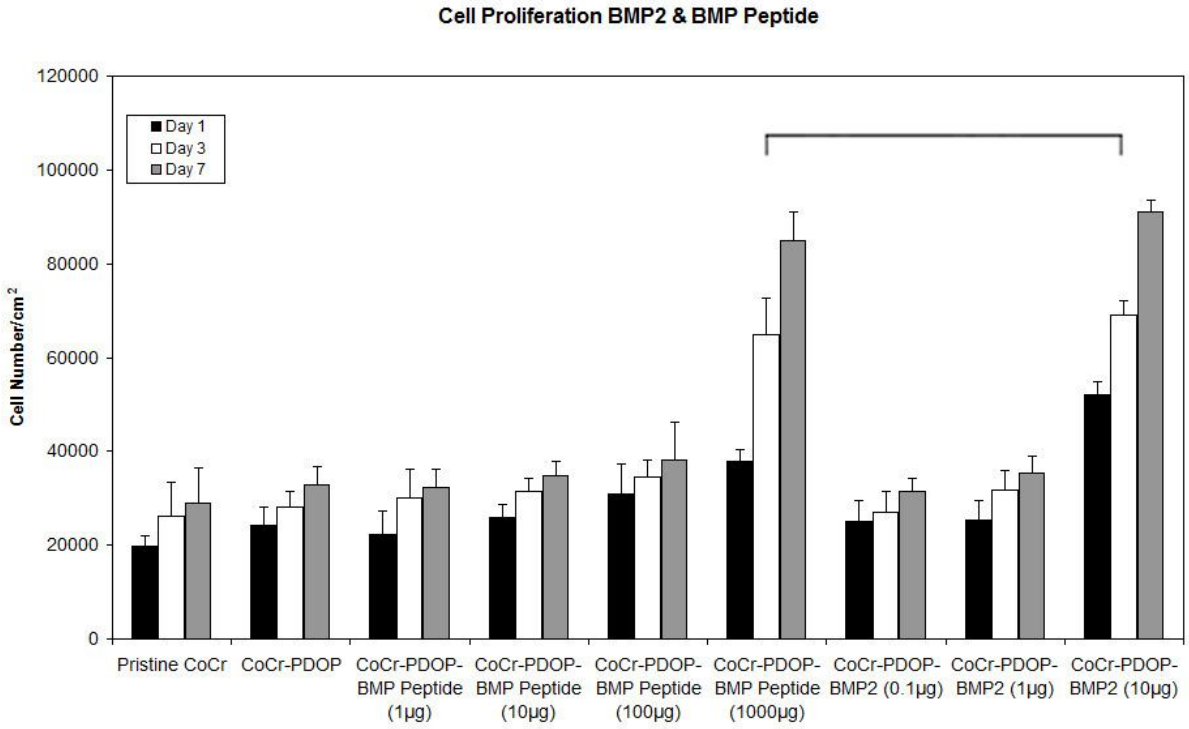


Figure 6.1 Comparison of MC3T3-E1 proliferation over 7 days on surfaces of control, pristine CoCr, CoCr-PDOP-BMP Peptide and CoCr-PDOP-BMP2 substrates at various concentrations on days 1, 3 and 7 (*n*=3).

Determination of bioconjugation of BMP Peptide

There was no positive signal on the pristine CoCr substrate (Fig. 6.2). A positive green fluorescence signal can be seen with the polydopamine-coated CoCr substrates

after reaction with OPA. Further coating with BMP peptide significantly increases the intensity of the signal.

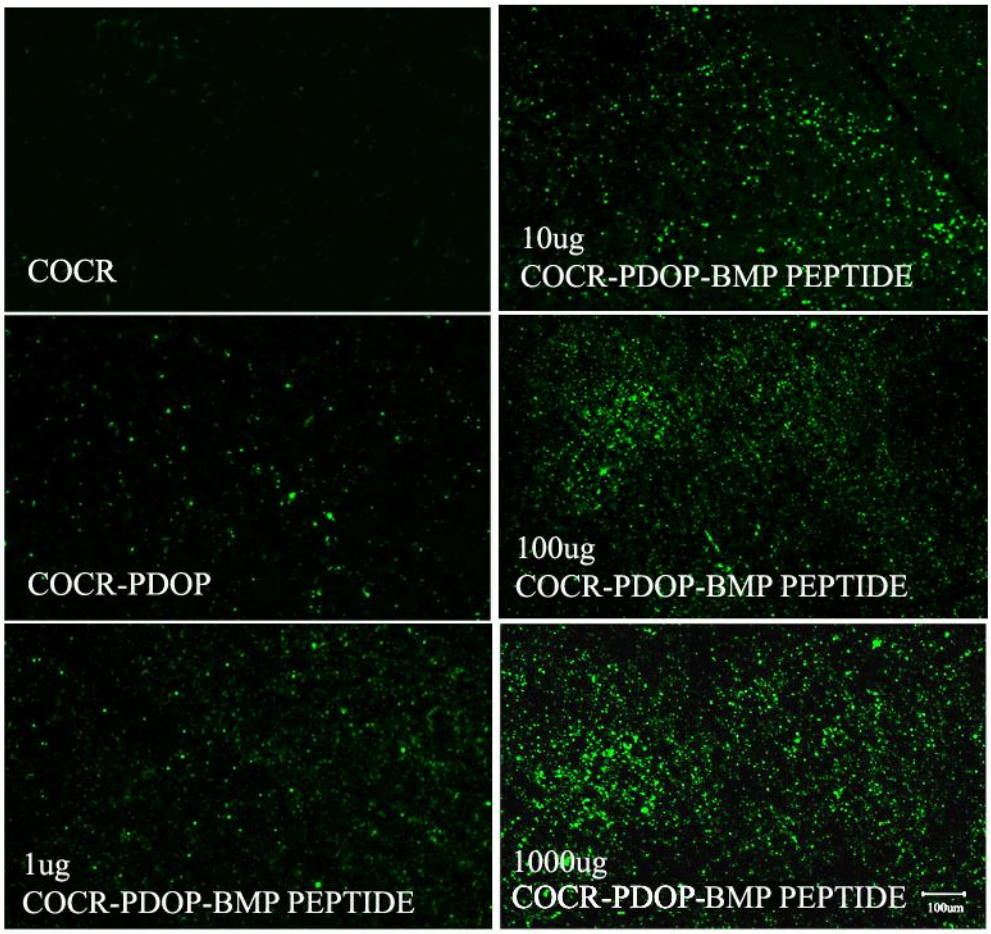


Figure 6.2 OPA images of pristine CoCr, CoCr-PDOP and CoCr-PDOP-BMP Peptide substrates at various concentrations. The scale bar represents 100 μm.

Concentration (μg/ml)	Absolute loading quantity (μg)	Intensity as determined by ImageJ (Mean)	Percentage bound over absolute quantity loaded
CoCr Negative Control	-	4.0±1	-
CoCr-PDOP	-	3.9±1	-
CoCr-PDOP-BMP	0.05	6.8±2	98.6

Peptide 1 µg			
CoCr-PDOP-BMP Peptide 10 µg	0.5	8.7±2	97.1
CoCr-PDOP-BMP Peptide 100 µg	5	12.0±3	95.3
CoCr-PDOP-BMP Peptide 1000 µg	50	19.3±2	93.8

Table 6.1 Binding Intensity of Cobalt Chromium with BMP Peptide as measured by ImageJ 1.44p Wayne Rasband, National Institutes of Health, USA. and converted to percentage over absolute quantity loaded.

Cytotoxicity

Significantly higher viability of MC3T3-E1 was observed on the CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates compared to both the pristine CoCr and CoCr-PDOP (Fig. 6.3).

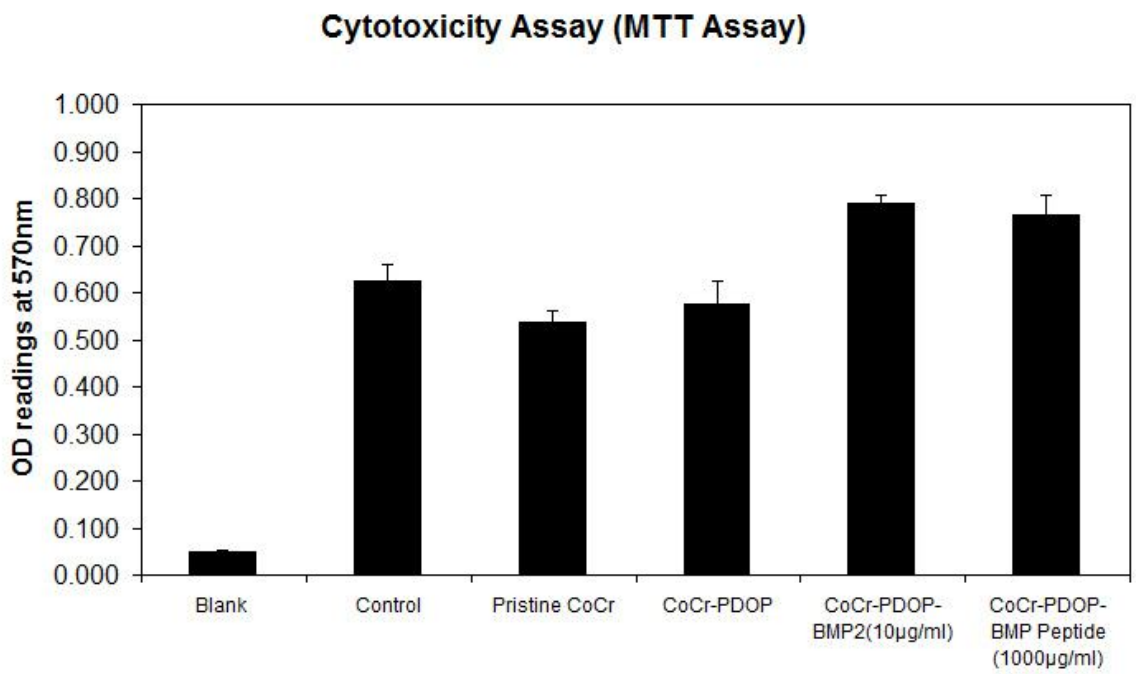


Figure 6.3 Cell viability measured by MTT assay on the pristine CoCr, CoCr-PDOP, CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates after 1 week (*n*=3).

Immunostaining with anti-phospho Smad1/5/8

The cells were responsive to BMP stimulation as seen by the activation and phosphorylation of Smad1/5/8 transcription factors (Fig. 6.4).

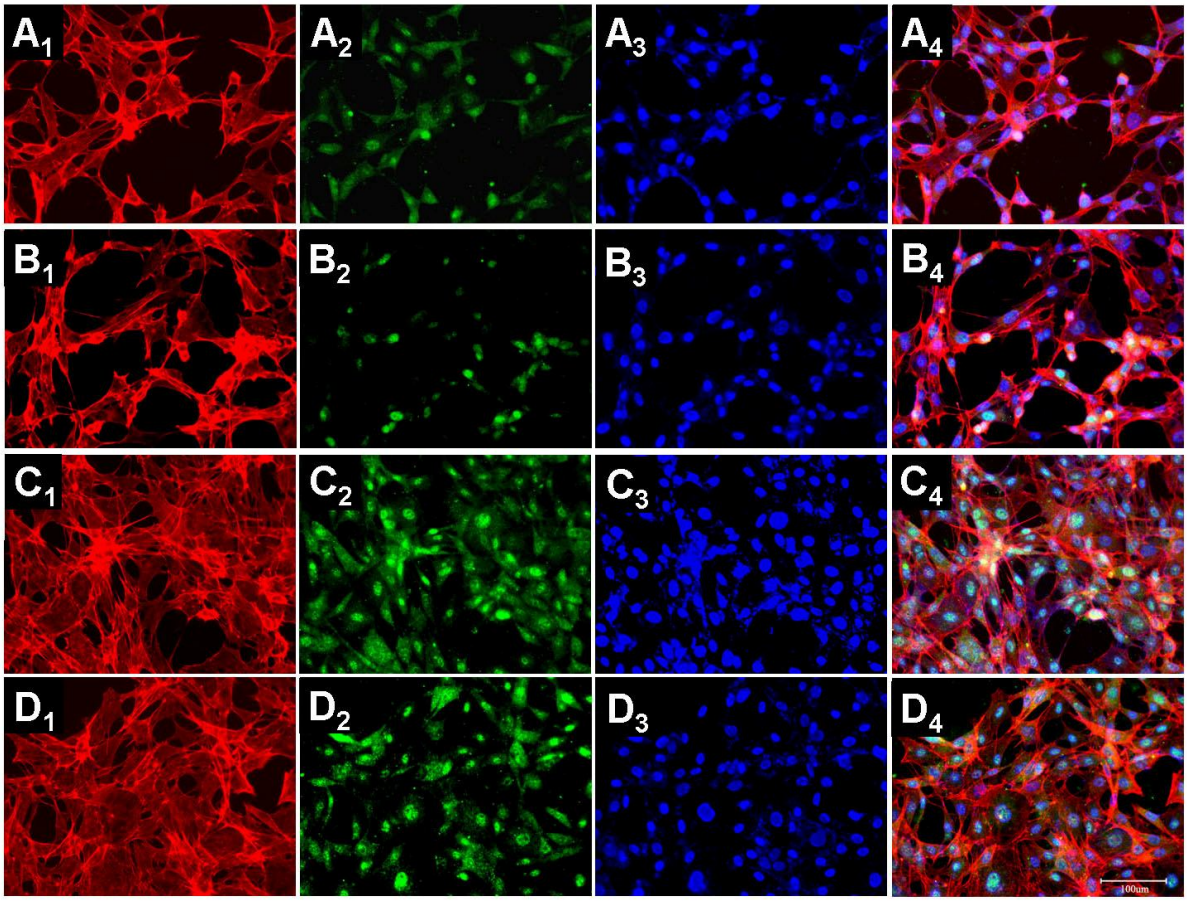


Figure 6.4 Confocal images of representative 3T3 cells on functionalized substrates. (A_1-A_4) pristine CoCr; (B_1-B_4)CoCr-PDOP; (C_1-C_4)CoCr-PDOP-BMP2 (D_1-D_4)CoCr-PDOP-BMP Peptide substrates. Red: F-actin; Green: phospho Smad1/5/8; Blue: DAPI. Scale bar: 100 μm.

Surface structure by SEM

SEM investigations revealing the structures of the pristine CoCr and the modified CoCr substrates (Fig. 6.5).

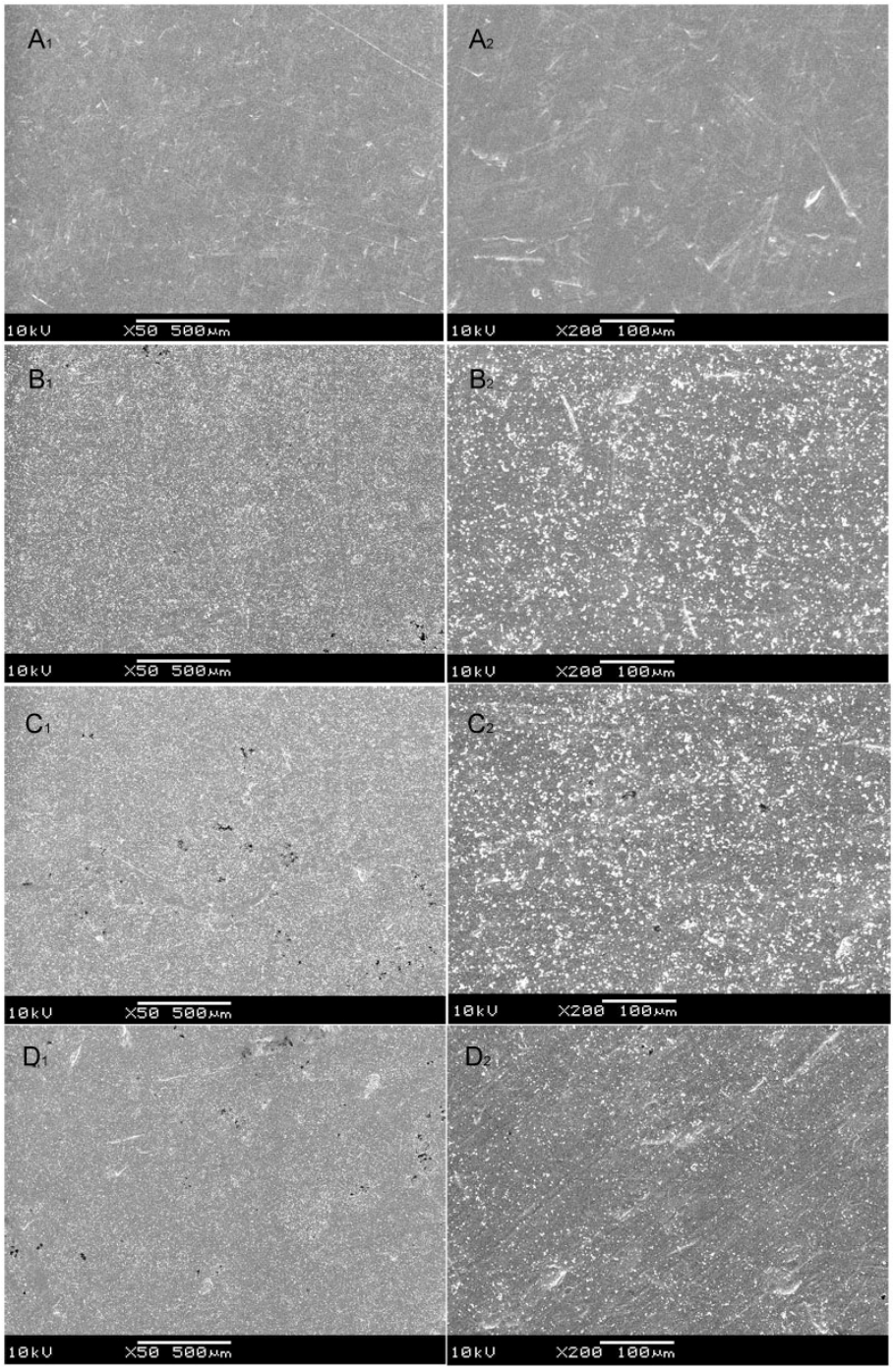


Figure 6.5 SEM images of (A) pristine CoCr, (B) CoCr-PDOP, (C) CoCr-PDOP BMP2 (10 µg/ml) and (D) CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates.

Surface texture by AFM

The surface roughness as determined by AFM (Fig. 6.6) was 139 ± 25 nm, 347 ± 48nm, 363 ± 55 nm and 351 ± 49nm for pristine CoCr, CoCr-PDOP, CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) respectively.

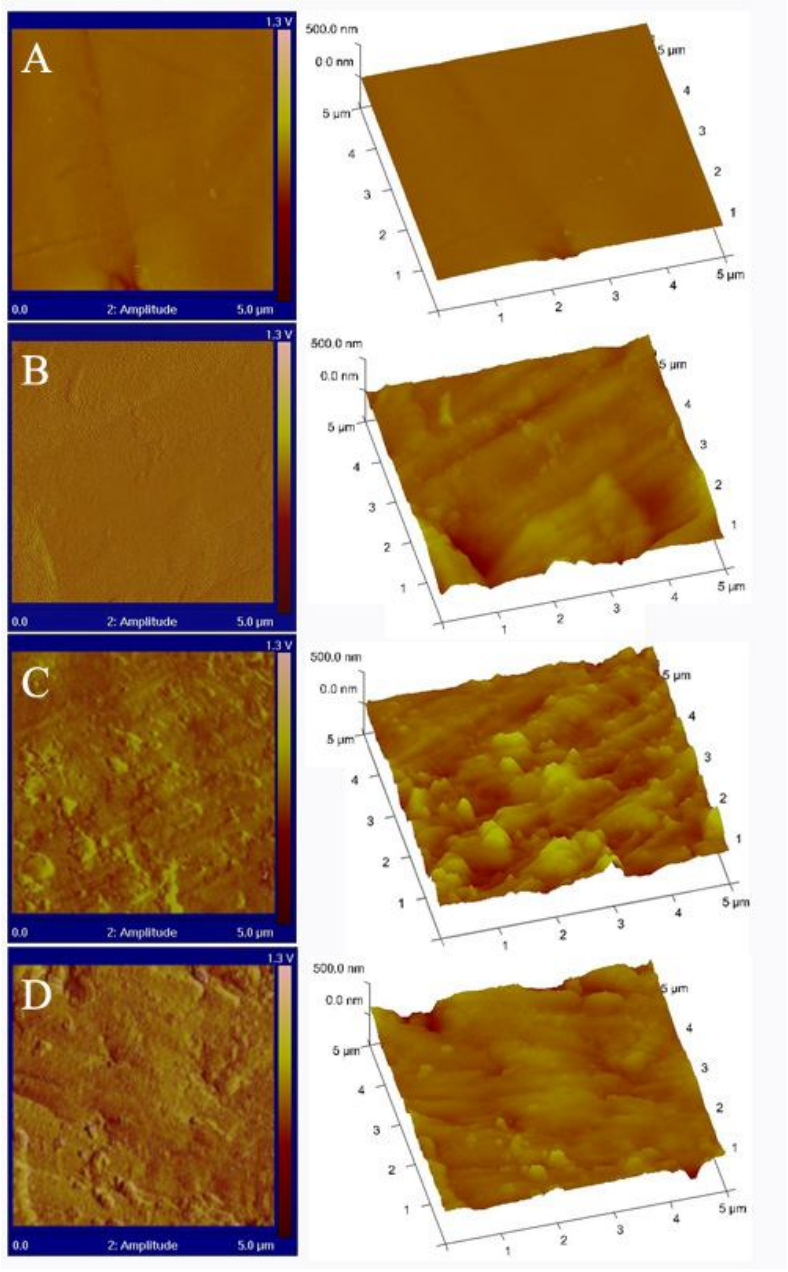


Figure 6.6 AFM images of (A) pristine CoCr, (B) CoCr-PDOP, (C) CoCr-PDOP BMP2 (10 µg/ml) and (D) CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates.

Cell adhesion

From the results (Fig.6.7) it is revealed that the number of osteoblasts on the CoCr-PDOP, CoCr-PDOP-BMP2(10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) were significantly higher than that on the pristine CoCr. There is an approximately increase of about 30% for the CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) compared to the pristine substrates.

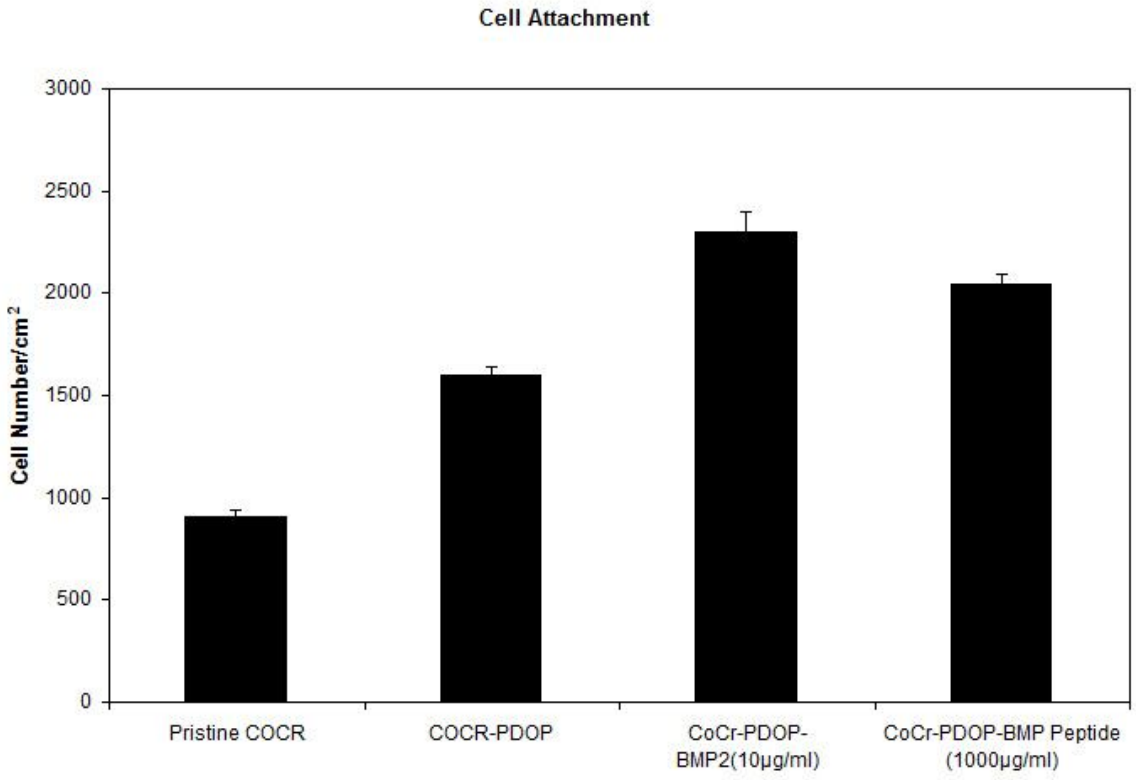


Figure 6.7 Number of adherent MC3T3-E1 per cm^2 on surfaces of pristine CoCr, CoCr-PDOP, CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates (n=3). (*) denote significant differences ($P < 0.05$) compared with the pristine CoCr.

Cellular morphology

The cellular morphology of MC3T3-E1 on different substrates after 24 h cultivation was investigated using immunochemistry staining. Cell spreading on CoCr-PDOP, CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) were enhanced compared with that on pristine COCR (Fig. 6.8). Most of the cells on CoCr-PDOP, CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates displayed a spindle shaped or elongated morphology whereas the cells on the pristine CoCr exhibited a stunted, less elongated morphology.

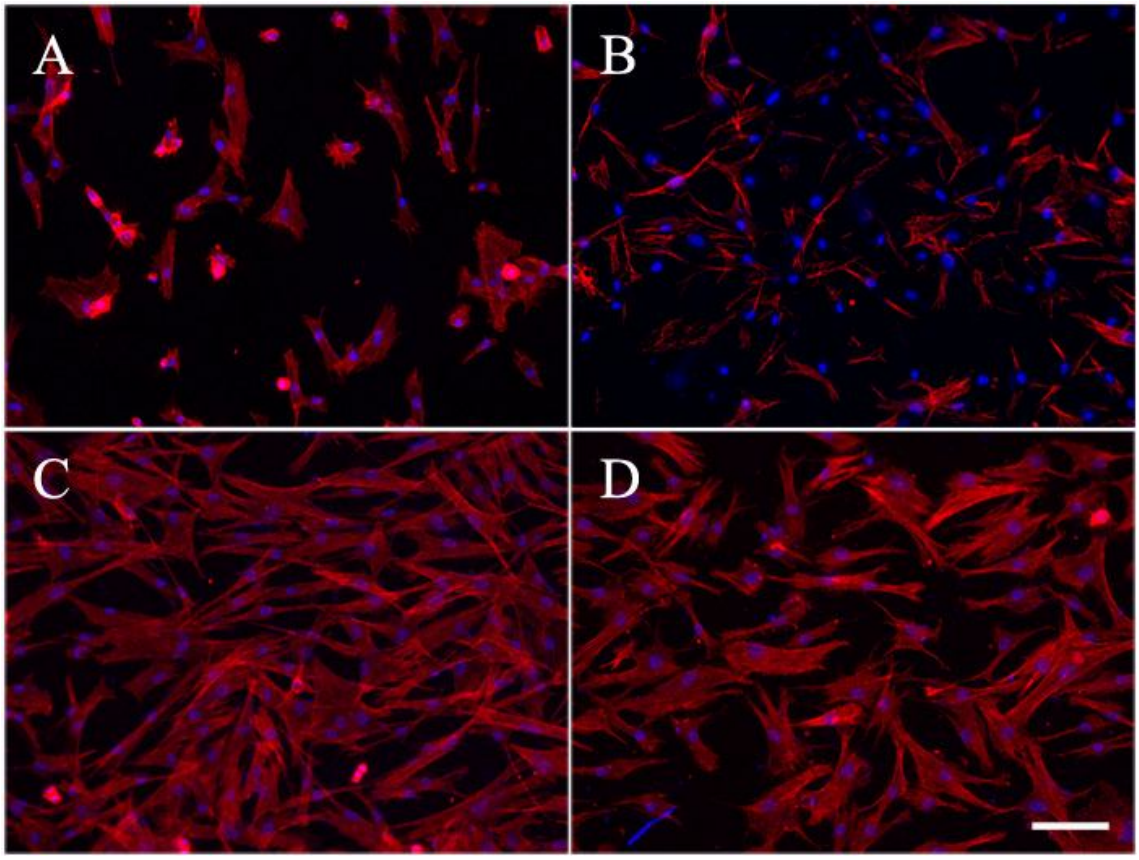


Figure 6.8 Analysis of cell morphology of MC3T3-E1 on surfaces of (A) pristine CoCr, (B) CoCr-PDOP, (C) CoCr-PDOP-BMP2 (10 µg/ml) and (D) CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates. The scale bar represents 100 µm.

ALP assay & calcium deposition

ALP activity was measured after the cells were cultured for 1 and 2 weeks on the substrates while calcium deposition was measured after the cells were cultured for 3 weeks on the substrates. From Fig.6.9 & Fig.6.10, it can be seen that ALP activity and calcium deposition of the CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates were significantly higher than those on the pristine CoCr and CoCr-PDOP substrates, with a roughly 2 fold increase in ALP activity after 2 weeks incubation and a 4 fold increase in calcium content after 3 weeks incubation compared to the pristine substrate.

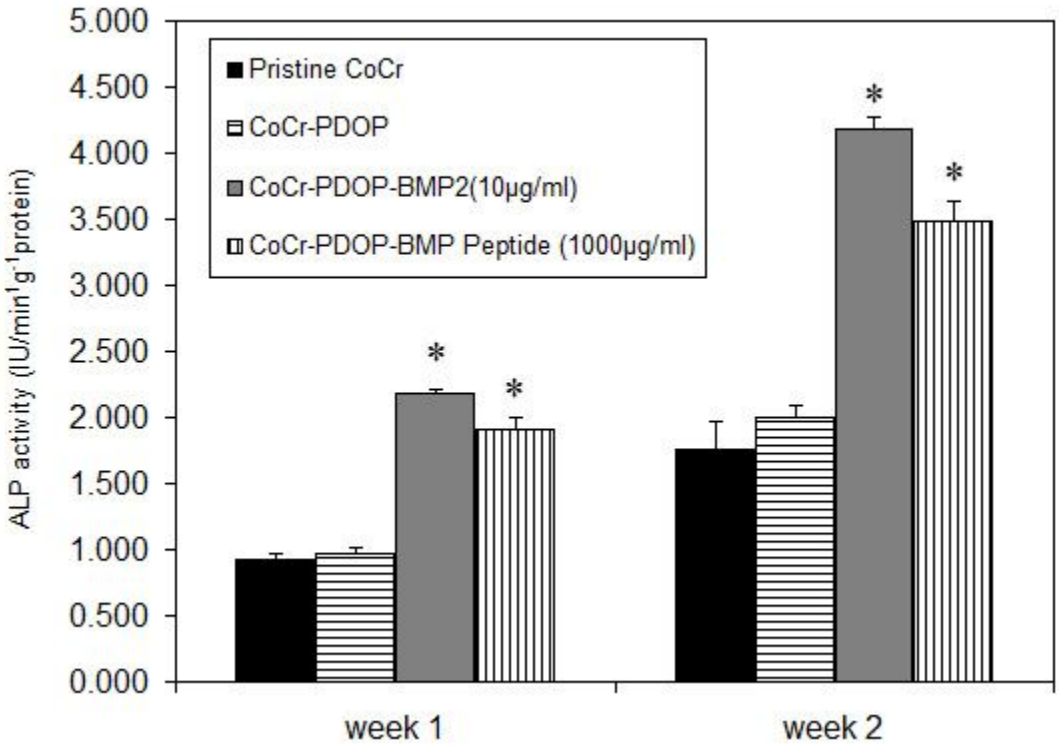


Figure 6.9 ALP activity of MC3T3-E1 seeded on pristine CoCr, CoCr-PDOP, CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates on week 1 and 2 (n=3). (*) denote significant differences ($P < 0.05$) compared with the pristine CoCr.

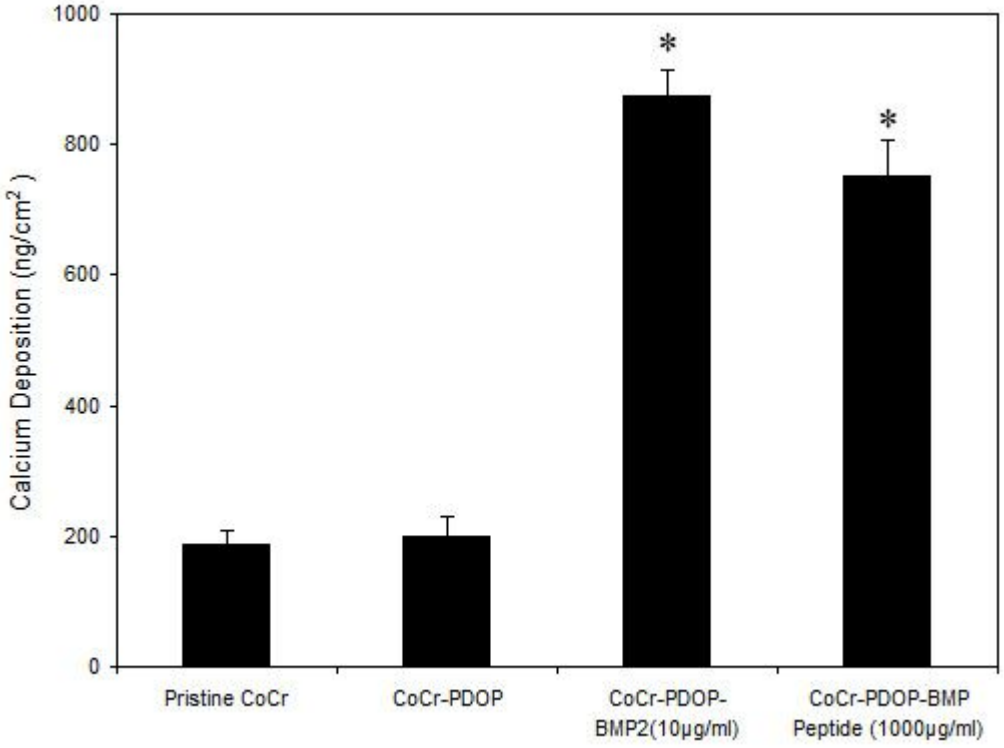


Figure 6.10 Calcium deposition of MC3T3-E1 seeded on pristine CoCr, CoCr-PDOP, CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates on week 3 (n=3). (*) denote significant differences ($P < 0.05$) compared with the pristine CoCr.

Alizarin red staining

Approximately about one third of the cells were positively stained in the CoCr-PDOP-BMP2 (10 µg/ml) and CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates compared to the pristine CoCr and CoCr-PDOP substrates (Fig.6.11).

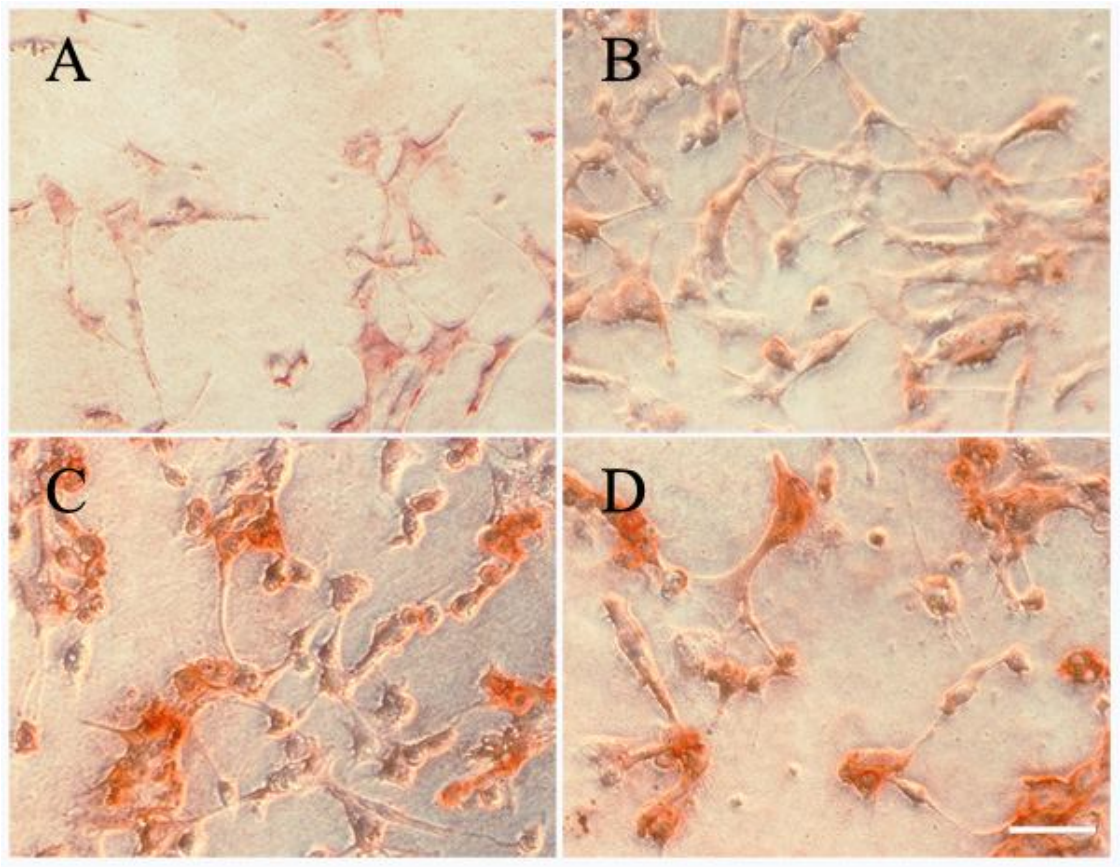


Figure 6.11 Alizarin red staining for the presence of calcium deposits of MC3T3-E1 seeded on (A) pristine CoCr, (B) CoCr-PDOP, (C) CoCr-PDOP-BMP2 (10 µg/ml) and (D) CoCr-PDOP-BMP Peptide (1000 µg/ml) substrates on week 3. The scale bar represents 50 µm.

6.4 Discussion

Although the immobilization of BMP2 on CoCr substrates can enhance osteogenesis, however the cost is enormous. BMP2 is expensive and a single intervention can cost as much as US$5000, and this has become a major issue for clinicians, patients and payers alike [142]. BMP peptide, derived from the knuckle epitope of BMP2 is known to be involved in the processes of differentiation, calcification and binds to BMP2 receptors on osteoblastic cells stimulating ALP activity [42]. There have been several studies attempting to covalently immobilize peptides on various biomaterials [143-145]. Despite these studies demonstrating the effect of peptide-coated surfaces on *in vitro* cellular responses, there is still a lack of studies on the osteogenic effects of peptides specifically immobilized on CoCr surfaces. The immobilization of BMP Peptide on such devices would enhance bone healing and regeneration by stimulating and recruiting host osteoblasts or osteoprogenitor cells.

Therefore in this study, we determined the effects of CoCr covalently grafted with BMP Peptide on osteoblastic cells. First we had to determine the equivalent functional dosages of BMP Peptide to BMP2 to elicit close or similar osteogenic responses. BMP Peptide and BMP2 is known to stimulate osteoblastic differentiation and proliferation [133, 146], therefore a comparison of MC3T3-E1 proliferation between BMP Peptide and BMP2 at various dosages of functionalization was performed. From Fig. 6.1 it was found that covalent immobilization of BMP Peptide at 1000 μg/ml would be close to BMP2 at 10 μg/ml for similar biological responses. Subsequently all further tests were performed at these 2 concentrations for comparison. Although the amount of synthetic peptide used

for the bioconjugation of BMP Peptide (1000 μg/ml) is higher than that compared to BMP2 (10 μg/ml), its use is till a viable option and translates to a substantial amount of savings in clinical applications.

To determine the distribution of the immobilized peptide, OPA assay was conducted to assess the surface distribution of the BMP Peptide on the polydopamine coated substrates. A very strong fluorescence was observed on the peptide-coated substrates due to reaction of OPA with the peptide moiety. These results (Fig. 6.2) showed the random dispersion of the immobilized peptide and demonstrated the ability to graft these amino acid groups to the biomimetic layer. The addition of the cys-tag for site specific conjugation with thiol directed chemistry conferred high binding efficiency of the peptide with the biomimetic layer, with over 90% for all the various concentrations used as shown in table 6.1.

MTT assay performed on the various substrates on day 7 of culture showed significantly higher viability of MC3T3-E1 on the CoCr-PDOP-BMP2 (10 μg/ml) and CoCr-PDOP-BMP Peptide (1000 μg/ml) substrates compared to both the pristine CoCr and CoCr-PDOP (Fig.6.3) substrates. The results suggest that CoCr-PDOP-BMP2 (10 μg/ml) and CoCr-PDOP-BMP Peptide (1000 μg/ml) substrates were not cytotoxic to the cells, and the immobilized bioactive factors on the surfaces can promote the survival of MC3T3-E1.

BMP signaling depends on Smad1, 5 and 8 which are activated in response to BMP treatment. Immunostaining performed revealed that the peptide-coated substrates were able to stimulate the osteoblastic cells as reflected by the upregulated phosphorylation of Smad1/5/8 (Fig. 6.4). Surface roughness affect osteogenesis [147] and

generally rough implant surfaces are known to be superior with regards to cellular attachment and osteogenesis [148]. The AFM results (Fig. 6.6) showed an increase in surface roughness after the covalent immobilization. Results of this study showed that the peptide-coated CoCr substrates significantly enhanced osteoblastic cell adhesion compared to unmodified CoCr substrates. With peptide attachment the surface roughness was close to that for CoCr-PDOP, but the changes in cellular attachment was markedly increased (Fig. 6.7), and the main factor is likely to be due to the added peptide. The biological activity of the immobilized peptide plays a critical role during implant integration as cells come into direct contact with the implant surface. The nature of this initial attachment and adhesion influences the cell's capacity to proliferate and differentiate [148] and ultimately the success or failure of the implant.

Morphological studies (Fig. 6.8) showed that the cells seeded on the pristine CoCr appear stunted and less elongated while cells seeded on CoCr-PDOP-BMP2 (10 μg/ml) and CoCr-PDOP-BMP Peptide (1000 μg/ml) substrates exhibited more spreading and increased bundles of actin microfilaments compared to both the pristine CoCr and CoCr-PDOP substrates. This is indicative of a generally healthier morphology and increased faster differentiation rate [133]. This was corroborated by the ALP activity and calcium deposition results, where we observed a 2-fold increase in ALP activity after 2 weeks and a 4-fold increase in calcium content after 3 weeks (Fig. 6.9, 6.10). It can be seen that ALP activity of the osteoblastic cells on the CoCr-PDOP substrates is at a similar level as the ALP activity on the pristine CoCr substrates throughout the 2 weeks period. On the other hand, osteoblastic cells cultivated on the peptide-coated substrates have a significantly higher ALP activity. Similar to the ALP results there is no significant difference in

calcium deposition between the pristine CoCr and CoCr-PDOP substrates while the calcium deposition on the peptide-coated substrates were increased significantly. Alizarin Red was used to visualize the extent of mineralization. In the presence of the BMP Peptide, mineralization is greatly enhanced as indicated by the dense coverage of the calcium deposits on the peptide-coated substrates (Fig. 6.10).

To ensure that the increased osteogenic effects observed were not confounded by the release of the immobilized BMP2 or BMP peptide into the culture medium, MC3T3-E1 cells were cultured on a permeable support (0.4 μm pore size, Costar®, Corning) in transwells with or without substrates immobilized with BMP2 or BMP Peptide for 3 weeks (results not shown). No significant differences were observed in cell viability, ALP and calcium deposition tests between the groups. This indicated that the enhanced osteogenesis observed was due to the immobilized factors and not the soluble forms being released into the culture medium.

6.5 Conclusion

In summary, we have shown that immobilization of BMP Peptide onto the surface of metal substrates may be a viable option in promoting osteogenesis and enhanced implant integration. By immobilizing BMP Peptide onto a metal substrate using a cysteine placement for site-specific conjugation with the biomimetic polymer film, we have demonstrated that the modified substrate promotes the differentiation and mineralization of osteoblastic cells. This enhanced osteogenesis is beneficial in accelerating wound healing and new bone tissue formation. Although results achieved with the BMP Peptide are slightly lower to those seen with BMP2, but that is at one tenth of the cost. The usage of BMP Peptide in implant functionalization is hence, a cheaper and viable alternative compared to BMP2, especially in instances where costs may be prohibitive.

Chapter 7

Conclusion of study

There is an ever growing need for orthopaedic advancement with the high prevalence and impact of musculoskeletal diseases. 50% of the world's population over 65 suffer from joint diseases and more than 25% of population over 65 require health care for joint related diseases. The instances for failed joint replacements associated with osteolysis and bone defects is increasing. There is an urgency to increase the success of bone implant fixation and the longevity of implant. Fixation of orthopaedic implants has been one of the most challenging and difficult problem faced by orthopaedic surgeons and patients. Fixation can often be achieved via direct biological fixation by allowing tissues to grow into the surfaces of the implants or with the use of bone cement acting as a grouting material. Whether cemented or cementless fixation are employed, the problems of micromotion and the generation of wear particles may eventually necessitate further surgery. Revision surgery poses increased risks like deep venous thrombosis, infection and dislocation, in addition to being an economic burden to the patient. Therefore the enhancement of implant integration would bring enormous benefits.

The basis of clinical strategies to manage bone defects would center around the fundamentals of orthobiological therapies based on osteoconduction (structure) and osteoinduction (growth factors). Therefore to increase the rate of implant integration and to reduce revision surgery, our study investigated these factors for improving bone regeneration. 1) Metallic alloys (Ti and CoCr) which provided the structural matrix for mechanical function, delivery of growth factors, cellular support and cell fixation. 2) Bioactive factors (VEGF, BMP2 and BMP peptide) that induce bone formation, increases

cellular proliferation and for facilitating early angiogenesis. These two factors have major impact in osteogenic enhancement of bone defects especially those with limited healing potential.

One of the main problems encountered with biomaterials for surface functionalizaton is the lack of reactivity and suitable binding groups on the surface. For more functions and flexibility, the concentrations of reactive groups such as amino, carboxyl and phenolic group of tyrosine have to be increased. This initial organic layer deposited on the biomaterials can then by used as a tether for bioactive factors which can be used for enhancement of the bone-implant interface. Another issue is the control of the retention and/or release of the bioactive factors from the implant surface. The easiest method often employed is physical adsorption, which provides little control over the delivery of the bioactive factors. Considering the risks of using soluble or non-covalently immobilized growth factors for therapeutic interventions, the use of covalently immobilized growth factors would provide significant advantages. Deleterious effects such as unwanted ectopic bone formation, high levels of growth factors in the local microenvironment and the risk of undesirable effects at locations beyond the implant site can be avoided.

Despite the good inherent bioactivity and biocompatibility exhibited by Titanium alloys, osseointegration with host tissue is still not definite, the lack of vascularity may cause implant failure at times. Cobalt Chromium alloys are one of the strongest metal in use today for orthopaedic implants, unfortunately osseointegrative properties are almost non-existence for Cobalt Chromium implants. Strategies were developed in this thesis to overcome the challenges of insufficient vascularity and osteogenesis faced by these

implant materials. Although it is not possible to have a universal means of surface functionalization, however our study has developed a viable technique which may be applied to applications where enhancing the host implant interface would be of crucial importance.

With the ever growing number of patients requiring orthopaedic reconstructions, a rapid translation of basic research for use in clinical disease diagnosis, prevention and treatment is crucial, employing medical research towards a patient-oriented approach. Structural reconstruction based implants have limitations associated with bone fixation and osteolysis. Therefore the development and evolvement of implant material with structural and biological potential to manage bone healing impairment and defects would be desirable. The control of surface function with immobilization holds great potential for creating biofunctional implant materials to provide for enhanced osseointegration. The studies performed in this thesis showed for the first time the effectiveness of using covalent bioconjugation of bioactive factors with polydopamine onto metallic substrates which can potentially be used for the development of orthopaedic devices for enhancing the chances of successful implant integration. This would assist patients in maintaining a good quality of life and remain independent longer with advancing years. Recent *in vivo* evaluation of polydopamine [149] has shown it to be non toxic which would further reinforce its usage in biomedical applications.

Chapter 8

Recommendations for future study

The work in this thesis has shown that it is possible to confer bioactivity to implant materials for enhancing osseointegration. Possible future work can look at the use of multiple biochemical cues to simulate revascularization which remains an inadequately resolved challenge. The presence of VEGF may not be sufficient and other promaturing factors such as angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) may be required for vascular integrity and development. Work can also be done to examine the angiogenic effects and the osteogenic effects of these functionalized substrates *in vivo*. A detailed study of the functionalized substrates in an *in vivo* setting will yield useful information such as response of bone to the implant, the rate of implant fixation and bone ingrowth which will be crucial for further improving the modification process of the substrates. An investigation into the immunological response after implantation can also be evaluated. The process of bone healing is intricately linked with immune responses therefore a study of the osteoimmunology of bone healing *in vivo* will provide vital information for improving the functionalization process of the substrates.

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