Influence of Surface Topographical Modifications of Titanium Implants on Bone Cell Responses

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Na Gui

2 July 2018
To my beloved parents

Ju Hua Zhao and Yu Yan Gui
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Executive Summary

Failures of orthopaedic implants often arise from poor osseointegration at the interface between the implant and the bone. Loosening of the implant is one of main reasons for implant failures. The surface of an orthopaedic implant plays an important role in determining bone cell functions such as cell morphology, adhesion, proliferation and differentiation. Consequently, a wide variety of surface topographical modifications have been developed to improve interactions with osteoblasts. In particular, engineering micro- or nano-structures on the implant surface holds promise to enhance bone cell functions. On the other hand, titanium (Ti) and its alloys have proved to be the materials of choice for orthopaedic implants, thanks to their outstanding biocompatibility, good corrosion resistance in bodily liquid, high strength-to-weight ratios and good mechanical properties. This thesis has addressed the role of surface topographical modifications at the sub-micro to micro scales in the regulation of bone cells. These surface topographical modifications include both random and ordered surface patterns on Ti surfaces. Surface characteristics including surface roughness, morphology and wettability have been evaluated using atomic force microscopy (AFM), surface profilometry, white light interferometry, focused ion beam/ scanning electron microscopy (SEM) and sessile-drop contact angle goniometry. Ordered groove arrays were fabricated using conventional micro/nano fabrication techniques including photolithography, reactive ion etching (RIE) and sputter coating. The biocompatibility of these surface patterns was evaluated by in vitro assessment using human fetal osteoblasts (hFOBs). Cell-surface topography interactions were studied using confocal microscopy and SEM. This thesis aims to provide critical experimental data for the future design of orthopaedic titanium implants for rapid osseointegration.
• **The influence of submicron porous and smooth ultrafine-grained Ti-20Mo surfaces on osteoblast responses**

Research has shown that both interconnected porous titanium surfaces and dense ultrafine-grained titanium surfaces can enhance bone cell responses. However, it remains elusive as to which surface features are more effective in regulating cell responses. They represent two distinctly different directions in surface engineering of a functional implant. This study compares the *in vitro* osteoblast responses to ultrafine-grained (grain size: 100 nm), coarse-grained (grain size: 500 μm), fine-porous (pore size: 155 nm) and coarse-porous (pore size: 350 nm) surfaces of Ti-20Mo alloy. A large amount of original experimental data was produced for each type of surface in terms of surface topography, chemistry, wettability, cell morphology, attachment, growth and differentiation. This study concludes that the coarse-porous surfaces provide the optimum topographical environment for osteoblasts. Combining ultrafine grains with abundant grain boundaries is not as effective as porous surfaces to improve cell growth and osteogenic capacity. Furthermore, pore features including size and depth play a more important role in cell growth and osteogenic capacity than smooth surfaces. These findings reveal that the osteoblasts can discern the differences in pore size and depth and responded differently.

• **Fabrication of titanium-coated microgrooves and their anisotropy in wettability**

The attainment of surface wettability is essential for the success of orthopaedic implants. Surface grooves are of particular importance to orthopaedic implants due to their similarities to collagen fibrils in geometry, which are the basic components of extracellular matrix (ECM, i.e., the cell living environment). Anisotropy in wettability can result from surface grooves. However, no information has been found on the anisotropy in wettability of Ti-coated grooves. Therefore, this study focuses
on the fabrication of Ti-coated microgrooves with various groove widths (5-20 μm) and the subsequent characterisation of the resultant anisotropy in wettability was compared with the Wenzel and Cassie models. The results show that significant anisotropy in wettability was found among these Ti-coated microgrooves. In particular, the degree of anisotropy (Δθ) is elevated with increasing groove width from 5 μm to 20 μm on the sub-cellular scale. The Wenzel model can appropriately predict the contact angles measured along the groove direction while the Cassie model offers a better fit for the contact angles measured perpendicular to the groove direction. The anisotropy of wettability influenced osteoblast spreading. Consequently, osteoblasts preferred aligning, rather than perpendicular to along the groove direction.

- **Osteoblast responses to titanium-coated microgrooves with sub-cellular scaled widths**

Ordered groove arrays have been widely used to modify orthopaedic implant surfaces due to their geometrical similarity with the groove-like collagen fibrils. In general, altering groove geometry has been proved to be an effective way of controlling osteoblast functions. However, the influence of groove geometry at the sub-cellular scale on osteoblast responses remains unclear. Groove width is crucial in regulating osteoblast functions. In this study, osteoblast responses to Ti-coated microgrooves with variable groove width on the sub-cellular scale from 5 μm to 20 μm were systematically investigated. The cell responses include cell morphology, cell-groove adhesion, the spatial arrangement of actin cytoskeleton, cell proliferation and osteoblastic capacity. Both FIB and SEM were used to investigate the osteoblast-groove adhesions, the first close-up study to understand how cells rest more comfortably on grooved surfaces. Full osteoblast-groove adhesion was achieved when the groove width reached 15 μm and beyond, while below 15 μm, the adhesion...
was gradually enhanced with increasing groove width. The cell spreading area and the cell width were found to be proportional to the groove width. However, the groove width over the range of 5-20 μm exert little influence on cell proliferation and cell differentiation compared to flat surfaces. Apart from the groove width, the groove geometry is another factor that can be tuned to facilitate cell adhesion. The favourable geometries for full osteoblast-groove adhesion include microgrooves with either vertical groove sidewalls (groove width: 15 μm, ridge width: 5 μm, groove depth: 2 μm) or slanted groove sidewalls (slope angle: 158.2°, groove width: 15 μm, groove open width: 25 μm, ridge width: 5 μm, groove depth: 2 μm). On this basis, the correlation between groove geometry and the osteoblast-groove adhesion has been redefined.

**Keywords:** orthopaedic implants, titanium, surface topographical modification, ultrafine grains, submicron porous surface, sub-cellular scaled microgroove, surface morphology, surface wettability, cell-groove interface, osteoblast responses
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Table 5.1 Dimensions of designed surface microgrooves.
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List of Abbreviations

AFM atomic force microscopy
ALP alkaline phosphatase
ANOVA analysis of variance
ASTM American Society for Testing and Materials
BCA bicinchoninic acid
BMPR2 bone morphogenic protein receptor type 2
BMSC bone marrow-derived mesenchymal stem cell
BSA bovine serum albumin
BSP-2 bone sialoprotein type 2
BSSA biosurface structure array
CFL capillary force lithography
DI deionized
DMEM/F12 Dulbecco’s modified essential medium and Ham’s F12 medium
DMSO dimethyl sulfoxide
DRIE deep reactive ion etching
EBL electron beam lithography
ECM extracellular matrix
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FA</td>
<td>focal adhesion</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fe</td>
<td>iron</td>
</tr>
<tr>
<td>FIB</td>
<td>focused ion beam</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>GP</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>hFOB</td>
<td>human fetal osteoblasts</td>
</tr>
<tr>
<td>hOB</td>
<td>human osteoblast</td>
</tr>
<tr>
<td>hMSC</td>
<td>human mesenchymal stem cell</td>
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<tr>
<td>He</td>
<td>helium</td>
</tr>
<tr>
<td>HF</td>
<td>high frequency</td>
</tr>
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<td>HPT</td>
<td>high pressure torsion</td>
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<td>H$_2$O$_2$</td>
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<td>integrated-coupled-plasma</td>
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<tr>
<td>IPA</td>
<td>isopropanol</td>
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<tr>
<td>LSM</td>
<td>laser confocal microscope</td>
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MAPK  mitogen activated protein kinase

MARC  multi architectural chip

MIF  metal ion free

Mo  molybdenum

MSCs  mesenchymal stem cells

Nb  niobium

NaCl  sodium chloride

OB  osteoblast

OCN  osteocalcin

OPN  osteopontin

OPS  oxide polishing suspension

PBS  phosphate buffered saline

PC  polycarbonate

PCL  polycaprolactone

PDMS  polydimethylsiloxane

PFA  paraformaldehyde

PLGA  polylacticglycolic acid

PLLA  polylactic acid

PMMA  polymethylmethacrylate

XXVIII
PS  polystyrene
Pt  platinum
PUA  polyurethane
RDG  Arg-Gly-Asp acid
RGD  Arg-Gly-Asp acid
RF  radio frequency
RIE  reactive ion etching
rMSCs  rat mesenchymal stem cells
Runx2  Runt-related protein 2
ROI  region of interest
Runx-2  runt related protein 2
SD  standard deviation
SEM  scanning electron microscopy
Si  silicon
$S_a$  average surface roughness
$S_q$  root mean square roughness
$S_z$  the maximum height of the surface
Ti  titanium
TiO$_2$  titania
Ta  tantalum

TGF-β  transforming growth factor-beta

TEM  transmission electron microscope

TiO₂  titanium dioxide

UV  ultraviolet

VASP  vasodilator-stimulated proteins

XPS  x-ray photoelectron spectroscopy

XRD  x-ray powder diffraction

3D  three-dimensional
Publications during Candidature

Peer-reviewed Journal Papers


Conference Presentations


Chapter 1

Introduction

1.1 Background

In Australia, about one out of every 200 citizens undertook hip, knee and shoulder replacement procedures in 2016 according to the Australian Orthopaedic Association [1]. The demand for orthopaedic implants is on the rise mainly due to the increasingly aging population worldwide and growing needs for a better life quality [2]. However, around 10% of implant failures occur during the first 10-20 years [3]. Consequently, a large number of patients are outliving their implants, in particular people below 55 are highly likely to undergo a revision surgery [1]. Compared to the initial surgery, a revision surgery is more difficult to accomplish and exhibits a lower success rate [4]. Hence, there is an urgent need to develop novel orthopaedic implants that can provide rapid and long-last osseointegration (i.e., strong and direct integration between the bone and the implant surfaces [5, 6]).

The main requirements for orthopaedic implants include: 1) good osseointegration; 2) good biocompatibility, i.e., non-toxicity and not causing any adverse reactions such as inflammation or allergy in the host body [4, 7]; 3) comparable mechanical properties. For instance, implants with higher stiffness than human bones prevent the stress from being transferred to the adjacent bones.
and this can cause undesirable bone resorption around the implant surfaces. This phenomenon is known as “stress shielding effect” [8].

Commonly used bone scaffolds include biopolymers, ceramics and biphasic calcium phosphate, metals and natural products [9]. Although ceramics such as hydroxyapatite (HA, Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) and biopolymers exhibit similar chemical compositions to natural bone and biodegradable property, they are not suitable for use as load-bearing applications due to their poor mechanical properties [9]. 316L stainless steel and cobalt (Co)-based alloys (e.g., Co-Cr-Mo) have long been used as orthopaedic implants [10]. However, Ni, Cr and Co elements released from 316L stainless steel and cobalt-based alloys were found to be toxic to human body [4].

In contrast, titanium (Ti) and its alloys are preferable for orthopaedic implants, as they exhibit excellent mechanical properties, biocompatibility and corrosion resistance [11]. Ti and its alloys naturally form 3-7 nm thick oxide film, i.e., TiO$_2$, which provides outstanding biocompatibility and corrosion resistance [10]. Up to now, commercially pure Ti (CP-Ti) and Ti-6Al-4V (in wt%) are widely used for biomedical devices. However, CP-Ti exhibits poor mechanical strength, which makes it not suitable for load-bearing applications, for instance, bone plates and screws [4, 12]. A potent method to improve the mechanical strength of CP-Ti is grain refinement [13]. Another concern from Ti-6Al-4V is the release of toxic Al and V ions [4]. The common issue for both CP-Ti and Ti-6Al-4V is that they have significantly higher Young’s modulus (100-112 GPa) compared to cortical bones (10-30 GPa). This has led to develop novel low-modulus Ti alloys without Al and V elements [14].
The bulk properties of Ti implants determine their mechanical performance, while surface properties are closely associated with bioactivity [15]. The surface of orthopaedic implant is in direct contact with the bone cells or tissues, therefore it plays a decisive role in the success of implants [16]. To ensure good osseointegration and improve bioactivity of Ti implants, surface topographical modification has emerged as a promising route to regulate cell responses [4, 17] and improve osseointegration [18]. In general, an implant surface can be tailored with various type of patterns including random or ordered topography [19]. The distinct advantage of random patterns is the simplicity and high-efficiency of fabrication, while the ordered patterns can quantify the influence of topographical cues on specific cell responses [19].

1.2 Problem statement

A substantial amount of research has addressed the modifications of titanium implant surfaces to enhance their functional outcomes. However, to date, the influence of surface topographies in particular with random porous and ordered grooves on osteoblast responses remains unclear. Grain refinement and the introduction of interconnected pores into surfaces are two distinctive directions in engineering an implant surface. Recent research has shown that both dense ultrafine-grained [20] and porous (interconnected pores) Ti and its alloys [21] [22] can enhance cell responses. However, it remains undefined to which surface features are more effective in improving cell responses. To our knowledge, no relevant comparative studies have been conducted on ultrafine-grained surfaces versus porous surfaces. Regarding ordered surface grooves, anisotropy in wettability can be induced, which is relevant to the responses of osteoblasts.
However, no information is available on the anisotropy in wettability of Ti-coated grooves and its influence on osteoblast spreading. Groove width has been identified as the most influential factor on regulating bone cell responses among the three crucial groove geometrical parameters including width, depth and ridge width [23]. However, little is known about the exact influence of groove widths at the subcellular scales on osteoblast functions. In addition, the favourable groove geometries for osteoblast functions remains undefined.

1.3 Research aim and objectives

The aim of this research is to improve the *in vitro* biofunctionality (i.e., enhanced osteoblast responses) of Ti orthopaedic implants through surface topographical modifications. In particular, this study will focus on furthering our understanding of the interactions between the osteoblasts and the patterns on implant surfaces under *in vitro* conditions. It will also provide crucial experimental data for future design of orthopaedic implants.

Specifically, the main objectives of this thesis include:

- To systematically compare the influences of submicron porous and smooth ultrafine-grained Ti-20Mo surfaces on osteoblast responses;
- To reveal the relationship between the anisotropy in wettability and groove geometric parameters, particularly concerned with the influence of the groove width, aspect ratio and space ratio on the anisotropy in wettability;
- To clarify the role of sub-cellular scaled groove widths in osteoblast functions including cell adhesions, actin cytoskeleton organisation, proliferation and differentiation;
• To determine favourable groove geometries for full osteoblast-groove adhesions and establish the quantitative relationship between the groove width and the degree of the cell-groove adhesion; and

• To clarify the influence of surface topography and wettability on the osteoblast attachment, adhesion, morphology, proliferation and osteogenic capacity.

1.4 Thesis outline

This thesis is composed of six chapters, combining publications and submitted manuscripts as follows:

Chapter 1 highlights the significance of surface topographical modifications of titanium orthopaedic implants. Subsequently, the problem statement, aims and specific objectives of this research are presented. In addition, the structure of the thesis is summarised with a brief description of each chapter.

Chapter 2 provides a comprehensive literature review on recent understanding of ordered and partially ordered surface topography on in vitro bone cell responses. The investigated partially ordered classes of patterns include dots, tubes, while ordered pattern types cover protrusions, pits, grooves, intricate matrices and hierarchical micro- to nano-topographies. Bone structures and interactions of bone cells with an implant surface at the micro-, submicro- and nano-scales have been reviewed in the introduction section. A series of biological cascades of cell-implant surface interactions and the analysis methods of osteogenic capacity are discussed in this work. Moreover, the review highlights the challenges to translate
Chapter 1

These research findings into the clinical implant applications. The findings of this work were published in the *Biomaterials Science*.

Chapter 3 compares how submicron porous and smooth ultrafine-grained Ti-20Mo surfaces influence osteoblast functions. Various characterisation techniques such as transmission electron microscopy (TEM), scanning electron microscopy (SEM)/ focused ion beam (FIB), atomic force microscopy (AFM), sessile-drop contract angle goniometry were used to quantitatively analyse grain structures, phase components, surface topography and wettability of the Ti-20Mo alloys. The resultant cell spreading, distribution of vinculin and organisation of actin cytoskeletons on these surfaces were observed using confocal microscopy. Moreover, the cell morphology, in particular with the interactions of filopodia with these surfaces, was studied using SEM. In addition, the cell growth and differentiation were quantitatively assessed by directly counting or enzyme-linked immunosorbent assay (ELISA) kit, respectively. The results of this work were summarised and published in the *Journal of Biomedical Research Part A*.

Chapter 4 presents the fabrication of Ti-coated microgrooves with various groove widths (5-20 μm) and the characterisation of the resultant anisotropy in wettability via measuring water contact angle. The water contact angles measured along and perpendicular to the grooves compared with the Wenzel and Cassie models. These microgrooves were fabricated using standard micro-fabrication techniques including photolithography, reactive ion etching (RIE) and sputter coating. The influence of anisotropic wettability on osteoblast morphology was discussed. The findings of this work were published in the *Journal of Applied Physics*.
Chapter 5 investigates the influence of groove width at the sub-cellular scale on osteoblast responses including the cell morphology, actin cytoskeleton organisation, proliferation and osteogenic capacity. Moreover, this chapter determines three boundary conditions to achieve full cell-groove adhesions and proposes the exponential model to predict the nil cell-groove adhesion. The osteoblast-groove adhesions were systematically studied using FIB-SEM. The cell morphology (spreading area, width, length and orientation angle) was quantitively analysed from the SEM images with Image J software. The actin cytoskeleton and the formation of focal adhesions were studies using confocal microscopy. This chapter is currently under review in the *Acta Biomaterialia*.

Chapter 6 concludes all the significant findings of this thesis and proposes some future directions in this area.

### 1.5 References


Chapter 1


Chapter 1


Chapter 2

Literature Review

2.1 Brief summary*

Implant surfaces play important roles in regulating protein adsorption and determining subsequent cell responses, including cell attachment, proliferation, migration and differentiation. With rapid developments in micro- and nano-fabrication methods and additive manufacturing (3D printing) technologies, precisely controlled patterns such as partially ordered or ordered patterns can now be generated on bone implant surfaces, rather than restricted to randomly roughened surfaces. Over the last two decades, much effort has been dedicated to manipulating cell responses through surface topographical modifications. This review discusses the recent developments and understanding of surface topography in prompting or enhancing desired cell responses, particularly the roles of ordered and partially ordered surface topography under in vitro conditions. In addition, the challenges to translate research findings into implant applications are addressed.

2.2 Introduction

Biological tissues interact with implant materials mainly at the interface, which affects both initial protein adsorption and subsequent cell responses, including cell proliferation, migration and differentiation [24, 25]. In the context of bone implants, implant failures are frequently attributed to poor osseointegration (i.e., poor direct contact between bone and the implant surface) [6]. On the one hand, a metal implant is inanimate. On the other hand, micromotion at the interface can result in fibrous encapsulation and particulate wear debris. These events, particularly the wear debris, can eventually lead to inflammation and destabilisation of the bone-implant interface and loosening of the implant [3].

2.2.1 Classifications of surface topography and their fabrication methods

In order to promote fast and long-lasting osseointegration and minimize the aforementioned adverse responses, a variety of surface modification methods have been utilized over the past decades for orthopaedic implant surface designs [26, 27]. Surface characteristics, more specifically, topography, chemistry, wettability and charge, are reported to influence implant integration [26-29]. Generally, cells respond to underlying surface topography through the contact guidance that alters cytoskeleton organization, migration and other cellular functions. Further, surface topography can be tailored to regulate cell orientation and cell movement in a preferred direction [30-32]. Therefore, there is a consensus that modifying surface topography can manipulate cell-implant interactions and achieve desired cell responses [33]. In terms of the degree of order, patterns on an implant surface can be categorized into three primary types: random, partially ordered and ordered. Random patterns are features where
limited or no control is exercised over orientation and geometry during the pattern fabrication process [34]. Examples include substrates with roughened [35-38], porous [39-41], or fibrous surfaces [39] that are produced by acid etching and/or sandblasting, electrospinning and polymer demixing. Randomly patterned surfaces are difficult to reproduce. For example, surfaces with the same surface roughness parameter $R_a$ often have distinctly different surface profiles [42]. Partially ordered patterns are features with controllable dimensions in a short-range array [43]. In comparison, periodically ordered patterns consist of precisely defined geometric features in long arrays such as grooves, pits, pillars or their combinations. Some of the most representative random [35-41, 43-45], partially ordered [46, 47] and ordered patterns [48-54] reported in the literature for bone implant surfaces are summarized in Table 2.1. Fig. 2.1 illustrates typical examples of partially ordered and ordered patterns.
Table 2.1 Topographical patterns on bone implant surfaces and their fabrication methods.

<table>
<thead>
<tr>
<th>Patterns</th>
<th>Pattern shape</th>
<th>Representative fabrication method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random</td>
<td>Roughened</td>
<td>Acid etching and/or sandblasting</td>
<td>[35-38]</td>
</tr>
<tr>
<td></td>
<td>Pore</td>
<td>Anodization, Laser writing</td>
<td>[39-41]</td>
</tr>
<tr>
<td></td>
<td>Fibre</td>
<td>Electrospinning</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td>Island/pit</td>
<td>Polymer demixing</td>
<td>[43, 45]</td>
</tr>
<tr>
<td>Partially ordered</td>
<td>Dot</td>
<td>Anodization</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>Tube</td>
<td>Anodization</td>
<td>[47]</td>
</tr>
<tr>
<td>Ordered</td>
<td>Protrusion</td>
<td>Photolithography and etching,</td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td>Pit</td>
<td>Photolithography/electron beam lithography (EBL) and etching, laser writing</td>
<td>[48, 50]</td>
</tr>
<tr>
<td></td>
<td>Groove</td>
<td>Photolithography/ EBL and etching, laser writing</td>
<td>[51, 52]</td>
</tr>
<tr>
<td></td>
<td>Intricate matrix</td>
<td>Additive manufacturing (3D printing)</td>
<td>[53, 54]</td>
</tr>
</tbody>
</table>

Fig. 2.1 Illustration of representative surface patterns for bone implants studied to date.
In fact, the complex extracellular matrix (ECM) that surrounding cells exhibits highly oriented topographical features in bone and other tissues at the nanoscale [55, 56]. Evidence also suggests that surfaces with ordered patterns can lead to enhanced metabolic activities [36] and osteogenic activity [57, 58]. With regards to random features, it remains challenging to quantify the effect of individual features on cell responses due to poor reproducibility of features with defined geometrical dimensions [30, 49, 59-62]. These findings have inspired an increasing research interest in understanding how ordered and partially ordered patterns affect cell responses for bone implant applications.

Recent advances in micro- and nano-technologies have facilitated fine control of partially ordered and ordered surface topographies [30]. More specifically, anodization [63, 64] and colloidal lithography [43] have been commonly used to fabricate partially ordered patterns. A variety of advanced methods have been employed to create ordered patterns, including photolithography and reactive ion etching (RIE) [49], nano-imprinting [65], electron beam lithography (EBL) [66], laser interference lithography [66], femtosecond laser lithography [50, 52, 67], three dimensional (3D) two-photon polymerization [68-70], block copolymer templates [71, 72], capillary force lithography (CFL) [56, 73], and additive manufacturing or 3D printing [27, 74, 75]. Details regarding the current microscaled and nanoscaled surface modification methods for bone implant applications are not the focus of this review, and they have been discussed in detail elsewhere [74, 76-82].
2.2.2 Bone structures

Bone is hierarchically arranged in structure, from macroscale, microscale to nanoscale [83, 84]. At the macroscale, cortical bone externally, and cancellous bone (trabecular bone) internally, provide mechanical support to the body and protect inner organs [83-85]. At the microscale, while cortical bone consists of lamellar structured osteons and Haversian canals [83], spongy cancellous bone, which is also lamellar bone, is composed of an interconnected porous network of trabeculae [84, 86]. Nanoscaled bone structures include collagen fibres, whose main components are well-organized collagen fibrils [83, 84].

2.2.3 Interaction of bone cells with an implant surface at different scales

Interactions of bone cells with an implant surface at different topographical scales are illustrated in Fig. 2.2. Load-bearing implants have been developed to include surface changes at the macroscale level. Such changes are designed to improve mechanical support and the strength of the prosthesis integration. Given that bone cells are microscaled in size, microscaled (1 μm ≤ dimension < 1000 μm) surface features are considered to be suitable to directly interact with bone cells [24, 87]. When reduced to the nanoscale (dimension ≤ 100 nm), topographic patterns attain the same scale of subcellular structures, e.g., membrane proteins (integrins) [87], filopodia [88], cytoskeletal proteins and their aggregates, which are two orders of magnitude lower than the cell level. Growing evidence is proving that surface topographies at both the microscale and nanoscale play a crucial role in regulating morphology, adhesion, differentiation, migration, proliferation and, eventually, the fate of cells [42, 47, 61, 89-93]. A principal advantage of nanoscaled features over microscaled features is that they offer a much larger surface area to adsorb
proteins, and more adhesion sites to integrins, as well as facilitating integrins to identify the proteins absorbed on the surface [61, 87, 94, 95]. Indeed, several studies have revealed that nanoscaled features are more effective in improving osteoconduction (i.e., bone growth on the implant surface [6]) than microscale ones [56, 91, 96, 97].

**Fig. 2.2** Illustration of interactions between bone cells and an implant surface at the micro-scale (1 μm ≤ dimension < 1000 μm), submicro-scale (100 nm < dimension < 1 μm) and nano-scale (dimension ≤ 100 nm). The figure is reprinted with permission from Ref [24].

To date, no clear and consistent conclusions have been reached regarding the optimum geometry and scale of the surface topography for bone cell responses is due to inconsistent experimental conditions, as well as the subjective nature of the evaluation methods [46, 94]. Here we review the influence of partially ordered and ordered surface topographies on the responses of bone cells. Specific topographical patterns to be discussed include dots, tubes, protrusions, pits,
grooves, intricate matrices and hierarchical topographies. The purpose is to further our understanding of the interactions of bone cells with implant surfaces under *in vitro* conditions. Since the responses of cells to surface topographies differ by cell types [30, 94, 98, 99], this review is mainly concerned with osteoblasts (i.e., bone forming cells) and bone marrow-derived mesenchymal stem and stromal cells (BMSCs). Among them, MSCs are multipotent stem cells, which can differentiate into distinct cell types, including osteoblasts, adipocytes and chondrocytes [61, 100, 101].

### 2.3 Cell-implant surface interactions

Interactions of bone cells with implant surfaces are highly dynamic processes. Understanding how cells interact with patterned surfaces is essential to clarify the role of partially ordered and ordered patterns. Since the processes involved vary in different stages, we discuss each below separately. The first event after the insertion of an implant in body is the adsorption of water molecules [102]. Subsequently, proteins from blood or serum bind onto the hydrated implant surfaces [99, 102-106]. The specific proteins adsorbed by an implant surface are determined by the surface properties, which in turn affect cell adhesion, migration, proliferation and differentiation [30, 33, 102, 104, 106-108]. Cells generally have no direct interactions with an implant surface. In fact, they respond to proteins bound on implant surfaces through transmembrane proteins, referred to as integrins [102, 104]. Cells sense topographical cues from implant surfaces as well as ECM through the protrusion structures of cells, i.e., lamellipodia and filopodia [33, 109]. In the beginning, filopodia are responsible for detecting favourable sites for attachment and for inducing subsequent
reorganization of cytoskeletons to further migrate cells [33, 88, 99, 107-110]. Once filopodia have adhered to desired sites, focal adhesions (FAs) form at the leading edges of the cells, which render linking the actin cytoskeleton to the ECM [33, 111]. At the microscale, a complex ECM consists of pre-coated ECM components and endogenously formed ECM, providing necessary microenvironment for cells [112]. Cell membranes and associated membrane proteins and receptors regulate cell function and the complex array of membrane molecules, particularly glycoproteins (GPs) that bind to GP binding sites in the glycocalyx are essential in connecting cells and implant surfaces [113]. The glycocalyx comprises glycoproteins, proteoglycans and glycolipids and each plays certain role in cell-cell adhesion and cell-surface adhesion [114]. Integrins are responsible for anchorage and communications between cells and ECM proteins (e.g. collagen-I and bone sialo proteins), and are involved in cascades of biological reactions [115]. As a consequence, they regulate cellular functions such as cell attachment [104]. Integrins adhere to their specific peptide ligands, for instance, Arg-Gly-Asp acid (RGD), which is located in the ECM proteins [115]. Fibronectin and vitronectin are cell adhesion proteins, which reside in the ECM and play a critical role in regulating cell attachment, morphology and migration [64, 104, 108]. From an intracellular perspective, integrins can bind to adhesion proteins such as talin, paxillin and vinculins. In addition, \( \alpha \)-actin, zyxin, myosin and vasodilator-stimulated proteins (VASP) are those key proteins that facilitate the reorganization of cytoskeletons and focal adhesions [116]. These processes collectively regulate the interactions of cytoskeletons with surface patterns [33, 116]. Vinculin is one such critical protein located in the FAs, whose spreading and intensity levels are frequently used as a marker to label the FAs.
Modifications in the FAs affect the arrangement of cytoskeletons and consequently influence genetic pathways [48, 95, 117]. The FAs can be quantified by measuring their size, length and number directly, as well as attachment sites through the gene expression profiling indirectly [46, 117-119]. Their size is correlated with cellular functions [48, 72, 117]. More specifically, a small adhesion size tends to stimulate cell proliferation instead of cell differentiation, while a large adhesion size signals the initiation of cell differentiation and depression of cell proliferation and migration [48]. In addition, the early activities of focal adhesion kinase (FAK) are essential for topography-induced cell differentiation [89]. More specifically, FAK phosphorylation plays an important role in transducing signals between integrins to cytoskeletons, which is critical for gene regulation in hMSC differentiation [89]. Fig. 2.3 illustrates the overall interactions of bone cells with an implant surface.

Actin cytoskeleton reorganizes itself to adapt to an extracellular environment, transducing mechanical signals into biochemical ones through a process called mechanotransduction [56, 95, 120-122]. The commitment of stem cells towards either the osteoblastic or adipogenic lineages is also regulated through mechanotransduction [52, 56, 61, 89, 120, 122-125]. The details with regards to mechanotransduction in cells have been extensively covered in recent comprehensive reviews [26, 126-132].
Fig. 2.3 Overall interactions of bone cells with an implant surface: A) adsorption of water molecules, B) protein adsorption, C) cell attachment, and D) molecular interactions at mature focal adhesions and contact points. Fig. 2.3 D) is reprinted with permission from Ref. [116]. Copyright © 2015 American Chemical Society.

The osteogenic capacity of a bone implant surface can be indicated by the cellular expression levels of alkaline phosphatase (ALP), type-I collagen, osteocalcin (OCN), osteopontin (OPN) and bone sialoprotein (BSP)-II. In addition, the capacity of bone mineralization and bone nodule formation can be used as markers for osteogenesis [42, 48, 56]. Also, osteoblastic genes Runt-related protein 2 (Runx2) [50, 101] and transforming growth factor-beta (TGF-β) [133] have been reported to play an important part in osteoblastic differentiation. Further details on the signalling molecules that are involved in bone regeneration have been discussed in a number of recent reviews [134-137].

It is generally accepted that cells probe the cues on an implant surface via water molecules, and the adhesion of water molecules, which is related to surface
wettability. Surface wettability can be fine-tuned through surface chemistry and topography [98, 99, 108, 138, 139]. Compared to a hydrophobic surface, a hydrophilic surface is capable of upregulating bone cell attachment,[140] cell spreading [140, 141], integrin expression [141], ECM protein (fibronectin) secretion [140], cell differentiation [35, 142], and the later-stage mineralization [143]. In addition, with increasing wettability to superhydrophilic surfaces, bone cells, irrespective of growth medium compositions, can adhere to an implant surface in the early stage (≤ 2 days) [144]. However, it should be noted that in the in vivo setting, evidence also exists to show that hydrophilicity exhibits only a marginal effect on osseointegration while surface topographic complexity has a far greater effect [145-147]. This further highlights the difference between in vitro studies and in vivo applications. Factors that affect the surface wettability of a metallic implant surface have been reviewed elsewhere [148, 149]. This review deals only with surface topography and correlated surface wettability in the context of cell-implant surface interactions.

2.4 Partially ordered and ordered patterns on an implant surface

Based on the influence of partially ordered and ordered patterns on cell functions, we here classify surface patterns into seven categories, namely, dots, tubes, protrusions, pits, grooves, intricate matrices and hierarchical micro- to nanotopographies. The influences of the partially ordered and ordered patterns on in vitro cell responses are summarized in Table 2.2 [42, 43, 46-48, 51, 56-58, 61, 64, 65, 72, 101, 117-119, 150-158].
Table 2.2 A summary of effects of partially ordered and ordered surface topographies on cell responses [42, 43, 46-48, 51, 56-58, 61, 64, 65, 72, 101, 117-119, 150-158].

<table>
<thead>
<tr>
<th>Pattern shape</th>
<th>Pattern material</th>
<th>Dimensions</th>
<th>Cell type</th>
<th>Main cell response</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dot</td>
<td>PMMA</td>
<td>140-2200 nm / groove width 0.184-4.3 μm / centre-to-centre spacing 11-45 nm / height/depth 11-45 nm</td>
<td>hMSCs</td>
<td>Increased FA formation on nanodots, bone nodules formed on 45 nm high dots</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>Ti/TiO₂</td>
<td>20-55.5 nm / groove width 30-115 nm / centre-to-centre spacing 8-100 nm / height/depth 8-100 nm</td>
<td>hMSCs</td>
<td>Increased cytoskeleton organization, FA size, expression of Runx-2, OCN and metabolites on 15 nm high dots</td>
<td>[46, 72, 101]</td>
</tr>
<tr>
<td></td>
<td>SiO₂</td>
<td>10/30 nm / groove width 50-120 nm / centre-to-centre spacing 20-50 nm / height/depth 20-50 nm</td>
<td>MSCs &amp; hOBs</td>
<td>Increased proliferation on 20 nm high dots, increased osteogenic differentiation of MSCs on 50 nm high dots</td>
<td>[150]</td>
</tr>
<tr>
<td>Tube</td>
<td>TiO₂</td>
<td>15-100 nm / diameter 3 x the diameter of the tube 100 nm / centre-to-centre spacing 40 nm / height/depth 40 nm</td>
<td>N. A. MSCs</td>
<td>Increased proliferation, formation of focal contacts, mineralization and OCN on 15 nm tubes, reduced cellular activity on diameter &gt; 50 nm tubes</td>
<td>[47, 151]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 nm / diameter 30-120 nm / centre-to-centre spacing 80 nm / height/depth 80 nm</td>
<td>MSCs</td>
<td>Increased cell adhesion, proliferation, ALP activity and Ca concentration</td>
<td>[152]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40-60 nm / diameter 80-120 nm / centre-to-centre spacing 80-120 nm / height/depth 80-120 nm</td>
<td>OBs</td>
<td>Increased expressions of ALP, type-I collagen, and Ca deposition</td>
<td>[153]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125 nm / diameter 200 μm / centre-to-centre spacing 200 μm / height/depth 200 μm</td>
<td>hFOB 1.19</td>
<td>Increased adsorption of fibronectin and vitronectin, cell attachment and proliferation</td>
<td>[64]</td>
</tr>
<tr>
<td>Protrusion</td>
<td>PUA</td>
<td>700 nm / diameter 1.2-5.6 μm / centre-to-centre spacing 1.2-5.6 μm / height/depth 1.2-5.6 μm</td>
<td>N. A. hMSCs</td>
<td>Increased osteogenic differentiation on sparse pillars than dense pillars</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nm / diameter 300 nm / centre-to-centre spacing 100 nm / height/depth 100 nm</td>
<td>Primary hOBs</td>
<td>Increased expression of ALP and RUNX2, and more stress fibres on the sparsest pillars</td>
<td>[42, 117, 154]</td>
</tr>
<tr>
<td>Pit</td>
<td>PMMA/PC</td>
<td>120 nm / groove width 300 nm / centre-to-centre spacing 100 nm / height/depth 100 nm</td>
<td>Primary hOBs</td>
<td>Inhibited cell adhesion, cytoskeleton organization and osteogenic-specific genes on highly ordered topography</td>
<td>[42, 117, 154]</td>
</tr>
<tr>
<td></td>
<td>Ti</td>
<td>N. A. / groove width 185-280 μm / centre-to-centre spacing 30 / 120 μm / height/depth 30 / 120 μm</td>
<td>Murine OBs</td>
<td>Increased migration and bone mineralization</td>
<td>[155]</td>
</tr>
<tr>
<td></td>
<td>PCL</td>
<td>20 / 30 / 40 μm / groove width 50 / 60 / 90 μm / centre-to-centre spacing 300 nm / height/depth 300 nm</td>
<td>Primary hOBs</td>
<td>Increased cytoskeleton spreading and FA formation</td>
<td>[48]</td>
</tr>
<tr>
<td></td>
<td>PDMS coated with fibronectin</td>
<td>3 μm × 3 μm / groove width 5 μm / centre-to-centre spacing 5 μm / height/depth 2/4 μm</td>
<td>Murine MSCs</td>
<td>Increased FA size, actin polymerization and osteogenic differentiation</td>
<td>[57]</td>
</tr>
</tbody>
</table>
2.4.1 Dots

Dots are surface features with their vertical dimension being smaller than, or similar to, their lateral dimension [46, 72], as shown in Fig. 2.4. The centre-to-centre spacing determines the density of the dots for a given diameter. Adjusting
anodization potential [46, 72] and etching time of the RIE process [150] can lead to the development of different dot heights on the substrate.

**Fig. 2.4** Side-view SEM images of nanodots on an implant surface with different heights: A) 15 nm, B) 55 nm, and C) 100 nm. Reprinted with permission from Ref [46]. Copyright 2009 Elsevier.

Sjöström et al. reported that BMSC cell spreading area, cytoskeleton organization and the secretion of OPN were inversely proportional to dot height from 15 nm to 100 nm [46]. Among all the dots investigated, BMSCs entailed the largest FAs on dots of 15 nm in height [46]. Similarly, the researchers from the same group confirmed that dots of 15 nm in height were most effective in inducing osteogenic differentiation compared to flat controls and shorter dots (height: 8 nm), where significant increments of large FAs (size: 20~30 μm) and the largest enhancement of OCN were observed [72]. Consistent with studies of Ref [46, 72], research has also revealed that dots of 15 nm in height tend to encourage more metabolites, which can be crucial in osteogenesis of MSCs, than higher dots (55 nm and 90 nm) [101]. Large FAs were more likely to enhance cytoskeletal tension, which helped to further upregulate mechanotransduction in osteogenic differentiation.
process [46, 72, 101]. However, Sjöström et al. did not clarify the underlying mechanisms.

In contrast, Fiedler et al. found that osteogenic differentiation of MSCs tended to occur more frequently on higher dots (50 nm) than on shorter ones (20 and 35 nm) [150]. Apart from pillar height, centre-to-centre spacing of dot arrays also showed influences on cell responses [150]. In addition to surface topography, cell adhesion and proliferation were also dependent on nature and origin of cells [150]. Dot heights in the range of 20-50 nm exerted no significant influence on the proliferation of MSCs. However, they observed that shorter dots (20 nm) enhanced the proliferation of osteoblasts as compared to higher dots (35 and 50 nm) [150].

Height and centre-to-centre spacing of dots are two decisive parameters in osteogenic differentiation of cells. As there is no detailed information about the influence of the surface wettability of dots on the functionality of a bone implant surface currently, more studies need to be conducted.

2.4.2 Tubes

Nanotubular structures of titania (TiO$_2$) produced on implant surfaces are often arranged vertically in a partially ordered fashion. These TiO$_2$ nanotubes are produced directly from the underlying titanium substrates via a simple and cost-effective anodization method [61, 100, 152, 159]. Owing to their hollow structure, TiO$_2$ nanotubes can serve as carriers to allow the transit of nutrients and proteins necessary for bone cell growth [61, 100]. Tube diameter is a decisive factor in promoting osteogenic differentiation of the MSCs and other cell
functions *in vitro* [47, 100]. Tube diameter and length can be controlled by adjusting anodization voltage, time, and electrolyte type including the pH value to achieve desired nanotubular structures that can facilitate osteogenic differentiation of cells [47] [61, 100]. In addition, annealing at temperatures in the range of 500-550 °C transforms amorphous TiO$_2$ nanotubes into the anatase phase of TiO$_2$ nanotubes, which have the potential to enhance surface hydrophilicity [160, 161].

Different TiO$_2$ nanotube diameters correspond to different functionalities. Park et al. studied TiO$_2$ nanotubes of different diameters (15 - 100 nm) on MSCs and osteoblasts [47, 151]. These studies concluded that the diameter in favour of osteogenic differentiation was 15 nm [47, 151]. They found that cell spreading and adhesion were impeded when the diameter was above 50 nm, and that integrin clustering and FA formation were entirely hampered on 100 nm diameter tubes [47]. Phosphorylation of FAK and ERK was the highest on 15 nm diameter tubes, which was essential for integrin clustering and activation [47]. This is because the lateral dimension of integrin heads is close to 15 nm. Hence, Park et al. have suggested that 15 nm diameter tubes can better stimulate signalling pathways to nuclei and consequently influence relevant cell responses, as shown in Fig. 2.5 [47, 151].

Nevertheless, not all experiments support this conclusion. For example, a detailed study has found that, in the absence of osteogenic additions, the 100 nm diameter TiO$_2$ nanotubes induced the highest osteogenic gene expressions of MSCs compared to those of smaller diameters (30, 50 and 70 nm) [61]. In addition, cell length reached 200 μm on 100 nm diameter TiO$_2$ nanotubular surfaces vs. about 20 μm on 30 nm TiO$_2$ nanotubular surfaces. Tubes of 100 nm in diameter were
not easy for protein aggregates to anchor on, thereby leading to a low cell density (in the early culturing period) and elongated cell morphology. These two developments resulted in enhanced osteogenic differentiation as reported by the authors [61]. Later on, researchers from the same group also confirmed that TiO$_2$ nanotubes with diameters of 70-100 nm promoted ALP secretions and entailed significantly elongated cell morphology, although cell population decreased compared to the case of thinner tubes (e.g., 30 or 50 nm diameter) [61].

**Fig. 2.5** Hypothetical model of the lateral spacing of focal contacts on nanotubes of different diameters. Top: Nanotubes larger than 70 nm diameter do not support focal contact formation and cell signalling, thus leading to apoptosis, bottom: A spacing of 15 nm seems optimal for integrin assembly into focal contacts, thus inducing assembly of actin filaments and signalling to the nucleus. Reprinted with permission from Ref. [47]. Copyright © 2007 American Chemical Society.
Ercan et al. introduced biphasic electrical stimulation to conventional anodization in the production of TiO$_2$ nanotubes [153]. The innovative process produced nanotubes of 40-60 nm in diameter and 80-120 nm in length. When tested *in vitro*, long-term osteoblast responses, including ALP activity, collagen synthesis and calcium deposition, were enhanced mostly on TiO$_2$ nanotubes fabricated by 15 V stimulation and anodization [153]. Electrical stimulation induced the largest cell density [153].

Despite extensive studies, the debate continues as regards to the optimum diameter of TiO$_2$ nanotubes for cell responses. It appears that the differences are caused by the phase state of the TiO$_2$ nanotubes (amorphous or anatase), cell types and cell densities applied [46, 61]. A major advantage of nanotubes is that they can be generated on an existing patterned surface regardless of whether it is smooth or roughened [64]. Furthermore, nanotubes can be used to fabricate hybrid patterns on an implant surface.

### 2.4.3 Protrusions

Protrusions are features that stand out from a surface with their vertical dimension clearly exceeding lateral dimension. As experimental data about the influence of surface protrusions on bone cell responses is limited, herein we include some data reported by Matschegewski et al. [49, 62, 162, 163] on human MG-63 osteoblasts for a basic view of the potential influence of surface protrusions. Typical protrusions include pillars and pillar-like features. Compared to flat surfaces, pillared surfaces exhibited lower hydrophilicity [49, 162]. Additionally, pillars show decreased surface free energy, especially the polar component of the surface free energy [49]. In consequence, the cell coverage area is often reduced on a
pillared surface vs. a flat surface, although a pillared surface has a larger surface area [49, 163]. Accordingly, both initial cell adhesion (after 5 - 10 min) and cell spreading (after 24 h) were severely impaired on pillar-structured surfaces [49]. Osteoblasts were found to adhere predominantly to the top and edge of pillars, but rarely to the sidewalls and bottoms of the pillars, as shown in Fig. 2.6 [49, 62, 163]. Actin cytoskeleton tended to aggregate on pillars with short stress fibers, which was probably related to decreased wettability of surface topography [49, 163]. An inhomogeneous distribution of vinculin could be one of the reasons for the decreased cell spreading on pillars [49]. Moreover, decreased expressions of β3 integrins, collagen I and BSP-II were found on micropillared surfaces compared to flat surfaces [49]. The authors proposed that reorganization of actins had played a critical role in determining the cell functions [49]. As these studies are concerned with early stage (up to 24 h) cell responses to micropillared surfaces [49, 62, 162, 163], further effort is needed to gain more insights into long-term responses of osteoblastic cells to micropillared surfaces.

Ahn et al. [56] demonstrated that osteogenesis of hMSCs were affected by the density of pillars of 700 nm in diameter. After culturing for 2 days, a higher density of nanopillars resulted in a smaller cell coverage area and more circular cell shapes. In contrast, the least dense pillars (5.6 μm centre-to-centre spacing) stimulated the production of ALP and Runx-2 significantly compared to surfaces with a higher pillar density (1.2 μm centre-to-centre spacing) and flat surfaces [56]. They concluded that surfaces with 5.6 μm centre-to-centre spacing pillars were more likely to induce osteogenic differentiation of hMSCs via regulating integrin signalling pathways than other surfaces concerned. Further experimental studies are needed to identify and understand the influence of surface protrusions
on MSC responses. The diameter and density of protrusions are both critical parameters when designing surface patterns.

![SEM images of cells attached on micropillared surfaces](image)

**Fig. 2.6** SEM images of cells attached on micropillared surfaces. Reprinted with permission from Ref. [49]. Copyright 2010 Elsevier.

### 2.4.4 Pits

The osteogenic capacity of a pitted surface can be adjusted by the diameter of pits [48] and the symmetry of pit arrays [42, 154]. At the microscale, pits of 30 μm and 40 μm in diameter were found to be more osteogenic than those of smaller diameter (20 μm), evidenced by the adhesion size and intensity as well as by the
expression level of OPN, as shown in Fig. 2.7 [48]. In each case, micropitted surfaces improved organizations of cell cytoskeletons compared to flat controls [48]. ERK 1/2 signalling pathway can be one such factor that has induced bone formation [48]. Biggs et al. [164] first reported that nanopits with 120 nm in diameter and 100 nm in depth arranged in a square order prevented osteoblast adhesion and spreading compared to randomly arranged counterparts. A possible reason was that such ordered nanopits failed to provide sufficient contact cues for cells to sense [164]. Later, Biggs et al. found that spreading and FA formation of primary HOBs were hampered on square ordered nanopits vs. near-square arranged nanopits [154]. Enlightened by these studies, Dalby et al. identified that the symmetry of nanopits was able to regulate MSC differentiation [42]. A slightly disordered surface up-regulated key genes for osteogenic functions, e.g., intracellular adhesion molecule 1 (ICAM1), integrin αM, integrin α1, collagens and OCN [42]. The authors explained that signalling pathway alterations are related to adhesion formation, which can influence cytoskeleton tension. Consequently, the organization of nuclei or Rho A signalling can be affected and so can the gene expression profile, either directly or indirectly [42]. Furthermore, Biggs et al. observed that pits of 120 nm in diameter that were organized in either square or hexagonal symmetry led to smaller adhesions than flat surfaces or those with random nanoislands or nanocraters [117]. Biggs et al proposed that this was caused by the combining effect of a lower expression level of Wnt/β-cadherin and a smaller number of signalling molecules [117].
**Fig. 2.7** Osteoblasts cultured on flat controls and micro-pitted surfaces with pit diameters of 20, 30 and 40 μm. Left column: osteopontin staining after 21 days of culture; red: actin; green: osteopontin. Higher intensity of osteopontin on pitted surfaces compared to flat controls, particularly for the 30 and 40 μm diameter pits. Right column: alizarin red staining after 28 days of culture; red: calcium; blue: cell morphology. Compared to 20 μm pits, larger pits induced large and mature nodules. Adapted with permission from [48]. Copyright 2011 Elsevier.
Interestingly, square micropitted (3 μm × 3 μm) surfaces were capable of prompting murine MSCs to differentiate into osteoblasts due to an enhanced expression level of osteogenic differentiation markers of ALP, OCN, COL1 and Runx2/Cbfa1 [57]. In addition, increased total FA areas were observed on such micropitted surfaces [57]. Also enhanced were actin polymerization and the traction force between cells and the micropitted surface [57]. By using Y-27632 and Blebbistatin, the authors found that an increase in the strength of both RhoA/ROCK and myosin II signalling pathway was responsible for the enhanced cell functions including FA formation, actin polymerization and osteogenic differentiation on micropitted surfaces, as shown in Fig. 2.8.

**Fig. 2.8** Mechanism of micropitted surface stimulating osteogenic acceleration. Black arrow: the intracellular RhoA/ROCK/NM 2 pathway between focal adhesion, actin fibril and osteogenic gene activation. Red arrow: the traction force by actin fibrils. Reprinted with permission from Ref. [57]. Copyright 2014 Elsevier.
2.4.5 Grooves

In terms of grooves, cell functions can be regulated through ridge width, groove width, groove depth, centre-to-centre groove spacing, and orientation of grooves. Owing to its anisotropic nature, cell morphology is typically elongated along the grooves. Tamiello et al. summarized the influence of each dimension of a groove on cell responses comprehensively, and concluded that the anisotropy of a groove affects cell responses [130]. In general, decreasing groove width or increasing groove depth encourages the formation of elongated cells [65]. Conversely, when the groove width is much wider (e.g., 100 μm) than the cell size, cells usually choose to avoid aligning themselves along the groove direction [118].

Submicron-grooved surfaces with a centre-to-centre spacing of 400 nm compared to wider grooves were found to enhance osteogenic differentiation of hMSCs [51]. Moreover, it was indicated that groove width in the range of 100 - 400 nm may offer the optimal scale for directing hMSCs towards osteogenic lineage [51]. Similarities in dimension or orientation between collagen fibrils and grooves were suggested to lead to promoted osteogenic differentiation of hMSCs [51]. Later, Cipriano et al. investigated cell morphology and cell adhesion on a variety of grooves with width ranging from 500 nm to 50 μm on titanium substrates [157]. Substrates with narrower grooves (500 - 750 nm) stimulated more MSCs to adhere to. Also, more cells were elongated along the narrower grooves than along wider grooves (especially the 50 μm wide ones), i.e., narrower grooves showed higher potential to induce osteogenic differentiation of MSCs [157]. In contrast, Yim et al. unveiled decreased expressions of integrins (α2, α6, αV, β2, β3 and β4) of human MSCs on submicron-grooved surfaces compared to unpatterned
surfaces [165]. Regulations of integrins, FAs and FAKs can be the cause of changes of cell functions [165]. FAKs and vinculins were distributed only at the leading parts of the hMSCs on grooved surfaces, whereas they were observed only on the peripheral and central parts of the hMSCs on flat surfaces [165]. In addition, the expression level of FA-associated and cytoskeleton proteins declined significantly on grooves [165].

Using nanogrooved surfaces, Lamers et al. demonstrated that osteoblasts were able to respond to patterns with a width of 75 nm and a depth of 33 nm [66]. Moreover, the up-regulated gene expressions of osteogenesis including ALP, OCN, BSP, COL 1 and Cbfa/Runx2 were found on nanogrooved surfaces compared to flat surfaces in the initial period [66]. Reducing the centre-to-centre spacing of nanogrooves resulted in a reduction in both the FA formation and the degree of cell alignment [66]. It was suggested that such grooves were too narrow to be recognizable by FAs [66]. However, on nanogrooved Ti-6Al-4V substrates, different cell types exhibited distinct responses, e.g., MSCs were more sensitive than human osteoblasts (HOBs) in terms of cell proliferation and cell viability [32].

Groove depth is another factor that can affect bone cell responses. An increase in the number of elongated osteoblasts was noticed along deep grooves (depth: 306 nm or 2046 nm) over shallow grooves (depth: 35nm) and flat surfaces [65]. Shallow grooves were less capable of stimulating changes in cell morphology than deep grooves. For example, the use of 2046 nm deep grooves enhanced the gene expressions of integrins, paxillin signalling and also the osteogenic makers [65]. As a result, mineralized bone matrices were formed through integrins binding to ECM molecules like collagen, fibronectin etc. However, no noticeable
difference in cell metabolic activity, viability and proliferation was detected on deep grooves, which suggests that no apoptotic pathways were stimulated by elongated cells [65].

Another key factor is the spacing ratio, defined as the ratio of groove width to ridge width. Spacing ratio plays an important role in promoting osteogenic differentiation of stem cells because it reflects the pattern density on the surface [55]. The effect of spacing ratio on human MSCs was demonstrated using grooves with ridge width of 550 nm and depth of 600 nm in Fig. 2.9. It was found that nanotopography with a spacing ratio of 1:3 encouraged osteogenic differentiation of human MSCs compared with a spacing ratio of 1:1 and 1:5, which was related to the expression levels of integrin β1 and N-cadherin [55].

Additionally, recent research by Yoon et al. indicates that the orientation of the 5 µm wide microgrooves significantly influenced the migration and cell coverage of osteoblasts [158]. Compared to parallel microgrooves, the radial microgrooves enhanced the cell coverage area in vitro noticeably. However, regardless of the orientation of the microgrooves, an up-regulation of Rac1, PI3K and pAkt proteins and a down-regulation of E-cadherin both contributed to enhanced migration and proliferation of osteoblasts [158].
Fig. 2.9 Osteogenesis of human mesenchymal stem cells (hMSCs) on grooved surfaces with spacing ratio of 1:1, 1:3 and 1:5. A) Alizarin Red S and B) Von Kossa staining of hMSCs cultured on the grooved and flat surfaces after 21 days of culture with osteogenic medium. The arrows indicate the formation of mineralized bones. C) Quantification of the degree of mineralization by Alizarin Red S staining. D) Quantification of cell viability of cells after 21 days. All values were normalized to the control. Error bars represent the standard deviation (SD) and mean (n=3 for each group). E) Representative immunofluorescent staining of osteocalcin (OCN) of human mesenchymal stem cells (hMSCs) on the grooved surfaces for 7 days in osteogenic medium. Higher expression of OCN was on 1:1 grooved surface than on 1:5 counterparts. Adapted with permission from [55]. Copyright 2013 Nature Publishing Group.
2.4.6 Intricate matrices

Intricate matrices are 3D structures, comprised of interconnected pores, protrusions, pits etc., made from polymer [77] and metals [166]. Fabrication methods include laser cutting, hydraulic press [166] and additive manufacturing [22, 81]. For instance, 3D intricate matrices with resolution down to 100 nm can be fabricated using two-photon polymerization, an advanced additive manufacturing technique for 3D microfabrication [54, 69]. The advantage of intricate matrices is to mimic 3D ECM, i.e. the natural cell environment [77, 167] and to provide spatial guidance for cells [168].

Raimondi et al. found that primary rat MSCs migrated and adhered to microscaled 3D niches spontaneously from the surrounding flat areas, as shown in Fig. 2.10 [54]. The largest cell numbers were observed on 20 μm high niches with varied pore sizes, compared to those with 80-100 μm high niches [54]. However, no information of whether the stem cells had differentiated into osteogenic lineage was given by the authors. Wang et al. demonstrated that ordered 3D titanium meshes promoted osteoblast differentiation, and conversely they depressed cell proliferation compared to 2D surfaces in the early stages of culture (around 5 - 6 days) [166]. This study further confirmed that the cell proliferation rate was inversely proportional to the degree of cell differentiation [166]. The combination of submicron roughness created by acid etching on a 3D structure resulted in better cell differentiation compared to unetched counterparts [166]. Compared to 2D surfaces, 3D scaffolds were able to prolong the osteoblast proliferation periods and also accelerate osteoblast maturation rates [166]. In
addition, signalling of integrins α2β1 and β1 was critical in MG63 maturation [166].

Fig. 2.10 A) SEM image of MG63 cells in the microscaled intricate matrices after 6 days of culture. The cells have proliferated to confluence on the flat surface surrounding the matrices. Cells first climbed the external walls of the matrix before invading into its internal structure. B) Top view of intricate matrices: in the central pore, a round cell nucleus, cytoskeletons spread in all directions to invade the internal structure of the intricate matrices. Adapted with permission from Ref. [54]. Copyright 2013 Elsevier.

2.4.7 Hierarchical patterned topography

As mentioned previously, bone has hierarchical structures and bone cells can interact with implant surfaces at different scales. Hierarchical surface topography combines micro- and nano-sized features together on the same implant surface. The use of a hybrid fabrication process allows the generation of hierarchical patterns on an implant surface. Photolithography can be used to produce microscaled patterns, followed by electrochemical anodization to create nanoscaled patterns on the same surface [169]. Capillary force lithography facilitated the fabrication of nanogrooves, while the micro-wrinkling method allowed bending nanogrooves to form microwaves on the surface [56]. A
A femtosecond laser technique has been developed to generate hierarchical structures on titanium alloys [52], allowing the production of high resolution features on the surface with limited thermal effects [50].

Dumas et al. reported that the direction of nanostructures in relation to microgrooves (i.e. parallel to or orthogonal to) exerted significant effects on the alignment of MSCs [52]. When nanostructures were parallel to the microgrooves, cells were more likely to orient themselves along the groove direction than in the case when the nanostructures were made to be orthogonal to the microgrooves [52]. Furthermore, the presence of 600 nm wide nanostructures alone, even without the microgrooves, was also capable of orienting the MSCs along the groove direction [52]. Recently, researchers from the same group fabricated surfaces comprised of micron pits (diameter: 30 µm, depth: 800 nm) with submicron ripples either inside or outside the pits, as well as surfaces with just single submicron ripple arrays (depth: 200 nm) [50]. All three types of surfaces were efficient to stimulate osteogenesis of MSCs and mitigate adipogenesis. Furthermore, hierarchical structures up-regulated more expression of Runx2 and OCN than submicron ripples alone [50].

Kim et al. revealed that hierarchical topography significantly increased surface hydrophilicity compared to un-patterned and nanopatterned-only surfaces on PLGA patches [56]. In addition, compared to single nanopatterns, hierarchical patterns exhibited higher adhesion to bone tissues. Multiscale topography manipulated cell morphology along the direction of patterns synergistically. This indicates that nanofeatures in hierarchical structures can play a more crucial role than microfeatures in osteogenesis of hMSCs [56]. The comparison of PLGA nanoscaled patterns and hierarchical patterns are shown in Fig. 2.11. In summary,
hierarchical patterns can increase cell responses synergistically compared to the single patterns aforementioned.

**Fig. 2.11** Cross-section SEM images of A) a surface with 350 nm wide grooves and B) a hierarchical surface (350 nm wide grooves on the 30 μm waved surface). Adapted with permission from Ref. [170]. Copyright 2014 Elsevier.
2.5 Concluding remarks

This paper has critically reviewed the recent studies on cell responses to both ordered and partially ordered patterns on implant surfaces under in vitro conditions. It should be pointed out that, such studies do not capture all the characteristics of bone implant surfaces in a real environment and significant differences may exist. As related in previous studies, that in vitro findings do not necessarily predict in vivo performance. Whilst in vivo data is not readily available regarding ordered and partially ordered surfaces, substantial published in vivo data is available for other topographically complex surfaces. It should also be highlighted that in vitro data is acquired to give some indication of biocompatibility, but the in vitro cell-to-synthetic substrate interactions are not always indicative of what to expect in the in vivo and true physiological setting. For this reason, whole animal modelling is required as the definitive next step.

Over the last two decades, substantial research has been conducted with a view to regulating cell responses through surface topographical modifications under in vitro conditions. The benefits of partially ordered and ordered surface topography in prompting or enhancing desired cell responses have been well documented. These include:

1) Pattern reproduction;

2) Quantification of cell responses to individual dimensions or features of surface patterns;

3) Capacity to amplify the favourable individual features and combine them to maximize implant osseointegration;
4) Capability to mimic the living environment of mammalian cells – this requires integration with the ECM.

However, as an emerging research area, several challenges remain to be addressed to translate the basic research findings into functional implants for robust clinical applications. In particular, the following aspects deserve some special attention in future surface designs for orthopaedic prostheses:

1) Hierarchical structures hold promise for future implant surface designs because of their demonstrated capabilities of providing cells with both micro- and nano-topographical stimuli [171].

2) 3D intricate matrices that mimic ECM can provide cells with a support structure that simulates the living environment, as in vivo. Consequently, additive fabrication may produce 3D intricate matrices that can promote cell lineage-specific development. This approach deserves further investigation.

3) Defined features, produced in conjunction with homogeneous functional coatings on an implant surface (e.g., antimicrobial zinc oxide, and more biocompatible tantalum oxide) may further enhance cell responses to improve implant integration.

4) A comparison of cell responses to different patterned surfaces is needed to gain a deeper understanding of the role of surface topography in enhancing cell responses. Justesen et al. concluded that the vertical dimension (Z) (i.e. height) exerted more effects on cell responses (morphology and mineralization) than the lateral dimensions based on a study of 13 micropatterned surfaces [172].
5) A particularly important issue is to increase the efficiency of fabricating ordered arrays on an implant surface and to lower the cost at the same time. Current mainstream methods for the fabrication of micro/nano surface patterns such as EBL and photolithography are time-consuming and costly. For example, in order to increase productivity, researchers have fabricated master substrates by exploiting EBL, followed by imprinting into polymers via hot embossing or injection moulding [42, 48, 117, 164]. As alternatives, high-throughput screening methods such as Biosurface Structure Array (BSSA) [173], the combined use of TopoChip and computer-aided algorithm [174], and Multi Architectural (MARC) chip [175] allow us to identify cell responses from a large quantity of samples efficiently.

6) Although many researchers have fabricated patterned surfaces on polymeric substrates, the mechanical strength of polymeric substrates is insufficient for load-bearing implant applications [176]. In comparison, biocompatible titanium alloys possess superior mechanical properties and they have been the focus of bone implant materials research [42]. Questions of how to efficiently convey the identified useful patterns from soft materials onto metallic implant surfaces without losing fidelity are yet to be fully answered.

7) The development of prosthetic devices requires in vivo assessment and this pre-clinical testing is crucial to determining the value of a particular prosthetic device and whether surface modifications are beneficial in terms of function and longevity of the prosthesis. Comprehensive pre-clinical testing is required in small animal models to assess integration and performance prior to clinical application, further providing valuable information about tissue maturation and physiological response to implanted devices. However, the
initial testing of any device will always be based on cell lines since it is known from extensive studies that cell responses and signalling pathways involved are reflected in the in vitro assessment.

Understanding the response of bone cells to partially ordered and ordered topographical stimuli can provide important insights into the design of bone-inspired surfaces. Owing to the wide variety of bone cell types, animal models, culture conditions (cell density, with/without osteogenic medium), and assessment methods utilized by different researchers, it is challenging to reach consistent and definite conclusions as regards the optimal surface topography for bone cell responses [99, 103, 150, 177]. In order to attain a comprehensive understanding of the relationship between the surface pattern and cell functions, multi-disciplinary knowledge on micro/nanofabrication, cell biology, microscopy analysis etc. is needed. Further in-depth structural and functional studies are required to understand the underlying mechanisms for bone cell responses to different surface topographies.

2.6 References


Chapter 2


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Chapter 3

Comparative study of the effect of submicron porous and smooth ultrafine-grained Ti-20Mo surfaces on osteoblast responses

3.1 Brief summary

The surface of an orthopaedic implant plays a crucial role in determining the adsorption of proteins and cell functions. A detailed comparative study has been made of the in vitro osteoblast responses to coarse-grained (grain size: 500 μm), ultrafine-grained (grain size: 100 nm), coarse-porous (pore size: 350 nm) and fine-porous (pore size: 155 nm) surfaces of Ti-20Mo alloy. The purpose was to provide essential experimental data for future design of orthopaedic titanium implants for rapid osseointegration. Systematic original experimental data was produced for each type of surfaces in terms of surface wettability, cell morphology, adhesion, growth and differentiation. Microscopic evidence was collected to reveal the detailed interplay between each characteristic surface with

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proteins or cells. Various new observations were discussed and compared with literature data. It was concluded that the coarse-porous surfaces offered the optimum topographical environment for osteoblasts and that the combination of ultrafine grains and considerable grain boundary areas is not an effective way to enhance cell growth and osteogenic capacity. Moreover, pore features (size and depth) have a greater effect than smooth surfaces on cell growth and osteogenic capacity. It proves that cells can discern the difference in pore size in the range of 100-350 nm.

**Keywords:** Ti-20Mo, ultrafine grains, submicron porous surfaces, osteoblast responses, filopodia

### 3.2 Introduction

Titanium (Ti) and its alloys are the materials of choice for orthopaedic implants due to their advantageous attributes including high biocompatibility, excellent corrosion resistance in bodily fluids, high strength-to-weight ratios, high fracture toughness and good fatigue properties [10, 178]. Up till now, commercially pure Ti (CP-Ti) and Ti-6Al-4V (in wt.% unless otherwise stated) remain the most widely used Ti alloys for biomedical applications (CP-Ti is mainly used for dental applications). However, these alloys have much higher Young’s modulus (~110 GPa) than cortical bones (10-30 GPa), leading to the so-called stress shielding effect [14]. Another concern on orthopaedic implants made from Ti-6Al-4V is the release of toxic Al and V ions from the surface [10]. Consequently, a significant effort has been made for the development of Al- and V-free low-modulus beta-type Ti alloys (β-Ti) [14], where β-Ti alloys refer to those that can retain 100% of the β-phase on water quenching due to the β-stabilizing effect of
alloying elements such as molybdenum (Mo), niobium (Nb), tantalum (Ta) and iron (Fe) [179]. The minimum amount of Mo required for this purpose is 10 wt.% [180]. On this basis, the so-called “Mo equivalency” has been commonly used to evaluate the β-phase stability in a Ti alloy for both alloy design and microstructural control [180].

As a non-toxic potent β-stabilizer, Mo has long been used as a principal alloying element for the development of biocompatible low-modulus β-Ti alloys [181]. In addition, Mo is an essential component of certain enzymes and therefore plays an important role in balancing the pH values in human body [182, 183]. As a result, binary Ti-Mo alloys have been studied extensively for biomedical applications [14, 184-186]. One such notable development is the composition Ti-15Mo, which is available commercially today in various product forms [187] under ASTM F2066 for high-strength bone implant applications [188]. Ti-15Mo is currently produced in two types of microstructures, namely, single β-phase and β + α [187]. The (β + α)-Ti-15Mo, which takes advantage of the metastability of its β-phase for α to precipitate due to the insufficient Mo content (15%), offers both high strengths (desired) and modulus (undesired) compared to the single β-phase Ti-15Mo. In order to ensure the achievement of a full β-phase Ti-Mo alloy under a broad range of processing conditions, rather than restricted to water quenching, it is necessary to further enhance the β-phase stability, i.e., to increase the Mo content. In this regard, metastable β-Ti-20Mo alloy has emerged as a promising choice with Young’s modulus of 91 GPa (as quenched) [183, 189-192], ultimate tensile strength of 992 MPa, yield strength of 494 MPa and elongation at fracture of 10 % (after cold-rolled treated) [193], which is desired for developing bone plates, spinal fixation devices and hip joints [14]. In particular, Bolat et al. have
recently shown that Ti-20Mo displayed excellent corrosion resistance in saline solutions [189], implying good potential for orthopaedic applications. Accordingly, Ti-20Mo is chosen as the experimental alloy for this study.

Rapid and long-lasting osseointegration is crucial to the clinical success of an implant [87]. The surface of an orthopaedic implant plays a crucial role in determining the adsorption of proteins and cell functions such as adhesion, proliferation and differentiation [127, 128]. The interactions occur from microscale down to nanoscale [87, 194]. As such, various approaches have been used to modify Ti surfaces for enhanced biofunctionality. For example, achieving an ultrafine-grained surface has proved effective in enhancing the biocompatibility with cells compared to the coarse-grained surface of the same alloy [20, 195-198]. In addition, an ultrafine-grained Ti alloy can effectively reduce the size or wall thickness of orthopaedic implants due to strengthening by grain refinement [13, 199]. With regards to the elastic modulus, which is an important implant property, in general, achieving an ultrafine grain size has no effect unless the grain size is reduced to about 20 nm below which the elastic modulus decreases with decreasing grain size [200-203]. However, it should be pointed out that for metastable β-Ti alloys such as Ti-Mo alloys, if the ultrafine grain size is achieved by severe plastic deformation and accompanied by significant formation of stress-induced ω-phase particles, then the elastic modulus can increase due to the ω-phase [204].

Another commonly used surface modification approach for implants is the introduction of an interconnected porous surface, which offers distinct advantages over dense surfaces. These include: (i) reduced Young’s modulus at the specific area of contact [10, 178, 205], (ii) enabling bone ingrowth [9, 206, 207], and (iii)
easy distribution of nutrients for cell growth [208]. Pore size and pore interconnectivity have been documented as the two main factors that need to be considered in the design of such a porous surface [10, 209].

Although both ultrafine-grained and interconnected porous surfaces can enhance cell responses, it remains elusive as to which surface features are more effective in regulating cell functions. A detailed literature survey has found no relevant *in vitro* comparative studies on ultrafine-grained surfaces vs. interconnected porous surfaces. Thus, this study systematically compares osteoblast cell responses to both ultrafine-grained (grain size: ∼100 nm) and submicron porous (pore size: 150-350 nm) surfaces of Ti-20Mo. The porous surface features ranging from 100-400 nm are selected because of their similarity in length scale to that of filopodia (100-300 nm) [210]. The purpose is to investigate how surface topography and wettability affect cell attachment, adhesion, growth and differentiation. The outcomes are expected to form an essential knowledge base for the design of Ti-20Mo alloy as orthopaedic implants.

### 3.3 Materials and methods

#### 3.3.1 Fabrication and preparation of substrates

The fabricated Ti-20Mo alloy substrates with a diameter of 10 mm and a thickness of 1 mm were used in this study. As-cast Ti-20Mo alloy samples were first solution-treated at 1273K for 48 h, followed by water quenching to produce metastable β-Ti-20Mo microstructures, denoted as coarse grained. To obtain the ultrafine-grained microstructure, high pressure torsion (HPT, a severe plastic deformation technique for grain refinement) was applied to the water-quenched
Ti-20Mo substrates under a pressure of 6 GPa for 10 turns, and the substrates thus produced are referred to as ultrafine grained. After HPT processing for 10 turns at a pressure of 6 GPa, homogeneous and equiaxed β grains were produced throughout the ultrafine-grained Ti-20Mo substrates [191]. Minor homogeneity limited to the core region of 0.5 mm in diameter (relative to 10 mm of the substrate; 95% homogeneity) has little effect on cell response statistically. This is consistent with previous studies showing that HPT processing with high applied pressure (≥ 5 GPa) and sufficient straining (more than 5 turns) to achieve homogeneous microstructures [211]. For further details regarding HPT technique the reader is referred to the published work by Xu et al.[191] and one comprehensive review by Zhilyaev et al. [212].

All the disks were ground first with SiC sandpaper from P320 to P4000 and then polished using a mixture of OPS colloidal silica (0.04 μm) and 10% hydrogen peroxide (H₂O₂). The prepared disks were cleaned with acetone, ethanol and distilled water in ultrasonic bath for 15 min at each step and dried with N₂ gas. Finally, for producing interconnected pores on the surfaces, the ultrafine-grained substrates were heat-treated (aging) at 550 °C for up to 60 min under Ar atmosphere followed by water quenching. The Kroll’s reagent (HF: 2ml, HNO₃: 6 ml, distil water: 92 ml) was then used to etch the polished substrates for 1 min. The creation of interconnected pores was achieved by removing the α (Mo-depleted) phase from the duplex structures via acid etching and only leaving the β (Mo-rich) phase [191]. The acid-etched substrates that were aged for 0 min (no holding) are denoted as fine porous. In addition, after prolonging the aging period to 60 min and acid etching, coarser pores were obtained on the surface, named as coarse porous. In this study, the coarse-grained Ti-20Mo (without HPT, aging and
acid-etching) is treated as a control. Table 3.1 summarizes the fabrication processes of all the substrates.

Table 3.1 Preparation methods, microstructural and surface features of Ti-20Mo alloy substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Preparation methods</th>
<th>Microstructural features [191]</th>
<th>Surface features</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solution treated at 1000°C for 48 h</td>
<td>Aging at 550 °C</td>
<td>Polish ing</td>
</tr>
<tr>
<td>Coarse grained</td>
<td>√</td>
<td>HPT 0 min</td>
<td>Acid etching 60 min</td>
</tr>
<tr>
<td>Ultrafine grained</td>
<td>√</td>
<td>HPT 0 min</td>
<td>Acid etching 60 min</td>
</tr>
<tr>
<td>Fine porous</td>
<td>√</td>
<td>HPT 0 min</td>
<td>Acid etching 60 min</td>
</tr>
<tr>
<td>Coarse porous</td>
<td>√</td>
<td>HPT 0 min</td>
<td>Acid etching 60 min</td>
</tr>
</tbody>
</table>

3.3.2 Grain structure and surface characterisation

X-ray diffraction (XRD) patterns of all Ti-20Mo substrates were measured using a Bruker D4 diffractometer, which is equipped with a Cu source. X-ray photoelectron spectroscopy (XPS) of all Ti-20Mo substrates was carried out using a Thermofisher Scienfitic K-Alpha system with an Al kα source and a spot size of
400 μm. The grain structures of both coarse-grained and ultrafine-grained surfaces were examined by a transmission electron microscope (TEM).

Surface morphology was evaluated using an ultra-high-resolution scanning electron microscope (SEM, FEI, Verios 460L). The measurement of the pore diameter distribution was analysed from measuring at least 100 pores on three different locations for each sample using both the line and ROI manager tools in Image J. Three samples were measured from each group. Focused ion beam (FIB, FEI, Scios) milling was employed to cut through the surface to observe the pore depths and profiles. Surface roughness characterised by the average roughness ($S_a$) and root mean square roughness ($S_q$) were measured using an AFM (Bruker) in the tapping mode. Each AFM analysis was performed over an area of 3 μm × 3 μm. To ensure the representativeness of the surface characteristics, three samples were selected from each group and three different areas were scanned in each sample.

Surface wettability was measured by the sessile-drop contact angle method (Theta Lite Optical Tensiometer, ATA Scientific). Two different solvents were applied: ultrapure distilled water and ethylene glycol. The contact angle was measured at 5 s after placing the droplet (2 μL) on the surface of each sample and repeated at three different areas of the sample. The tests were applied to three different samples selected from each group. The surface free energy ($\gamma$) and its components were calculated using the Owens-Wendt (OW) method, given in Eq. 1 [213]:

$$\gamma_L (1 + \cos \theta) = 2 \sqrt{\gamma_L^d \gamma_S^d} + 2 \sqrt{\gamma_L^p \gamma_S^p}$$  \hspace{1cm} (1)

where $\gamma_L$ is the surface tension of the liquid; $\gamma_S$ is the surface energy of the solid; and $\gamma_S$ is the sum of the dispersion component ($\gamma^d$) and the polar component ($\gamma^p$).
\( \gamma_L, \gamma', \text{ and } \gamma_d \) for water are 72.8, 51.0, and 21.8 mJ/m\(^2\), respectively [213], and 48.0, 19.0, and 29.0 mJ/m\(^2\), respectively for ethylene glycol.

### 3.3.3 Cell culture

Human fetal osteoblast line (hFOB 1.19) was used in this study (ATCC, CRL-11372). They were cultured in the medium of mixed Dulbecco’s Modified Eagle’s and Ham’s F12 (DMEM/F12, Thermofisher Scientific), added with 10 % (v/v) fetal bovine serum (FBS, Interpath Services) and 0.3 mg/ml Geneticin selective antibiotics G418 (Thermofisher Scientific) at 34°C in a humidified atmosphere of 5% CO\(_2\). The complete growth medium was replaced every 3 days and confluent cells were subcultured using TrypLE™ Express Enzyme (Invitrogen). When cells were confluent, a total of 3×10\(^5\)/200 μl cells were seeded on each disk in a 24-well culture plate for the study of cell number and differentiation. For imaging cell morphology, cells were seeded at a density of 2×10\(^5\)/200 μl on each disk in a 24-well plate. The culture medium was replenished every 3 days.

### 3.3.4 Immunofluorescence observation of cell morphology and focal adhesions

After culturing for 8 days, the cell-seeded disks were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde solution in PBS for 10 min. Cells were permeabilized with 0.02 % (v/v) Triton X-100 in PBS for 90 s and blocked with 2 % (wt/v) bovine serum albumin (BSA) in PBS for 30 min. Vinculin was immunostained by incubating cells in 1:50 dilution of mouse monoclonal IgG antibody (anti-vinculin) (Santa Cruz Biotechnology) in blocking
buffer (i.e., the BSA) for 1 h. Subsequently, cells were washed and probed with goat-anti-mouse secondary antibody Alexa Fluor 594 conjugate (Invitrogen, 1:500) in blocking buffer for 45 min. Following extensive washing steps to remove free secondary antibody, actin filaments were stained with 1:40 dilution of Oregon Green 488 phalloidin in blocking buffer for 20 min. Cell nuclei were stained with Hoechst 33342 (2 μg/ml in PBS) for 10 min. Finally, all disks were mounted onto cover glasses with gold anti-fade mounting medium and imaged using a confocal microscope (N–STORM, Nikon). Images were collected at three locations on each substrate using a confocal microscopy. Moreover, the distribution of vinculin, cell spreading area and cell elongation ratio (the ratio of cell long axis and short axis) were quantified using Image J (version 1.6) [214]. For each substrate, approximately 25 cells were analysed with two replicates. The confocal images taken using an objective of 40× were utilized to quantify the effect of surface features on the length of FAs. The captured immunofluorescence images were first split into single-channel greyscale images. Afterwards, all greyscale images of FAs were adjusted in terms of brightness and contrast and then thresholded, both particle analysis and ROI manager tools were employed to evaluate the length of FAs on each substrate [154, 215]. For measuring cell spreading area and elongation ratio of osteoblasts, polygon and line tool and ROI manager of Image J were used.

3.3.5 Cell morphology using SEM

Cell morphology was studied on various Ti-20Mo disks using a SEM. After culturing for 1 day, cell-seeded disks were rinsed with sodium cacodylate and then fixed with 0.1M cacodylate buffer that contains 2 % (v/v) paraformaldehyde
and 2.5 % (v/v) glutaraldehyde for 30 min. They were dehydrated in a graded series of ethanol (from 30% to 100%) [216]. All substrates were chemically dried with 100% hexamethyldisilazane (Sigma-Aldrich) for 20 min. Prior to SEM imaging, all samples were sputter-coated with platinum (Pt). Secondary electron images were collected with an accelerating voltage of 2 kV. For quantifying the length of filopodia and cell spreading areas, SEM images were analysed using line and polygon tool and ROI manager of Image J on two samples from each group.

### 3.3.6 Cell number and alkaline phosphatase (ALP) activity

Cell numbers were measured by directly counting using a haemocytometer. After culturing for 8 days, all the disks were transferred to other sterilized 24-well plates. After washing with PBS once, the adhered cells were detached from the surfaces of the disk samples with 200 μl of TrypLE™. The cell suspensions were collected in eppendorf tubes and placed on ice before centrifuging at 1000 rpm for 5 min. Adhered cell number was determined by counting using the standard trypan blue exclusion method.

ALP activity is an early osteogenic differentiation indicator, measured using an ALP fluorescence detection kit (Sigma-Aldrich). The principle of this kit is to hydrolyze \( p \)-nitrophenyl phosphatase to \( p \)-nitrophenol at 37 °C [217]. Cells were cultured on disks for 8 days at an initial seeding density of \( 3 \times 10^5 \) cells/ well. The adhered cells were lysed with 0.2 % (v/v) Triton X-100 for 10 min and cell lysates were collected in eppendorf tubes and centrifuged at 12,000 rpm for 5 min at 4 °C. Supernatants of cell lysates were transferred into new eppendorf tubes, stored at −80°C until used. Cell lysates were used to measure the ALP activity.
Fluorescence intensity was measured at an excitation wavelength of 360 nm and an emission wavelength of 440 nm using a microplate reader (SpectraMax Paradigm, Molecule device). The total protein content was determined by a BCA protein assay kit (Pierce) following manufacturer’s instructions and the absorbance was measured by a microplate reader. The ALP activity was normalized by the total protein content.

3.3.7 Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), with post hoc testing using Turkey’s multiple comparison tests (GraphPad Prism). Data were expressed in the form of mean value ± standard deviation (SD). A value of \( p < 0.05 \) was considered significant.

3.4 Results

3.4.1 Grain structure and surface characteristics

The grain structures of coarse-grained and ultrafine-grained substrates were characterised using either a SEM or a TEM, as shown in Fig. 3.1. The average grain size of the coarse-grained substrate is 507 ± 87 μm, while that of the ultrafine-grained substrates is 104 ± 22 nm. Average roughness \( S_a \) and root mean square roughness \( S_q \) (0.3-1.1 nm) of coarse-grained and ultrafine-grained substrates are listed in Table 3.2. Both coarse-grained and ultrafine-grained substrates were smooth and flat at the nanoscale. Fig. 3.2 shows XRD profiles of the four Ti-20Mo substrates. Both the coarse-grained and ultrafine-grained substrates are composed of β phases, while both the fine-porous and coarse-grained substrates...
porous substrates consist of a mixture of α and β phases. Fig. 3.3 shows submicron interconnected pores in the surface layers of both fine-porous and coarse-porous surfaces. The coarse-porous substrate (aged at 550 °C for 60 min, Figs. 3.3E-G) contained coarser pores, of a mean diameter of 350 ± 96 nm compared to 155 ± 38 nm in the fine-porous substrate (aged at 550 °C for 0 min, Figs. 3.3A-C). This is because increasing aging time from 0 min (fine porous) to 60 min (coarse porous) leads to an increase in the size of α precipitates [191] which are subsequently etched away to form coarser pore cavities. The average pore sizes (~155 nm for fine-porous and ~350 nm for coarse-porous surfaces) are far larger than the average sizes of the α phase (<100 nm for fine-porous and ~200 nm for coarse-porous surfaces) [191], indicative of a complete removal of α phases. In addition, surface composition analysis using XPS showed a substantial increase in Mo from 18 wt.% for coarse-grained and ultrafine-grained surfaces to more than 30 wt.% for fine-porous and coarse-porous surfaces (Table 3.3). This also suggests that the entire α phase and a portion of the β matrix have been removed by etching to form the porous surface structures of the fine-porous and coarse-porous substrates. Moreover, the analysis on the cross section of the porous surface structures using a dual beam FIB/SEM (Fig. 3.8) revealed an average pore depth of 0.95 ± 0.12 μm for the fine-porous surfaces, which was nearly half that of 1.95 ± 0.25 μm for the coarse-porous surfaces. Table 3.2 summarises the pore size and the thickness of the porous surface layer for both the fine-porous and coarse-porous surfaces.
Fig. 3.1 (A) SEM micrograph of the coarse-grained substrate with an average grain size of 500 µm, (B) TEM bright-field micrograph of the ultrafine-grained substrate with an average grain size of 100 nm. Yellow dashed line indicates the grain boundary in the ultrafine-grained substrate.

Fig. 3.2 XRD profiles of the coarse-grained, ultrafine-grained, fine-porous and coarse-porous surfaces of Ti-20Mo.
**Fig. 3.3** Topography analyses of Ti-20Mo porous surfaces. SEM images of fine-porous surfaces at (A) low and (C) high magnifications; (B) AFM images and (D) pore size distribution of fine-porous surfaces. SEM images of coarse-porous surfaces at (E) low and (G) high magnifications; (F) AFM images and (H) pore size distribution of coarse-porous surfaces.
Table 3.2 Surface average roughness (Sa) and root mean square roughness (Sq) values of coarse-grained and ultrafine-grained substrates; pore size and the thickness of the porous surface layer for the fine-porous and coarse-porous substrates (For surface roughness, three areas were measured on each sample and each area was measured to be 3 μm × 3 μm).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sa (nm)</th>
<th>Sq (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse grained</td>
<td>0.64 ± 0.37</td>
<td>1.11 ± 0.61</td>
</tr>
<tr>
<td>Ultrafine grained</td>
<td>0.33 ± 0.19</td>
<td>0.72 ± 0.41</td>
</tr>
</tbody>
</table>

Pore size (nm) | Thickness of the porous surface layer (nm)

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine porous</td>
<td>155 ± 38</td>
<td>950 ± 120</td>
</tr>
<tr>
<td>Coarse porous</td>
<td>350 ± 96</td>
<td>1950 ± 250</td>
</tr>
</tbody>
</table>

Table 3.3 XPS surface composition analysis for the studied Ti-20Mo substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ti (wt.%)</th>
<th>Mo (wt.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse grained</td>
<td>81.81</td>
<td>18.17</td>
</tr>
<tr>
<td>Ultrafine grained</td>
<td>81.83</td>
<td>18.17</td>
</tr>
<tr>
<td>Fine porous</td>
<td>67.84</td>
<td>32.16</td>
</tr>
<tr>
<td>Coarse porous</td>
<td>64.22</td>
<td>35.78</td>
</tr>
</tbody>
</table>

Fig. 3.4 shows the contact angle (θ) values of ultrapure distilled water measured on each surface and the corresponding water droplet morphology. Coarse-grained and fine-grained surfaces demonstrated similar contact angles (58.23 ± 6.28° vs. 61.66 ± 7.76°). However, much lower contact angles were observed for both fine-porous (27.86 ± 4.11°) and coarse-porous (39.33 ± 8.24°) surfaces. Table 3.4 summarises the surface free energy values obtained for each surface. Both the total surface free energy and its relevant polar component of the fine-porous and coarse-porous surfaces are about twice the respective values of coarse-grained and ultrafine-grained surfaces. In contrast, a substantial grain size reduction from 500 μm to 100 nm led to merely a marginal increase of 24% in total surface free energy.
energy (i.e., better wettability) (Table 3.4). Being porous was therefore much more effective in enhancing the wettability than grain refinement.

**Fig. 3.4** Water contact angle of different Ti-20Mo surfaces and water droplet morphology (mean ± SD, * p < 0.05 vs. coarse-grained surfaces, # p < 0.05 vs. ultrafine-grained surfaces, & p < 0.05 vs. fine-porous surfaces).

**Table 3.4** Surface free energy of different Ti-20Mo surfaces (mean ± SD, * p < 0.05 vs. coarse-grained substrates, # p < 0.05 vs. ultrafine-grained substrates).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dispersive component $\gamma^d$ (mJ/m$^2$)</th>
<th>Polar component $\gamma^p$ (mJ/m$^2$)</th>
<th>Total surface free energy $\gamma$ (mJ/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse grained</td>
<td>7.01 ± 1.29</td>
<td>25.71 ± 5.39</td>
<td>32.72 ± 5.69</td>
</tr>
<tr>
<td>Ultrafine grained</td>
<td>2.69 ± 1.23*</td>
<td>37.83 ± 6.97</td>
<td>40.52 ± 6.63</td>
</tr>
<tr>
<td>Fine porous</td>
<td>1.21 ± 0.41*</td>
<td>79.10 ± 4.65*#</td>
<td>80.31 ± 2.94*#</td>
</tr>
<tr>
<td>Coarse porous</td>
<td>5.40 ± 1.93</td>
<td>73.95 ± 7.15*#</td>
<td>79.35 ± 2.37*#</td>
</tr>
</tbody>
</table>
3.4.2 Cell morphology and adhesion

The osteoblast morphology on each substrate surface was investigated using both SEM and confocal microscopy at different times of culturing. Fig. 3.5 shows representative SEM images of osteoblasts on each surface after culturing for 1 day. Cells spread more broadly on both coarse-grained and ultrafine-grained surfaces than on fine-porous and coarse-porous surfaces. In addition, both the cell nuclei and grown cells were nearly spherical on fine-porous and coarse-porous surfaces. Figs. 3.5 (B, D, F, H) are higher magnification views of the filopodia corresponding to Figs. 3.5 (A, C, E G). Filopodia integrated themselves well into both fine pores and coarse pores on the surfaces.

Fig. 3.6 show a closer view of the interactions between the filopodia and pores on the fine-porous surfaces. The filopodia interacted with submicron pores (~155 nm) intensively at different depths and eventually grew into these pores (indicated by arrows in Fig. 3.6). In order to further gauge the interactions between osteoblasts and implant surfaces, a quantitative measurement of cell morphology was conducted using Image J. Both the average length of filopodia and the average cell spreading area were quantified from SEM observations (Fig. 3.7). Filopodia extended furthest on smooth surfaces, reaching 14.62 ± 5.00 μm on ultrafine-grained surfaces and 13.64 ± 6.46 μm on coarse-grained surfaces vs. 3.88 ± 1.37 μm on fine-porous surfaces and 2.89 ± 2.11 μm on coarse-porous surfaces. A similar trend in cell spreading area was observed (Fig. 3.7B), i.e., much smaller spreading areas on porous surfaces. On this basis, Fig. 3.8 exhibits representative observations of the cross sections of cells grown on porous surfaces, revealing strong interfacial connections between the cells and the porous surfaces.
Fig. 3.9 shows representative confocal images for vinculin, actin and nuclei of osteoblasts on each type of surfaces after 8 days of culturing. In general, actin cytoskeletal stress fibres were well developed and vinculins were found distributed on the leading edges of cells as well as at the centre of cells on all the substrate surfaces. Compared to the round cell shapes after culturing for 1 day (Fig. 3.5E and 5G), cells became less circular or more elongated on fine-porous and coarse-porous surfaces after extending culturing period to 8 days (Fig. 3.9). In addition, cells on fine-porous and coarse-porous surfaces were noticeably elongated (elongation ratio ~ 3) compared to those on coarse-grained and ultrafine-grained surfaces (elongation ratio < 2) (Fig. 3.10A). To identify how grain sizes and pore features influence the formation of focal adhesions (FAs), the lengths of FAs were quantified using Image J, as shown in Fig. 3.10B. Cells on fine-porous surfaces were featured by the largest FAs with the average length of 2.50 ± 1.60 μm, followed by coarse-porous surfaces (2.23 ± 1.48 μm), ultrafine-grained surfaces (2.07 ± 0.87 μm) and coarse-grained surfaces (2.05 ± 0.88 μm). In addition, a higher proportion of large and mature FAs (the length ≥ 5 μm) were present on the fine-porous (6.16%) and coarse-porous (6.00%) surfaces than on the coarse-grained (2.89%) and ultrafine-grained (1.84%) surfaces.
Fig. 3.5 SEM images of osteoblasts grown on various Ti-20Mo surfaces after culturing for 1 day. (A-B): coarse-grained surfaces, (C-D): ultrafine-grained surfaces, (E-F): fine-porous surfaces and (G-H): coarse-porous surfaces. Left column: low-magnification SEM images, right column: high-magnification SEM images. Yellow arrows indicate that filopodia interacted with Ti-20Mo surfaces.
Fig. 3.6 Interactions between filopodia and pores on the fine-porous surfaces of Ti-20Mo.
Fig. 3.7 (A): filopodia length and (B): cell spreading area of osteoblasts after culturing for 1 day on different Ti-20Mo surfaces (mean ± SD, *: p < 0.05 vs. coarse-grained surfaces, **: p < 0.001 vs. coarse-grained surfaces, #: p < 0.05 vs. ultrafine-grained surfaces, ##: p < 0.001 vs. ultrafine-grained surfaces, &: p < 0.05 vs. fine-porous surfaces).
Fig. 3.8 Cross section images of osteoblasts grown on porous surfaces of Ti-20Mo substrates after culturing for 1 day: (A) fine-porous and (B) coarse-porous surfaces.
**Fig. 3.9** Confocal microscopy images of osteoblasts grown on all substrates after culturing for 8 days. Cells were stained with phalloidin (green) for actin filaments, Hoechst (blue) for nuclei, anti-vinculin (red) for focal adhesions.
Fig. 3.10 (A): elongation ratio (cell long axis/short axis) and (B): the length distribution of focal adhesions on various Ti-20Mo substrates after culturing for 8 days (mean ± SD, *: p < 0.05 vs. coarse-grained surfaces, #: p < 0.05 vs. ultrafine-grained surfaces).
3.4.3 Cell number and differentiation

Fig. 3.11A shows the number of adhered cells on each substrate surface after culturing for 8 days. Both fine-porous and coarse-porous surfaces were more effective in enhancing cell growth than ultrafine-grained surfaces. Furthermore, the normalised ALP activity, which refers to the ALP activity with respect to the protein content, showed clear differences (Fig. 3.11B). The ALP activity was measured in the absence of any osteogenic medium and represented the level of osteogenic capacity. Coarse-porous surfaces displayed the highest intracellular ALP activity, followed by coarse-grained surfaces, while fine-porous and ultrafine-grained surfaces resulted in low ALP activity. Reducing grain size from 400 μm (coarse-grained) to 100 nm (ultrafine-grained) thus failed to enhance the ALP activity. Rather, it led to the lowest ALP activity. A statistical difference ($p=0.03$) was observed in ALP activity between coarse-porous and ultrafine-grained substrates, while no statistical difference ($p>0.05$) was observed between all other groups.
Fig. 3.11 (A): total cell number and (B): alkaline phosphatase (ALP) activity normalised to protein content of osteoblasts cultured on different Ti-20Mo surfaces for 8 days. (mean ± SD, * p < 0.05 vs. coarse-grained surfaces, #: p < 0.05 vs. ultrafine-grained surfaces).
3.5 Discussion

It is clear from the aforementioned comparative study that osteoblasts responded differently to Ti-20Mo surfaces featured with ultrafine grains (submicron) and submicron interconnected pores including cell morphology, adhesion, number and the production of ALP. The effects of ultrafine grains and submicron interconnected pores will be further evaluated below with respect to cell response to surface wettability and length scales of the topographical features.

Surface wettability is one of the decisive factors that dictate the adherence of proteins on an implant surface and thereby affect cell attachment in the early stages [149]. Hydrophilic surfaces often enhance initial cell spreading and osteoblastic differentiation of mesenchymal stem cells [38, 141]. In this study, the water contact angles were found to be similar (58.23 ± 6.28° vs. 61.66 ± 7.76°) for ultrafine-grained and coarse-grained smooth surfaces of Ti-20Mo. Estrin et al. reported similar observations for ultrafine- and coarse-grained smooth surfaces of CP-Ti [20]. However, different observations were made with 316L stainless steel, where ultrafine-grained (grain size: 320 nm; θ=52°) smooth surfaces were found to be more hydrophilic than coarse-grained smooth surfaces (grain size: 22 μm; θ=78°) [218]. This discrepancy might be due to the different properties of the surface oxide films (TiO$_2$ vs. Cr$_2$O$_3$). On the other hand, fine-porous and coarse-porous surfaces of Ti-20Mo were found to be much more hydrophilic than ultrafine-grained and coarse-grained smooth surfaces of Ti-20Mo. This can be attributed to capillary-driven water penetration into the surface pores [219]. It was reported that increasing pore diameter from 85 nm to 400 nm converted the nanoporous alumina surface from hydrophilic (θ=70°) to hydrophobic (θ=132°)
This is consistent with our observations shown in Fig. 3.4, where increasing average pore size from 155 nm to 350 nm increased the water contact angle from 28° to 39°. In addition, the ratio of pore depth-to-diameter can also affect the infiltration of water into the pores, which involves expelling the residual gas deep in the interconnected pores [219]. In that regard, deep pores are not always preferred for maximising surface wettability.

Filopodia, which are normally located at the leading edges of cells, are responsible for sensing favourable microenvironments (e.g., suitable proteins) for cells to adhere to [88, 109]. The locations of such microenvironments determine both the direction and distance of subsequent cell migration, which, in turn, involve rearrangement of the cytoskeletons [88, 109]. As shown in Fig. 3.6, osteoblasts sensed and interacted with pores as small as about 150 nm, and some filopodia eventually grew into them, equivalent to being anchored there. It has been suggested that nutrients and proteins that reside in such pores can attract filopodia to extend into the pores [220]. Consequently, the need for filopodia to sense suitable proteins that exist laterally on the porous surfaces was reduced, and this can explain the reduction in filopodia length on porous surfaces observed in this study (Fig. 3.5F, Fig. 3.5H, Fig. 3.7A). Zhu et al. reported that both submicron (500 nm) and micron (2 μm) pores were capable of anchoring filopodia, benefiting subsequent cell attachment [221]. Previous studies indicated that submicron pores (~263 nm) were more efficient in facilitating filopodia penetration than nanopores (76 nm) [222, 223] as they were comparable in size to filopodia (100-300 nm) [194]. Another study reported that both osteoblast adhesion and proliferation on surfaces decreased with increasing pore size from submicro-scaled (0.2 μm and 0.4 μm) to micro-scaled (3 μm, 5 μm and 8 μm).
However, larger pores with a pore size of 5 µm or 8 µm tended to stimulate ALP activity [224]. In this regard, the pore size is important and should be controlled to mimic the length scale of the filopodia for easy detection. This shows the complexity in topographical design of biofunctional surfaces.

In the present work, after culturing for 1 day, osteoblasts adapted themselves to both fine-porous and coarse-porous surfaces and displayed round morphology with limited spreading (Fig. 3.5 E-H and Fig. 3.7B). This finding is consistent with a number of studies showing that no matter what cell types were used (human osteoblasts [225], SaoS-2 cells [221], rat mesenchymal stem cells (rMSCs) [226] and human dental pulp stem cells [227]), cell spreading has been hindered by topographical features on concave surfaces such as nanoscaled (30-60 nm), submicron (120-500 nm) and micron (2 µm) pores/pits. The reduced cell spreading on porous surfaces can be attributed to the influence of pore depth. In the early stages of attachment, cells tend to conform to the pore shape, leaving cytoskeleton actins in a stressed state [228]. In order to reduce the stress, cells choose to minimise their interfacial contact with the surface, which accordingly reduces cell spreading [228]. As coarse-porous surfaces are deeper than fine-porous surfaces, the cell-surface contact was more restricted. Consequently, cell spreading on coarse-porous surfaces (458.8 ± 235.5 µm²) was clearly less than that on fine-porous surface (630.2 ± 327.6 µm²) as shown in Fig. 3.7B. On the other hand, robust attachments of osteoblasts were found on both fine-porous and coarse-porous surfaces (Fig. 3.8), suggesting close cell interactions with pores through ingrowth. This is an indicator of strong cell adhesion and fixation to the porous surface and is thus highly desired for long-time implantation [229].
contrast, the absence of stable anchoring sites on a smooth surface appears to facilitate cells spreading.

FAs link intracellular actin cytoskeleton structures to extracellular matrices (ECM), transduce signals to the nuclei and regulate the expressions or pathways of related genes [230]. According to Fig. 3.10B, a higher proportion of large FAs (marked with vinculin proteins) were present on both fine-porous and coarse-porous surfaces than on ultrafine-grained and coarse-grained smooth surfaces, revealing that rougher surfaces tend to facilitate the formation of FAs. Gentile et al. proposed that the tips of the protrusions on rough surfaces acted as initial sites for the contact of cells [231]. Progressively, the cell membrane extended into the vicinity of the protrusion tips and finally wrapped around the tips to promote the formation of FAs [231]. Thus, in the present study, the size and geometry of the ligaments of the interconnected porous surface structure play a crucial role in providing a large number of sites for the formation of FAs.

Both cell growth and osteogenic capacity are important for long-term osseointegration. In this study, a substantial increase in grain boundaries was found to be much less effective for cell growth than having a submicron porous surface. Oh et al. reported that enhanced cell growth was correlated to higher wettability and larger area on the surface [232]. As shown in Fig. 3.4 and Table 3.4, owing to their porous characteristics, the surface energies of both fine-porous and coarse-porous surfaces were twice that of the dense coarse-grained and ultrafine-grained smooth surfaces. An increased number of cells adhered to both fine-porous and coarse-porous surfaces can thus be ascribed to the greatly improved wettability and increased surface area. Bello et al. showed that larger FAs were associated with a higher number of cells on nanoporous CP-Ti surfaces.
(pore diameter: 20 ± 5 nm) [233]. In addition, at the initial stage of cell culturing, a smaller cell spreading area on porous surfaces allows more cells to anchor on porous surfaces than dense coarse-grained and ultrafine-grained surfaces [226].

As no osteogenic medium such as dexamethasone, ascorbic acid, vitamin D₃ or β-glycerophosphate was introduced to the culture medium, the observed ALP activity was solely determined by the surface topography [48, 232], and this, in turn, affected cell morphology. As shown in Fig. 3.11B, coarse-porous surfaces produced significantly enhanced intracellular ALP activity compared with ultrafine-grained smooth surfaces. The enhanced ALP activity was found relevant to cell morphology. Oh et al. demonstrated that highly elongated MSCs on 100-nm diameter nanotubes led to increased osteogenic gene expressions [232]. The noticeable cell elongation on the coarse-porous surfaces was probably linked to the enhanced ALP activity (Fig. 3.10A). In addition, Biggs et al. demonstrated that the enhanced formation of FAs upregulated intracellular ERK/MAPK signalling, which ultimately promoted osteoblastic differentiation in HOBs [225]. Thus, the presence of high proportion of large FAs could lead to the enhanced ALP activity on the coarse-porous surfaces (Fig. 3.11B). Besides, in general, water molecules and proteins adhere to the implant surface prior to cells interacting with an implant [106]. The adsorption of proteins to a surface is determined by the surface features and rougher surfaces tend to adsorb more protein aggregates than flat surfaces [106]. As one of ECM-located proteins, fibronectin (FN) plays an important role in stimulating osteogenic differentiation and bone formation [234]. Coarse-porous surfaces provided more ligament areas than fine-porous surfaces for FN adsorption and also for integrins to cluster on them [228]. Neither ultrafine-grained nor coarse-grained smooth surfaces provide
such surface areas. As a result, coarse-porous surfaces offered the most favourable *in vitro* microenvironment for osteoblasts. Such an optimum topography may benefit osseointegration *in vivo*. However, further *in vivo* studies are needed to determine the final implant functionality [235]. An effective design is to introduce a coarse-porous surface layer into an ultrafine-grained titanium implant where the latter offers high strength for lightweighting without increasing modulus while the former ensures desired cell responses.

### 3.6 Conclusions

The effect of surface topographical features on *in vitro* cell responses including cell morphology, adhesion, growth and differentiation has been studied by comparing coarse-grained (grain size: 500 μm), ultrafine-grained (grain size: 100 nm), coarse-porous (pore size: 350 nm) and fine-porous (pore size: 155 nm) surfaces of Ti-20Mo alloy. The following conclusions can be drawn:

1) The coarse-porous surface offered the optimum topographical environment for osteoblasts.

2) After culturing for 1 day, both the coarse-porous and fine-porous surfaces restrained the extension of filopodia and the spreading of cells due to the anchoring effect introduced by the submicron pores.

3) With increasing culturing period to 8 days, both coarse-porous and fine-porous surfaces enhanced the formation of large and mature focal adhesions compared with either coarse-grained or ultrafine-grained surfaces, which resulted in good adhesion of osteoblasts to the porous surfaces of Ti-20Mo.
4) Substantial grain refinement from 400 µm to 100 nm exerted limited influence on filopodia extension and cell growth.

5) Both coarse-porous and fine-porous surfaces exhibited significant surface wettability compared with coarse-grained and ultrafine-grained surfaces. However, surface wettability does not completely define the biofunctionality of a porous metallic surface. Rather, pore features (size and depth) were found to play an important role in regulating cell responses.

These findings collectively suggest that osteoblasts are more responsive to submicron pores (pore size: 155 or 350 nm) than submicron grains. In addition, cells can discern the difference in pore size and depth and respond differently. Therefore, controlling porous features of an orthopaedic titanium implant is essential to achieve desired functionality for rapid osseointegration.

3.7 References


Chapter 3


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filopodia with distinctive nanoscale protrusions by osteogenic cells, Acta Biomater. 60 (2017) 339-349.


Chapter 4

Fabrication and anisotropic wettability of titanium-coated microgrooves

4.1 Brief summary*

Surface wettability is critical in a variety of key areas including orthopaedic implants and chemical engineering. Anisotropy in wettability can arise from surface grooves, which are of particular importance to orthopaedic implants because they can mimic collagen fibrils that are the basic components of the extracellular matrix (ECM). Titanium (Ti) and its alloys have been widely used for orthopaedic and dental implant applications. This study is concerned with the fabrication of Ti-coated microgrooves with different groove widths and the characterisation of the anisotropy in wettability through measuring water contact angles, compared with both of the Wenzel and Cassie models. Experimental results revealed that there existed significant anisotropy in the wettability of Ti-coated microgrooves and the degree of anisotropy (Δθ) is increased with increasing groove width from 5 μm to 20 μm. On average, the contact angle measured parallel to the groove direction (θ∥) was about 50-60° smaller than that

measured perpendicular to the groove direction ($\theta_\perp$). In general, the Wenzel model predicts the contact angles along the surface groove direction reasonably while the Cassie model offers a better fit for the contact angles perpendicular to the groove direction. Osteoblast spreading was affected by the anisotropy in wettability, which occurred preferably along, rather than perpendicular to, the groove direction. These findings are informative for design of Ti implant surfaces when anisotropy in wettability matters.

**Keywords:** titanium, microgroove, surface topography, wettability, anisotropy

### 4.2 Introduction

Titanium (Ti) and its alloys exhibit high strength-to-weight ratio, high fracture toughness, excellent corrosion resistance, outstanding biocompatibility and good fatigue properties [11]. Consequently, they have found critical applications in a wide variety of sectors including chemical engineering, pharmaceutical manufacturing, marine and deep sea engineering, filtration of various types of liquid-solid mixture, orthopaedic implants, etc. [236]. In most of these applications, the surfaces of Ti components or devices are kept in contact with a liquid, where wettability is important for the anticipated functionality. Typical examples include open porous Ti used as corrosion-resistant filters and bone implants for enhanced osseointegration [236].

A number of studies have shown that surface topography, especially patterned grooves, can noticeably affect the wettability of a solid surface including the isotropy of wettability [237-239]. One area of particular interest is the surface design of orthopaedic implants. Wettability can play a critical role in cell
responses to microgrooved Ti substrates [142]. Grooves on such implant surfaces are capable of, e.g., mimicking collagen fibrils that are the basic components of the cell living environment, namely the extracellular matrix (ECM) [56]. Ding et al. have demonstrated that cells behaved similarly to viscous liquids in the initial stage of spreading on grooved surfaces [240], with their semimajor axis closely aligned with the groove direction. This observation suggests that further scrutinizing anisotropic wettability of a liquid can improve our understanding of cell response to grooved surfaces.

In principle, surface wettability can be tuned by changing either surface chemistry [241] or surface topography [242] or a combination of both approaches [243]. For instance, the chemical treatment such as plasma treatment of CHF₃, CF₄ or O₂ via reactive ion etching (RIE) can modulate anisotropic wettability of surfaces with submicron-scaled grooves [241]. On the other hand, anisotropic wettability can also be manipulated via changing the aspect ratio of groove patterns (i.e., the groove depth-to-width ratio) [244]. It requires no additional chemical treatments, thereby avoiding the introduction of surface contamination [244]. With regards to Ti surfaces, it has been shown that enhancing surface wettability by simultaneously altering surface chemistry and surface topography can promote *in vitro* osteoblast (bone-forming cell) responses [142]. Owing to the anisotropy in groove geometry, cells generally elongate and align along the groove direction [130], as illustrated in Fig. 4.1. As this is an area that is still not fully understood, here we aim to further unravel the underlying correlation between the anisotropic wettability and the grooved patterns, particularly the dependency of anisotropic wettability on groove width, aspect ratio and space ratio.
Fig. 4.1 Illustration of cell responses to substrate anisotropy. When cells are cultured on surface grooves, the cells tend to align along the groove direction. Focal adhesions are protein assemblies, which are essential in regulating cell responses and a few microns in size (green). Basal actin fibres (pink) and actin cap fibres (blue) also align along the groove direction. The side view (bottom) illustrates the arrangement of actin cap fibres and basal actin fibres. Reproduced from Ref. [130] under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0).

### 4.3 Pattern design and basic theoretical models for wetting of a grooved surface

Fig. 4.2 shows a schematic drawing of the designed surface microgrooves with ridge width denoted as $a$, groove width as $b$ and depth as $d$. Changing one of these groove features can potentially influence surface wettability. As pointed out
earlier, this study is particularly interested in enhancing osteoblast responses. Considering the compatibility of grooved patterns with osteoblasts, here a series of groove width in the range of 5 to 20 μm were selected because the average size of a typical osteoblast is about 20-30 μm [87] and more pronounced cell responses are triggered when groove widths are comparable with cell size [92]. The minimum groove width is chosen to be 5 μm in relation also to the typical osteoblast size of 20-30 μm as being too narrow risks developing significant incomplete contact with osteoblasts [23]. The groove depth of 2 μm was selected based on two findings. One is that grooves of 2 μm deep can stimulate the expression of osteogenic markers (i.e., the markers of osteogenic capacity for a bone implant surface) to a greater extent than shallower grooves (35 nm and 306 nm) [65]. The other is that they can induce more significant cell elongation than deeper grooves (e.g., 4.8 μm) [245]. This suggests that in general, 2 μm-deep grooves are appropriate to exert contact guidance for cells [245].

Fig. 4.2 Schematic drawing of microgrooves on a surface, a: ridge width, b: groove width, d: groove depth.

Ridge width is another parameter of the grooved surface topography. It is fixed at 5 μm, in relation again to the osteoblast size of 20-30 μm to avoid turning them into sharp edges for osteoblasts, where groove edges can hinder the migration of a
liquid or a viscous object [237, 246]. Table 4.1 lists the detailed dimensions of the designed microgrooves. The aspect ratio and space ratio (groove width to ridge width) vary from 1:2.5 to 1:10 and 1:1 to 4:1, respectively. In addition, in order to produce reliable statistic data, each grooved pattern is fabricated on an area of 1.05 cm × 1.05 cm, which has the capability of hosting a total of $2.5 \times 10^5$ human fetal osteoblast cells.

Table 4.1 Dimensions of designed surface microgrooves.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ridge width (a) (μm)</th>
<th>Groove width (b) (μm)</th>
<th>Groove depth (d) (μm)</th>
<th>Pitch (a+b) (μm)</th>
<th>Aspect ratio (d/b)</th>
<th>Space ratio (b/a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>10</td>
<td>1:2.5</td>
<td>1:1</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>10</td>
<td>2</td>
<td>15</td>
<td>1:5</td>
<td>2:1</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>15</td>
<td>2</td>
<td>20</td>
<td>1:7.5</td>
<td>3:1</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>20</td>
<td>2</td>
<td>25</td>
<td>1:10</td>
<td>4:1</td>
</tr>
</tbody>
</table>

Both Wenzel and Cassie states [247, 248] are commonly used to address the relationship between macroscopic surface roughness (here, geometric dimensions of grooves) and the contact angle of a water droplet, as shown in Fig. 4.3 [249]. In the Wenzel state, a water droplet completely fills into the grooves and eventually contacts the bottom of the grooves and its contact angle is described as:

$$\cos \theta_w = r_w \cos \theta_0$$  \hspace{1cm} (1)

where $r_w$ is the ratio of the actual wetted surface area to the planar surface area, $\theta_0$ is the intrinsic contact angle on a smooth surface and $\theta_w$ is the contact angle in the Wenzel state. In the groove geometry, $r_w$ is defined as [238]:

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However, in the Cassie model, a water droplet sits on top of the grooves with air entrapped underneath the droplet. In this case, the contact angle is described as:

$$\cos \theta_C = f (\cos \theta_0 + 1) - 1$$  \hspace{1cm} (3)$$

where $f$ is the ratio of the actual wetted surface area to the planar surface area, $\theta_C$ is the contact angle in the Cassie model. In the given groove geometry, $f$ is defined as [238]:

$$f = \frac{a}{a + b}$$  \hspace{1cm} (4)$$

Fig. 4.3 Wetting behaviour of a water droplet on rough surfaces: A) Wenzel model and B) Cassie model. Reproduced from Ref. [249] with permission from The Royal Society of Chemistry.
4.4 Materials and pattern fabrication

Fig. 4.4 shows the flow chart of the fabrication process of Ti-coated microgrooves on Si substrates, including photolithography, reactive ion etching (RIE) and sputter coating.

Fig. 4.4 Standard micro-processing steps for the fabrication of Ti-coated microgrooves on Si substrates.

4.4.1 Photolithography

Si wafers of 100 mm in diameter (Phosphatase/boron doping, <100> orientation, 525 ± 20 μm thickness, Silicon Materials Inc.) were cleaned sequentially in acetone and isopropanol (IPA) in an ultrasonic bath for 3 min each. Subsequently, they were rinsed with deionized (DI) water and dried with N₂ gas and then dehydrated by baking at 120 °C for 2 min and cooled down to room temperature.
Next, they were spin-coated with the positive photoresist AZ1512HS (Merck Performance Materials GmbH) for 45 s at 4000 rpm, followed by baking at 100 °C for 50 s to give a 1.3-μm thick layer, measured using a stylus profilometer (Dektak XT, Bruker). The photoresist layer was exposed to UV radiation in Karl Suss MA6 mask aligner for 9 s (exposure power: 12.22 mW/cm²). The exposed resist layers were subsequently immersed in AZ 726 metal ion free (MIF) developer for 10 s. Finally, all substrates were hard baked on a hotplate at 100 °C for 50 s.

### 4.4.2 Reactive ion etching (RIE) and sputter-coating

In order to completely remove the photoresist residue, a descum recipe of using oxygen plasma to blast the substrate for 10 s was performed in a Bostch etching system (PlasmaPro 100 Estrelas Deep Silicon Etch System, Oxford). Then the patterns on the photoresist were transferred to the underlying Si substrates by running a Si deep reactive ion etching (DRIE) recipe. Experimental parameters included the use of SF₆ and C₄F₈ ionized gas respectively at 35 sccm and 40 sccm, 25 W high frequency (HF) forward power, 650 W integrated-coupled-plasma (ICP) forward power, helium (He) backing pressure at 10 Torr. This set of parameters produced grooves with a depth of ~2 μm into the Si substrates. After DRIE, the remaining photoresist on the substrate was stripped using oxygen plasma for 80 s.

To improve the biocompatibility of the microgrooved surface, both microgrooved and blank Si wafers were sputter-coated with a 40-nm thin Ti film. The coating was realised using radio frequency (RF) magnetron sputtering from a metallic Ti
target (pressure of $5 \times 10^{-3}$ Torr, RF power of 200 W, Ar atmosphere, room temperature).

### 4.4.3 Surface characterisation

Surface morphology was analysed using an ultra-high-resolution scanning electron microscope (SEM, Verios 460L, FEI). SEM imaging was acquired at an accelerating voltage of 5 kV and a beam current of 25 pA. The true dimensions of each designed groove were measured from three replicates. To evaluate the cross sections of the microgrooves as well as the quality of the Ti coating on the grooves, substrates were cleaved to allow cross-sectional SEM imaging. A white light interferometer (ContourGT, Bruker) was used to characterise the three-dimensional (3D) surface morphology and surface roughness including the average roughness ($S_a$), the root mean square roughness ($S_q$) and the maximum height of the surface ($S_z$). To further analyse the roughness at the nanoscale of both the ridge and groove surfaces, atomic force microscopy (AFM, Bruker) was used in a tapping mode.

The static water contact angle ($\theta$) was measured using a sessile-drop contact angle goniometer (Theta Lite Optical Tensiometer, ATA Scientific). The value of $\theta$ was measured at 5 s after placing Milli-Q water droplets (2 µl) at three different areas on the substrate. All measurements were performed under ambient conditions and repeated three times. The degree of anisotropy in wettability is defined as $\Delta \theta = \theta_{\perp} - \theta_{//}$, where $\theta_{//}$ and $\theta_{\perp}$ are contact angles measured along and perpendicular to the groove direction, respectively [237, 238].
4.4.4 Cell culture and cell morphology analysis

*In vitro* assessments were conducted on micro-grooved substrates using human fetal osteoblast line (hFOB 1.19, ATCC, CRL-11372). The osteoblast cells were cultured in a mixture of Dulbecco’s Modified Eagle’ medium and Ham’s F12 medium (DMEM/F12, Thermofisher Scientific), supplemented with 10 % (v/v) fetal bovine serum (Interpath Services) and 0.3 mg/ml Geneticin selective antibiotics G418 (Thermofisher Scientific). Cells were incubated at 34 °C in a humidified atmosphere that contains 5% CO₂. The entire medium was replaced every three days and confluent cells were passaged using TrypLE™ Express Enzyme (Invitrogen). Cells between the 4th and 7th passages were used in this study.

After culturing for three days, the cell-seeded substrates were transferred into new six-well plates and washed with sodium cacodylate. Then the cells were fixed with 0.1M sodium cacodylate solution that contained 2 % (v/v) paraformaldehyde and 2.5 % (v/v) glutaraldehyde for 30 min. Afterwards, all substrates were dehydrated through a graded series of ethanol (30, 50, 70, 90, 95 and 100%). Finally, they were chemically dried with 100% hexamethyldisilizane (Sigma-Aldrich) for 20 min. The dried substrates were sputter-coated with platinum (Pt) prior to SEM analyses, which were performed at a voltage of 2 kV and a beam current of 0.8 nA (FEI, Verios 460L).
4.5 Results and discussion

4.5.1 Surface morphology of grooved patterns

Representative SEM micrographs of Ti-coated microgrooves of different groove widths are shown in Fig. 4.5A-D. Their cross-sectional views (Fig. 4.5E) revealed that these grooves are rectangular, and their side walls are nearly perpendicular to the groove bottom (~92°). The achieved parallel microgrooves shown in Fig. 4.5 are indicative of the high reliability of the micro-fabrication process employed. Table 4.2 summarises the measured dimensions of these grooves. The average thickness of the Ti coating on the ridge surface was ~56 nm, which was slightly higher than 41 nm measured on the groove bottom surface but doubled that of 27 nm on the groove sidewall. This is acceptable as long as the entire surface is coated with Ti.

A 3D view of each grooved substrate is shown in Fig. 4.6A-6D. According to 2D and 3D observations shown in Figs. 4.5 and 4.6, the grooved patterns achieved are consistent with our designs. The deviations from designed widths (5-20 μm) mainly arise from the developing or exposure time of the photoresist, while the deviations from the designed depth (2 μm) are mainly from the DRIE time. Figs. 4.6E-F show AFM analyses of the ridge surfaces and groove bottom surfaces of Substrate A, respectively. The ridge and groove surfaces exhibited equivalent average $R_a$ values ($1.38 \pm 0.07$ for ridge and $1.59 \pm 0.08$ nm for groove). Fig. 4.7 compares surface roughness measured for grooved surfaces and non-grooved flat substrate surfaces. The variations of $S_a$ (the average roughness), $S_z$ (the maximum height of the surface) and $S_q$ (the root mean square roughness) are limited to a small range for all four Ti-coated grooved surfaces, although the groove width
varies significantly. This provides an important experimental basis for investigating the influence of surface grooves on wettability.

![SEM micrographs of Ti-coated grooves on Si substrates. A: Substrate A, B: Substrate B, C: Substrate C, D: Substrate D and E: cross sections of Substrate A.](image)

**Fig. 4.5** SEM micrographs of Ti-coated grooves on Si substrates. A: Substrate A, B: Substrate B, C: Substrate C, D: Substrate D and E: cross sections of Substrate A.

**Table 4.2** Measured dimensions of fabricated Ti-coated microgrooves (μm).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ridge width (a)</th>
<th>Groove width (b)</th>
<th>Groove depth (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.06 ± 0.12</td>
<td>4.81 ± 0.19</td>
<td>2.26 ± 0.19</td>
</tr>
<tr>
<td>B</td>
<td>5.14 ± 0.22</td>
<td>9.60 ± 0.27</td>
<td>2.05 ± 0.25</td>
</tr>
<tr>
<td>C</td>
<td>5.07 ± 0.13</td>
<td>14.90 ± 0.18</td>
<td>2.10 ± 0.24</td>
</tr>
<tr>
<td>D</td>
<td>4.98 ± 0.08</td>
<td>19.43 ± 0.22</td>
<td>2.22 ± 0.83</td>
</tr>
</tbody>
</table>
Fig. 4.6 3D images for Ti-coated grooves using a light interferometer. A: Substrate A, B: Substrate B, C: Substrate C, D: Substrate D, E: AFM analysis on the groove surfaces of Substrate A and its corresponding surface height profile plotted along the black line and F: AFM analysis on the ridge surfaces of Substrate A and its corresponding surface height profile plotted along the black line.
Fig. 4.7 Surface roughness values at the microscale of Ti-coated grooved and non-grooved flat surfaces. $S_a$: the average roughness, $S_z$: the maximum height of the surface, $S_q$: the root mean square roughness.
4.5.2 Surface wettability

Fig. 4.8 shows a side view of the water droplets formed on both grooved surfaces and on a non-grooved flat surface. The latter exhibited isotropic water droplets which are essentially hemispherical. In contrast, droplets on the grooved surfaces were more elongated along the groove direction vs. its perpendicular direction. Fig. 4.9 compares the measured $\theta_{\|}$ and $\theta_{\perp}$ with the calculated contact angles using both Wenzel and Cassie models for all grooved surfaces. The non-grooved flat Ti-coated surface has a contact angle of 84.0 ± 1.6° (without Ti coating, the flat Si substrate has a contact angle of 38.3°). Overall, increasing groove width tends to increase wetting anisotropy. For example, $\theta_{\perp}$ decreased marginally from 126.7 ± 6.5° to 122.3 ± 4.2° with increasing groove width from 5 to 15 μm but then increased sharply to 141.4 ± 5.9° with further increasing groove width to 20 μm (Fig. 4.9). A similar trend was found for $\theta_{\|}$ as shown in Fig. 4.9. The measured $\theta_{\|}$ was close to that calculated from the Wenzel model, while $\theta_{\perp}$ were close to that predicted from the Cassie model. In other words, these models can be used to predict the degree of anisotropy in wettability on Ti-coated grooves. In particular, the wetting anisotropy ($\Delta \theta$: ~60.8°) was distinct for the grooved surfaces with a groove width of 20 μm where the measured $\theta_{\perp}$ was very similar to the calculated contact angles using the Cassie model. Moreover, with increasing groove width to 20 μm, the measured $\theta_{\|}$ gradually approached the contact angle of 84.0° for the non-grooved flat surfaces. As surface chemistry (high purity Ti) and surface roughness (Fig. 4.7) are similar for all grooved surfaces, it can be concluded that the observed anisotropic wettability is a result of anisotropic geometry of grooves. From Fig. 4.10A, the anisotropy in wettability ($\Delta \theta$) gradually increased from 51.8 ± 9.5° to 52.8 ± 8.5° with increasing space ratio from 1:1 to 2:1, and
then increased to $60.8 \pm 6.3^\circ$ at the space ratio of 4:1. The opposite trend was observed for the anisotropy in wettability ($\Delta \theta$) vs. the aspect ratio as shown in Fig. 4.10B.

**Fig. 4.8** Optical images of water droplets on four Ti-coated grooved surfaces and a non-grooved flat surface. Upper row: $\theta_{\perp}$ (perpendicular to grooves) and bottom row: $\theta_{\parallel}$ (parallel to grooves). (A, E): Substrate A, (B, F): Substrate B, (C, G): Substrate C and (D, H) Substrate D. I: a non-grooved flat surface.

**Fig. 4.9** Contact angles for the Ti-coated microgrooved and flat surfaces. Note that the Ti-coated flat surface has a contact angle of $84.0 \pm 1.6^\circ$. 

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Fig. 4.10 The relationship between the space ratio (A), aspect ratio (B) and the anisotropy in wettability of the Ti-coated microgrooved surfaces.
As proposed by Chung et al. and Xia et al., anisotropic wettability was affected mainly by the anisotropic surface geometry [237, 246]. On the one hand, water droplets spread along the groove direction, and on the other hand they were pinned at the groove edges in the direction perpendicular to the grooves [237, 246]. In other words, the three-phase contact line was continuous along the groove direction but discontinuous in the direction perpendicular to the grooves [250]. It is easier for water droplets to spread along the groove direction as the energy barrier for spreading is usually much lower than in the direction perpendicular to the grooves [246, 250]. Chung et al. further suggested that the three-phase contact line can be more influential on anisotropic wettability than surface roughness [246].

Based on the above observations and discussion, an effective way to enhance surface wettability is to minimise or avoid the discontinuity of the three-phase contact line of a water droplet in the direction perpendicular to grooves [237, 239]. In that regard, it is desirable to increase the ridge width and/or reduce the groove depth [239]. For instance, it is challenging to achieve deep penetration of a water droplet into narrow and deep grooves or those with high-aspect ratios [239, 246], due to a substantial increase in energy barrier for spreading with increasing groove depth [246]. To cope with such situations, the contact angle tends to increase and the water droplet finally reaches the Cassie state [246, 251].

In addition to the groove aspect ratio, the ridge slope (measured by $\alpha$) can also play an important role in influencing surface wettability by affecting the energy barrier for spreading [246]. For instance, rectangular grooves (i.e., a 90° slope) tended to hinder the spreading of a water droplet with air entrapped underneath [237]. Decreasing the ridge slope can lead to increased surface wettability [239].
Our results show that $\theta_{//}$ is much smaller than $\theta_{\perp}$ for all fabricated grooves, which is consistent with the above discussion. For grooved patterns with fixed ridge width and groove depth, increasing groove width from 5 to 20 μm means a reduction of aspect ratio (1:2.5 to 1:10) but an increase of space ratio (1:1 to 4:1). This leads to an increase in discontinuity of the three-phase contact line. Therefore, the contact angle in the direction perpendicular to grooves was close to that calculated using the Cassie model. On the other hand, the calculated free energy profile in the direction perpendicular to grooves displayed various high-energy metastable states, while the energy profile in the direction parallel to grooves and for non-grooved flat surfaces exhibited a minimum global free energy [240, 246]. This further suggests that water droplets that spread along the groove direction and on non-grooved flat surfaces are in a stable low-energy state, i.e., the Wenzel state [246, 252]. Consequently, the measured contact angle along the groove direction was close to that calculated using the Wenzel model (Fig. 4.9).

4.5.3 Influence of anisotropic wettability on osteoblast morphology

The SEM micrographs shown in Fig. 4.11 revealed significant differences in cell alignment and morphology between microgrooved and non-grooved flat surfaces. The osteoblasts spread randomly on the flat surfaces in polygonal shapes. In contrast, cells became elongated along the groove direction on all microgrooved surfaces. Moreover, cells tended to conform to the entire groove width as a result of the contact guidance by grooves.
Fig. 4.11 SEM micrographs of osteoblasts cultured on grooved and non-grooved flat surfaces coated with Ti after culturing for 3 days. (A) Substrate A, (B) Substrate B, (C) Substrate C, (D) Substrate D, (E) flat substrate.

It is generally accepted that a hydrophilic ($\theta < 90^\circ$) surface is in favour of cell spreading, differentiation and protein adsorption compared with a hydrophobic ($\theta > 90^\circ$) surface [35, 140, 141, 253]. Under in vitro conditions, osteoblasts tend to align and migrate along the groove direction [30, 32]. As mentioned earlier, cells behave similarly as viscous liquids do in the initial stage of spreading so that the low energy barrier allows the ease of cell spreading along the groove direction. This differs from the scenario of cell spreading along the direction.
perpendicular to the grooves, where cell spreading was largely restrained within one or two microgrooves, especially for narrow grooves (e.g., Substrate A). These observations are informative for the design of Ti bone implant surfaces when anisotropy in wettability is critically considered. However, more experimental data on in vitro and in vivo studies is needed to further clarify the detailed influence of each groove parameter on osteoblast responses.

4.6 Conclusions

This study has investigated the effect of groove geometry on anisotropic wettability. Ti-coated microgrooves with groove widths ranging from 5 to 20 μm at a constant ridge width of 5 μm and a fixed depth of 2 μm were successfully fabricated and characterised. Wettability experiments with water droplets revealed that the Ti-coated surface grooves exhibited significant anisotropy in wettability. On average, the contact angle measured parallel to the groove direction \( \theta_{//} \) was about 50-60° smaller than that measured perpendicular to the groove direction \( \theta_{\perp} \). The degree of anisotropy increased with increasing groove width. The Wenzel model can be used to predict the water contact angle along Ti-coated surface grooves, while the Cassie Model to predict the contact angle perpendicular to the grooves. Together they can reasonably predict anisotropy in wettability of Ti-coated grooves. The anisotropy of wettability can significantly affect in vitro cell responses, where cells tend to spread preferentially along the microgrooves. The experimental findings of this study provide the first-hand information for understanding the effect of Ti surface grooves on anisotropic wettability for various applications, including orthopaedic Ti implants.
4.7 References


Chapter 5

Osteoblast responses to titanium-coated sub-cellular scaled microgrooves

5.1 Brief summary*

Inspired by the groove-like natural structures of collagen fibrils, groove patterns have been frequently used to modify orthopaedic implant surfaces. In particular, both in vivo and in vitro studies have confirmed the positive influences of micro-grooved titanium compared to flat titanium surfaces. However, the exact influence of groove width, which is the most important groove geometrical factor, at the sub-cellular scale on regulating osteoblast responses remains unclear. Full cell-substrate adhesion is a prerequisite for rapid and robust osseointegration and therefore for mitigating or minimising subsequent loosening of orthopaedic implants. This work investigates osteoblast responses to titanium-coated microgrooves over the groove widths range of 5-20 μm (sub-cellular scale). More specifically, the adhesion characteristics at the osteoblast-groove interfaces were systematically studied using focused ion beam (FIB) and scanning electron microscopy (SEM). Direct evidence was produced for the first time to show that

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full cell-groove adhesion was achieved when the groove width reaches 15 μm and beyond, while below 15 μm, the adhesion was gradually increased with increasing groove width. The cell spreading area and the cell width were found to be proportional to the groove width. However, groove width over the range of 5-20 μm showed only limited influence on cell proliferation and differentiation compared to flat surfaces. Apart from the groove width, three boundary conditions for attaining full cell-groove adhesion have been defined. Finally, a quantitative relationship is proposed between the groove width and the degree of the osteoblast-groove adhesion, which predicts nil osteoblast cell-groove adhesion at the groove width of 2.4 μm.

**Keywords:** titanium, groove width, sub-cellular scale, osteoblast cell-groove adhesion

### 5.2 Introduction

In 2016, about one out of every 200 Australians underwent hip, knee and shoulder replacement surgeries according to the Australian Orthopaedic Association [1]. Statistical data have consistently shown that loosening of the implant is one of main reasons for revision surgeries of primary knee, hip and shoulder replacements performed in Australia [1]. With a growing and ageing population worldwide, there is an increasing need to develop orthopaedic implants that can facilitate fast and robust osseointegration (i.e., stable fixation between bone and implant surfaces [254]) and that can last sufficiently long [255]. Titanium (Ti) and its alloys have been widely used in both orthopaedic and dental applications due to their excellent biocompatibility, corrosion resistance and mechanical properties [74]. However, as artificial metal implants, they lack
natural osteoconduction capabilities (i.e., capabilities for direct bone growth on their surfaces [254]) [256]. Hence, a range of surface topographical modification techniques have been used to improve osseointegration of Ti implants [257]. In particular, it has been shown that partially ordered or ordered surface topography can enhance bone cell responses more effectively than randomly organised surface features [30, 235, 258].

Compared with smooth implant surfaces, micro-scale rough surfaces can allow bone cells to grow into and entangle with the rough landscape [91, 259], which can help to interlock the new bone to reduce the risk of implant loosening. Crafting ordered microgrooves is an effective way of roughening an implant surface with well-controlled topography [65, 142]. More importantly, microgrooves can play an immediate contact guidance role for cells, for example, to facilitate bone cells to align and migrate along the grooves, which can accelerate wound healing [31, 217] or improve osseointegration [18]. This is because the elongation of osteoblasts determines the orientation of the collagen matrix, which is closely associated with bone regeneration on the implant surfaces [260]. In addition, the elongated phenotype of mesenchymal stem cells (MSCs) on micro-grooved surfaces tend to differentiate towards osteogenic lineage [261]. Another attribute is that groove geometries bio-mimic the natural structures of collagen fibrils, which are the main components of extracellular matrix (ECM) [55, 96, 257] (Fig. 5.1). It has also been well documented that cell functions can be regulated by altering groove geometry including groove width [55, 261], depth [65], orientation [158] and groove depth-to-width ratio (aspect ratio) [262]. In particular, in vivo studies by Frenkel et al. revealed that micro-grooved commercially pure titanium (CP-Ti) (groove width: 12 μm, groove
depth: 12 μm) exhibited more robust bone-implant interface than smooth CP-Ti surfaces, which impeded bone resorption [263]. Recent work by Klymov et al. further showed very encouraging results, where Ti-coated micro-grooved cylindrical epoxy resin implants significantly upgraded the trabecular bone volume compared to randomly roughened surfaces in a rat model [18].

**Fig. 5.1** Geometrical similarities between mineralised collagen fibril bundles and groove patterns. (a) Transmission electron microscopy (TEM) image of mineralised collagen fibril bundles [264]. (b) Scanning electron microscopy (SEM) image of grooved structures of natural extracellular matrix in human bone [55]. (c) SEM image of Ti-coated microgrooves [265].

Of the three principal groove geometrical parameters (width, depth, ridge width), Kabaso et al. demonstrated by simulation that groove width is far more influential in determining osseointegration than the other two [23]. Groove width is inversely proportional to groove pattern density, defined as the ratio of ridge width to groove width [266]. Consequently, changing groove width could dramatically change groove pattern density. For example, increasing groove width from 0.55 μm to 2.75 μm at a fixed ridge width of 0.55 μm will reduce groove pattern density from 1:1 to 1:5. This led to an immediate weakened tendency of cell alignment and migration along the groove direction [266]. In
another study, Kim et al. identified that osteogenic differentiation of human mesenchymal stem cells (MSCs) was enhanced most on grooves with a pattern density of 1:3 at the width of 0.5 μm [55].

The above well-documented influence of groove width highlights the necessity for an in-depth fundamental understanding of the exact role of groove width at the sub-cellular scale in affecting osteoblast responses. It can allow the design of favourable groove geometry for enhanced osteoblast functions. The lack of an in-depth fundamental understanding is largely due to the absence of first-hand quantitative experimental observations of the osteoblast-groove adhesion process. The purpose of this study is twofold: to systematically investigate the influence of groove width at the sub-cellular scale on osteoblast adhesion, spreading, actin cytoskeleton organisation, proliferation and differentiation, and to define the theoretical groove geometries for complete cell-groove adhesion.

5.3 Materials and methods

5.3.1 Fabrication and characterisation of substrates

The groove width is chosen to be in the range of 5-20 μm because cells are more responsive to grooves at the sub-cellular scale, i.e. ≤ 20 μm [92] [118]. The methods used for fabricating Ti-coated silicon microgrooves have been detailed elsewhere [265]. Fig. 5.2 illustrates a grooved substrate surface while Table 5.1 summarises the pattern designs to be coated with titanium (see Ref. [265] for the Ti-coating process). The average thickness of Ti-coating on the ridge surface was 56 nm, which was 15 nm thicker than that on the groove bottom surface and almost twice that on the groove sidewall [265]. Smooth Ti-coated substrates (R_s=...
9.90 ± 6.81 nm) were used as pattern controls. Prior to cell culture, all substrates were cleaned with acetone, ethanol and distilled water in an ultrasonic bath for 15 min in each medium. Then all substrates were sterilized under UV light in a biohazard hood for 30 min and placed into each well of 6-well plates.

![Illustration of a microgrooved substrate surface](image)

**Fig. 5.2** Illustration of a microgrooved substrate surface, *a*: ridge width, *b*: groove width, *d*: depth.

**Table 5.1** Surface microgrooves fabricated in this study.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ridge width (a) μm</th>
<th>Groove width (b) μm</th>
<th>Groove depth (d) μm</th>
<th>Pitch (a+b) μm</th>
<th>Space ratio (b/a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>10</td>
<td>1:1</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>10</td>
<td>2</td>
<td>15</td>
<td>2:1</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>15</td>
<td>2</td>
<td>20</td>
<td>3:1</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>20</td>
<td>2</td>
<td>25</td>
<td>4:1</td>
</tr>
</tbody>
</table>

5.3.2 *In vitro* cell responses

Human fetal osteoblast cells (hFOB 1.19, ATCC, CRL-11372), in their log phase of growth, were seeded onto each substrate at an initial density of 2.5×10^5 cells/cm^2, following the same procedures detailed in [265]. On the third and seventh days, the live and total cell numbers in the growth medium as well as on
each substrate were assessed using the trypan blue exclusion method [267] in order to determine the cell viability, which is defined as follows:

\[
\text{Cell viability} = \frac{\text{live cell number}}{\text{total cell number}} \times 100\%
\] (1)

A minimum of three replicated substrates were used for each set of data reported. In order to identify and understand the cell-groove interactions, a lower cell density of cells (3×10^4/cm^2 to avoid cell-cell contacts) was cultured on each substrate for three days. The protocol of fluorescent staining for vinculin, nuclei and actin filaments has been reported previously [267]. The stained cells were examined with an inverted confocal laser scanning microscope (Nikon). The alkaline phosphatase (ALP) activity was evaluated using an ALP fluorescence detection kit (Sigma-Aldrich) after seven days of culture. The results were expressed as a normalised quantity from the total protein content that was assessed using a bicinchoninic acid (BCA) protein assay kit (Pierce) following the manufacturer’s protocols.

The cell morphology was characterised using scanning electron microscopy (SEM) and the cell-groove interfaces were evaluated using a dual-beam gallium (Ga) focused ion beam (FIB)-SEM system (Scios, FEI). Areas of interest were cut by FIB milling from the cell-seeded substrates for detailed analyses. Firstly, only those cells that spread their main bodies along the grooves were chosen. Secondly, FIB milling was applied to the widest section of the cell along the groove (i.e., the minor axis shown in Fig. 5.3a) to reveal the cell-groove adhesion in the groove cavity (see Fig. 5.3b). The substrate was tilted perpendicular to the FIB to produce a cross-sectional cut of the selected cell-groove assembly. To avoid cell damage from the FIB milling, a 200-nm thick protective platinum (Pt)
layer was deposited using an in-chamber gas injection system at a beam current of 100 pA. Rough FIB milling was first performed at an ion beam current of 7 nA. To remove the curtaining artefacts and produce a clean cross-section, a finishing current of 100 pA was used to polish the rough-milled cross-section. The acceleration voltage applied to the Ga ion beam was 30 kV for all steps. SEM imaging was taken at an acceleration voltage of 2 kV, an electron beam current of 0.2 nA and a tilting angle of 45°.

5.3.3 Cell morphology analyses

*Image J* (Version 1.6, National Institute of Health, Bethesda, MD) [214] was used to analyse the cell morphology using tilted SEM images. The spreading area of each selected cell was measured manually by fitting with an ellipse, as shown in Fig. 5.3a. The length and width of the fitting ellipse represent the major and minor axes of the cell, respectively [268]. The ratio of the cell major axis to its minor axis is used to measure the degree of cell elongation along the grooves. The cell orientation angle (θ, Fig 5.3a) is defined as the angle between the cell major axis and the groove direction [269]. Cell orientation angles of less than 10° represent the situation that the cells are aligned along the grooves while the angle of 90° represents random cell orientation on surface grooves. *Matlab* (R2017b, Mathworks) was used to analyse the proportion of cell orientation angle on grooved surfaces. A minimum of 50 cells on each substrate were measured and the measurement were repeated three times. To quantify the gap between the cell and groove cavity following Ref. [270], the degree of the cell-groove adhesion (A%) is characterised as follows [270]:

\[
A\% = \frac{\text{Cell body cross section area}}{\text{cross section of groove cavity}} \times 100\% \quad (2)
\]
As shown schematically in Fig. 5.3b, when $A = 1$, the cell fully adheres to the groove cavity with no gap left, while no cell-groove adhesion occurs when $A = 0$. The value of $A$ was determined from the cell-groove cross-section images (FIB-SEM) using the freehand and region of interest (ROI) manager tools of Image J.

![Diagram](image)

**Fig. 5.3** Illustrations of (a) cell morphology and (b) cross section of a cell-groove contact.

### 5.3.4 Statistical Analysis

All data were analysed using GraphPad Prism 7 (GraphPad Prism software). Statistical analysis was performed using either one-way or two-way analyses of variance (ANOVA), followed by the Turkey post hoc tests. Data were expressed in the form of mean value ± standard deviation (SD). Statistical significance was considered at $p < 0.05$.

### 5.4 Results

#### 5.4.1 Osteoblast morphology analysis using SEM

The SEM micrographs, shown in Fig. 5.4 (taken at a tilt angle of 45°), revealed significant differences in cell alignment and morphology between microgrooved
and smooth surfaces. The osteoblast cells spread randomly on smooth substrates (Fig. 5.4a). In contrast, cells are elongated and aligned along the groove direction on the microgrooved surfaces (Fig. 5.4b-e). An additional feature is that the cells were capable of straddling the ridge to connect to their neighbours. Moreover, they tended to conform to the entire groove width due to both the contact guidance role from the grooves and the appropriate sub-cellular groove width dimension. In contrast, cells spread randomly on smooth surfaces (Fig. 5.4). In the case of Substrate A, which has the narrowest groove width, cells tended to attach themselves to the ridge surface rather than to occupy the narrow groove cavity. As indicated by the arrows in the right column of Fig. 5.4 (b-e), filopodia had managed to reach out to sense the adjacent topographical features on each microgrooved substrate surface. For example, they have stretched themselves across the ridges.

To investigate the effect of the groove width on osteoblast cell shape and orientation, a quantitative assessment was conducted using Image J to analyse the cell morphology. The results are shown in Fig. 5.5. The average cell spreading area on smooth substrates was $1740.0 \pm 624.9 \, \mu m^2$. A significant reduction ($p < 0.01$) was observed on microgrooved substrate surfaces, particularly on Substrates A ($536.1 \pm 190.1 \, \mu m^2$), B ($486.9 \pm 224.8 \, \mu m^2$) and C ($826.1 \pm 351.4 \, \mu m^2$), which have narrow grooves. The same observation was made with cell width as shown in Fig. 5.5b. However, groove width exerted no significant influence on cell length (Fig. 5.5c). Consequently, an increase in cell elongation ratio was observed with decreasing groove width (Fig. 5.5d). The cell orientation angle (Fig. 5.3a) is similar with respect to different groove widths ($\theta < 30^\circ$, Fig. 5.5e), suggesting that the role of contact guidance is significant in each case.
Fig. 5.4 SEM micrographs of osteoblast cells cultured on Ti-coated smooth and microgrooved surfaces after 3 days. (a) Smooth substrate, (b) Substrate A (5 µm), (c) Substrate B (10 µm), (d) Substrate C (15 µm), (e) Substrate D (20 µm). Left column: low-magnification SEM images, right column: high-magnification SEM images. Yellow arrows indicate the filopodia.
Chapter 5

5.4.2 Cell-groove interface

To reveal the adhesion details between the cells and the grooves, systematic FIB-SEM cross-sectional examination was applied to microgrooved substrates after 3 days of culture, and representative results are shown in Fig. 5.6. The bright surface layer is the protective Pt coating. Fig. 5.6a shows an osteoblast cell on a microgrooved substrate surface with the groove width of 5 μm. The cell grew in the groove with filopodia extended to each ridge of the groove. The judgement
based on Fig. 5.6a is quite positive in regard to the growth of this cell. However, the corresponding cross-sectional FIB-SEM image revealed that the cell is essentially detached from each sidewall of the groove (see blue arrows) and the contact is restricted to the central strip of the groove bottom. Increasing the groove width from 5 μm to 10 μm led to a substantial increase in the cell-groove contact at the bottom of the groove (Fig. 5.6a vs Fig. 5.6b). Further increasing the groove width to 15 μm (Fig. 5.6c) resulted in a near full contact between the cell and the groove bottom (Fig. 5.6c). At the groove width of 20 μm, near full cell-substrate adhesion was also attained (Fig. 5.6d). These FIB-SEM observations revealed that groove width played a crucial role in regulating cell-groove adhesion. Table 5.2 lists the quantitative analyses of the cell adhesion. With increasing groove width from 5 μm to 20 μm, the degree of cell-groove adhesion (A%) increased from 53.07% to 98.55%. Another 2 μm of groove depth proved to be able to allow the cell to grow into the groove cavity.

**Table 5.2 Quantitative analyses of the cell adhesion.**

<table>
<thead>
<tr>
<th>Substrate (Groove width)</th>
<th>Cross-sectional area of the cell membrane growing into the groove cavity (μm²)*</th>
<th>Entire cross-sectional area of the groove cavity (μm²)</th>
<th>Degree of cell-groove adhesion (A%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (5 μm)</td>
<td>5.31 ± 0.69</td>
<td>10.0</td>
<td>53.07 ± 6.87</td>
</tr>
<tr>
<td>B (10 μm)</td>
<td>17.87 ± 1.09</td>
<td>20.0</td>
<td>89.34 ± 4.70</td>
</tr>
<tr>
<td>C (15 μm)</td>
<td>29.57 ± 0.17</td>
<td>30.0</td>
<td>98.56 ± 0.56</td>
</tr>
<tr>
<td>D (20 μm)</td>
<td>39.42 ± 0.30</td>
<td>40.0</td>
<td>98.55 ± 0.76</td>
</tr>
</tbody>
</table>

* Averaged from measurements across five different cell-groove cross sections.
Fig. 5.6 Representative cell-groove interfaces investigated by the SEM/FIB technique. (a) Substrate A (5 µm), (b) Substrate B (10 µm), (c) Substrate C (15 µm), (d) Substrate D (20 µm). Left column: SEM images of selected grooves. Right column: Cross sections of
osteoblast cells along each dashed line marked out in the left-column images. Blue arrow indicates the gaps formed between the cells and the groove sidewalls.

5.4.3 Actin cytoskeleton organisation and the formation of focal adhesions

Actin cytoskeletons are essential for cells to adapt to topographical cues [271]. Confocal microscope analyses revealed that the actin cytoskeletons were well-developed as stress fibres and aligned along the grooves after culturing for 3 days (Fig. 5.7). In contrast, those on the flat substrates were randomly distributed. There is a clear tendency that cell elongation increases with decreasing groove width from 20 μm to 5 μm, consistent with that shown in Fig. 5.5d. Focal adhesions are multi-protein structures that connect ECM to intracellular cytoskeleton, and have significant influence on cell migration and spreading [33]. After being visualised by immunostaining of vinculins (Fig. 5.7), focal adhesions were found closely aligned along the groove direction on microgrooved substrates.
**Fig. 5.7** Confocal images of osteoblast cells on microgrooved and smooth surfaces after 3 days of culture. (a) Smooth surfaces, (b) Substrate A (5 µm), (c) Substrate B (10 µm), (d) Substrate C (15 µm), (e) Substrate D (20 µm).
5.4.4 Cell proliferation and ALP activity

Fig. 5.8a compares cell proliferation while Fig. 5.8b compares cell viability on microgrooved and smooth surfaces after 3 and 7 days of culture. The total number of osteoblast cells on each substrate showed a clear increase from 3 to 7 days of culture (Fig. 5.8a, \( p < 0.01 \) for Substrates A, B, C and smooth surfaces, \( p < 0.0001 \) for Substrates D, Turkey’s multiple comparisons test). However, microgrooved surfaces, irrespective of the groove width studied, showed no definable influence (\( p > 0.05 \)) on the total number of cells (Fig. 5.8a) compared with smooth surfaces after either 3 or 7 days of culture. The cell viability value for all substrates fell in the range of 69-77% after 3 or 7 days of culture (Fig. 5.8b). No significant difference was observed between microgrooved and smooth surfaces.

The total cellular protein expression level on each substrate is shown in Fig. 5.8c by focusing on the situation after 7 days of culture. No statistically significant difference (\( p > 0.05 \)) was observed. The same was observed with the ALP activity as shown in Fig. 5.8d. This further indicates that groove width in the range of 5-20 \( \mu \text{m} \) has limited influence on cell proliferation and early-stage osteoblastic differentiation.
Fig. 5.8 (a) Cell proliferation and (b) Cell viability on microgrooved and smooth surfaces after 3 and 7 days of culture. (c) Total protein adsorption and (d) ALP activity normalised to protein content, of osteoblasts cultured on both microgrooved and smooth surfaces for 7 days (3 replicates for each set of data). Blue lines indicate the protein adsorption and ALP level of smooth surfaces.

5.5 Discussion

5.5.1 The influence of groove width on osteoblast responses

The experimental study presented above provides a detailed assessment of the influence of titanium-coated microgrooves with sub-cellular scaled groove width on osteoblast responses. The statistical results are summarised in Table 5.3.
Table 5.3 Summary of hFOB 1.19 cell responses to titanium-coated microgrooves*

<table>
<thead>
<tr>
<th>Groove width</th>
<th>Cell contact</th>
<th>Cell elongation</th>
<th>Cell area</th>
<th>Proliferation</th>
<th>ALP activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μm</td>
<td>Smaller</td>
<td>Increase ((p&lt;0.01))</td>
<td>Decrease ((p&lt;0.01))</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>10 μm</td>
<td>Smaller</td>
<td>Increase ((p&lt;0.01))</td>
<td>Decrease ((p&lt;0.01))</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>15 μm</td>
<td>NSD</td>
<td>Increase ((p&lt;0.05))</td>
<td>Decrease ((p&lt;0.01))</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>20 μm</td>
<td>NSD</td>
<td>NSD</td>
<td>Decrease ((p&lt;0.05))</td>
<td>NSD</td>
<td>NSD</td>
</tr>
</tbody>
</table>

* All data are compared to Ti-coated smooth surfaces; NSD: no significant difference.

Filopodia with 250-400 nm in diameter are thin protrusions of a cell membrane [110]. They are responsible for sensing the mechanical (topography and rigidity) and chemical cues from the extracellular environment [210, 235, 272]. As shown in Fig. 5.4, filopodia can extend and orient themselves in response to all those 2-μm deep microgrooves with different groove widths. Previous studies have found that groove depth is another important geometric factor in determining contact guidance [273] and osteoblastic functions [65]. This study identifies that microgrooves with a depth of 2 μm are appropriate to orient osteoblasts along the groove direction.

The results of cell morphology (Figs. 5.4, 5.5 and 5.7) suggested that both osteoblast attachment and spreading were affected by groove width. Our FIB-SEM observations shown in Fig. 5.6 revealed full cell-groove adhesion in grooves with groove width of 20 μm. This is because a 20-μm wide groove is comparable to the average size of an osteoblast [274]. Consequently, the space is sufficient for osteoblasts to settle on the groove bottom rather than to bridge over
the groove ridges. Such developments can immediately affect cell spreading, elongation and orientation. For example, Teixeira et al. observed less cell spreading on grooved surfaces with groove width ranging from 400 nm to 4000 nm than on smooth surfaces. Narrow grooves on Substrate A (5 µm) and Substrate B (10 µm) offer no sufficient space for cells to cram into them. As a result, cells resort to groove ridges for contact guidance [275].

As revealed in Fig. 5.8a, microgrooves with a width in the range of 5-20 µm showed no statistically important influence on cell proliferation and early-stage osteoblastic differentiation. This agrees with previous studies using different types of cells [55, 142, 261, 276]. For instance, no clear difference in MSC proliferation was observed on microgrooved (groove width = ridge width: 2-15 µm, groove depth: 2 µm) and smooth chips [261]. In a similar study, Den Braber et al. reported that microgrooves (groove width = ridge width: 2-10 µm, groove depth: ~500 nm) were unable to enhance fibroblast proliferation [276]. Moreover, V-shaped microgrooves on CP-Ti surfaces (groove width: 15 µm to 90 µm) resulted in no clear changes in osteoblast adhesion compared with smooth substrates [142]. In this study, no significant difference was observed in the average ALP activity on microgrooved and smooth surfaces (Fig. 5.8c). This is contrary to the observations reported by Refs [142, 277], which showed that microgrooves with a groove width of 15-90 µm significantly stimulated the level of osteogenic expression compared with smooth surfaces [142, 277]. This discrepancy is likely due to the absence of osteogenic supplements in the culture growth medium such as ascorbic acid or β-glycerophosphate [261, 278]. It is noted that the alteration in cell size and shape can lead to regulation of specific genes during bone formation [279, 280]. Further studies are needed to reveal
whether other related genes are regulated by surface topographies. This information is crucial to develop better orthopaedic implants.

5.5.2 Favourable groove geometries for full cell-groove adhesion

An incomplete cell-groove adhesion with clear gaps at the bone-implant interface risks leading to implant loosening or micromotion towards ultimate failure [3]. In addition, metallic wear particles can amass in such gaps, impeding osseointegration at the bone-implant interface [3, 281]. Hence, it is essential to eliminate any gaps between a groove cavity and osteoblast cells [3]. Based on the decisive role of groove width in cell-groove adhesion observed in this study, we can define the favourable groove geometries for full cell-groove adhesion in Fig. 5.9. The premise is that if a groove is wide enough (e.g., \( \geq 15 \mu m \)) to allow an osteoblast cell to settle in it, full cell-groove adhesion can always be expected irrespective of the groove sidewall being vertical or slanted. In fact, a slanted groove sidewall with a slope angle (\( \alpha \)) greater than 90° can provide extra surface for cell adhesion (a narrow opening with \( \alpha < 90° \) hinders complete cell adhesion).

To facilitate full cell-groove adhesion, the groove opening width can be chosen to be the maximum average width of the osteoblast cells observed on microgrooved surfaces, which is 25 \( \mu m \) (Fig. 5.5b) while the groove bottom width can range from 15 to 20 \( \mu m \) according to Fig. 5.6. The slope of the groove sidewall (\( \alpha \)) then becomes critical for attaining full cell-groove adhesion. The critical values are determined to be at \( \alpha_{critical} = 158.2° \) for a bottom width of 15 \( \mu m \) and at \( \alpha_{critical} = 141.3° \) for a bottom width of 20 \( \mu m \), as shown in Fig. 5.9. Full adhesion can occur when \( 90° \leq \alpha \leq \alpha_{criticals} \) while beyond \( \alpha_{criticals} \) cells may become isolated in
each groove without communication with those in their neighbouring grooves, which can inhibit osteogenesis.

Fig. 5.10 plots the average degree of cell-groove adhesion (Table 5.2) versus groove width. The best curve fit gives

$$\Psi = 1 - 2.066 \cdot e^{-\frac{b}{3.373}}$$

(3)

in which $\Psi$ is the degree of cell-groove adhesion ($0 \leq \Psi \leq 1$) and $b$ is groove width. The exponential describes the experimental data remarkably well ($R^2=0.9987$). Eq. (3) predicts nil cell-groove adhesion ($\Psi=0$) at a groove width of 2.42 \( \mu \text{m} \), which is about 5-10\% of the average width of a typical osteoblast cell (30-50 \( \mu \text{m} \) in diameter). This suggests that when a groove width is noticeably smaller than the width of an osteoblast cell, the cell will not choose to interact with the groove cavity. It is noted that a single cell adhesion state cannot represent the final or overall osseointegration state. From differentiation and formation of osteoblast to final osseointegration, the entire process involves a series of complex biochemical reactions including further osteoblast proliferation, production of osteoid and extracellular matrix, mineralisation of the extracellular matrix and apoptosis of osteoblasts. However, this study has provided first-hand quantitative experimental information on how microgrooves at the subcellular-scale can influence osteoblast adhesion in the early period of \textit{in vitro} culture.
Fig. 5.9 Full cell-groove adhesion on microgrooves with vertical groove sidewalls or slanted groove sidewalls. Blue line indicates the slanted groove sidewall with a slop angle of 158.2°, a groove bottom width of 15 µm and a groove open width of 25 µm. Green line indicates the slanted groove sidewall with a slope angle of 141.3°, a groove bottom width of 20 µm and a groove open width of 25 µm. Blue dashed line indicates the vertical groove sidewall with a slope angle of 90°, a groove bottom width of 15 µm.

Fig. 5.10 Cell-groove adhesion (%) versus groove width (µm). Solid circles are experimental data on the average degree of cell-groove adhesion.
5.6 Conclusions

The influence of groove width at the sub-cellular scale (5-20 μm) on osteoblast morphology, adhesion, actin cytoskeleton organisation, proliferation and osteogenic capacity has been systematically evaluated. The following conclusions can be drawn:

1. The degree of the osteoblast cell-groove adhesion increased exponentially with increasing groove width and reached full adhesion at the groove width of 15 μm. The exponential model predicts nil osteoblast cell-groove adhesion at the groove width of 2.42 μm, which is about 5-10% of the average width of an osteoblast cell. It is therefore essential to design micro-grooved titanium implant surfaces with groove width comparable to the cell size, in order to achieve full cell-groove adhesion. Full adhesion is important to mitigate or minimize loosening of the orthopaedic implants.

2. Both the cell spreading area and cell width were proportional to the groove width (5-20 μm), while the cell length was not affected. The groove width showed only limited influence on cell proliferation and differentiation compared with flat surfaces, despite the substantially increased surface area (up to 40%).

3. Three boundary or critical conditions have been defined for full osteoblast cell-groove adhesion. The first is feature by vertical groove sidewalls (α=90°) with groove width = 15 μm, groove depth = 2 μm and ridge width = 5 μm. The second consists of slanted groove sidewalls with α = 158.2°, groove bottom width = 15 μm, groove open width = 25 μm, groove depth = 2 μm and ridge width = 5 μm. The last one, which is similar to the
second one, also consists of slanted groove sidewall but with $\alpha = 141.3^\circ$, groove bottom width = 20 μm, groove open width = 25 μm, groove depth = 2 μm and ridge width = 5 μm. Beyond these three conditions, osteoblast cells could become entrapped inside the groove cavity, leading to disconnected cell-cell communications.

These findings provided important new guidelines for future design of orthopaedic implants. Further in vivo studies are needed to validate and/or amend these insights.

### 5.7 References


Chapter 6

Summary and future work

6.1 Summary

The research presented in this thesis addressed the influence of surface topographical modifications of titanium and its alloys on osteoblast responses, with a special focus on the introduction of either random or ordered patterns to the implant surface. Starting with an informative literature review, presented in Chapter 2, we discussed the current understanding of the role of ordered and partially ordered surface topography in bone cell interactions and functions. The challenges to translate research findings into implant applications were also addressed. In order to further elucidate differences in osteoblast response to ultrafine-grained and submicron porous surfaces, a comparative study was conducted in Chapter 3. The study subsequently addressed the fabrication of titanium-coated sub-cellular scaled microgrooves and the correlated anisotropy in wettability, which were discussed in detail in Chapter 4. Chapter 5 clarified the exact influence of groove width on osteoblast responses through a systematic experimental study. Additionally, the favourable groove geometries to achieve full cell-groove adhesion were determined and a quantitative relationship was defined between the microgroove width and the degree of the osteoblast-groove adhesion.
The major conclusions of each chapter are highlighted as follows:

- Among coarse-grained (grain size: 500 µm), ultrafine-grained (grain size: 100 nm), coarse-porous (pore size: 350 nm) and fine-porous (pore size: 155 nm) surfaces of Ti-20Mo alloy, the coarse-porous surface provided the optimum topographical cues for osteoblasts. After culturing for one day, the extension of filopodia and the spreading of cells were limited on both the coarse-porous and fine-porous surfaces. However, with increasing culturing period to eight days, the formation of large and mature focal adhesions was enhanced on both coarse-porous and fine-porous surfaces compared with either coarse-grained or ultrafine-grained surfaces. This further led to good adhesions of osteoblasts to both porous surfaces. On the other hand, substantial grain refinement from 500 µm to 100 nm showed only limited influence on the extension of filopodia and the osteoblast growth. Compared to submicron pores, the osteoblasts are more responsive to submicron pores.

- Compared with coarse-grained and ultrafine-grained surfaces, both coarse-porous and fine-porous surfaces displayed significantly higher surface wettability. However, the biofunctionality of a porous metallic surface is not solely dependent on the surface wettability. Rather, pore size and depth were found to be a more crucial factor on regulating osteoblast responses. Moreover, these findings indicate the osteoblasts can distinguish the pore size and depth and make corresponding responses. Therefore, in order to achieve enhanced biofunctional outcomes, it is essential to control pore features of an orthopaedic titanium implant.
• Titanium-coated microgrooves over the groove widths range of 5-20 µm (groove depth: 2 µm, ridge width: 5 µm) displayed significant anisotropy in wettability. The degree of anisotropy (Δθ) increased with increasing groove width from 5 µm to 20 µm. Generally, the water contact angle measured parallel to the groove (θ∥) was about 50-60° smaller than that measured perpendicular to the groove (θ⊥). The Wenzel model can be used to predict the contact angle along the groove, and the Cassie model can be employed to predict that perpendicular to the groove. Consequently, osteoblast spreading was significantly influenced by the anisotropy of wettability, where the osteoblasts preferred aligning along, rather than perpendicular to the groove.

• The cell spreading area and cell width were proportional to the groove widths over the range of 5-20 µm, while the cell length was not affected. Additionally, the groove width exerted only limited influence on the cell proliferation and early differentiation compared to flat surfaces, despite the significantly increased surface area (up to 40%).

• This thesis has provided direct evidence for the first time that the degree of the osteoblast-groove adhesion increased exponentially with increasing groove width and reached full adhesion at the groove width of 15 µm. The nil osteoblast-groove adhesion is predicted to occur at the groove width of 2.42 µm, which is about 10% of the average width of an osteoblast. Therefore, in order to achieve full cell-groove adhesion, it is crucial to design micro-grooved titanium implant surfaces with groove width comparable to the cell size.
Three different critical conditions have been defined for full osteoblast-groove adhesion. The first consists of vertical groove sidewalls with $\alpha = 90^\circ$, groove width = 15 $\mu$m, groove depth = 2 $\mu$m and ridge width = 5 $\mu$m. The second is featured by slanted groove sidewalls with $\alpha = 158.2^\circ$, groove bottom width = 15 $\mu$m, groove open width = 25 $\mu$m, ridge width = 5 $\mu$m and groove depth = 2 $\mu$m. The last groove geometry, which is similar to the second one, also is featured by slanted groove sidewall but with $\alpha = 141.3^\circ$, groove bottom width = 20 $\mu$m, groove open width = 25 $\mu$m, ridge width = 5 $\mu$m and groove depth = 2 $\mu$m. Beyond these three boundary conditions, osteoblasts could be entrapped inside the groove cavity, resulted in disconnected cell-cell communications.

6.2 Recommendations for future work

Further studies are needed to address some outstanding issues for future design of titanium orthopaedic implants. These include:

1. The role of groove depth in influencing osteoblast response

Groove depth is another key factor that can influence the osseointegration of implants. There is still controversy on the exact influence of groove depth on osteoblast responses. It is also important to understand whether groove depth plays a more significant role than groove width in affecting osteoblast proliferation and differentiation. This information can provide useful guideline for modifying surface topography of titanium implants.

2. Combination of micro- and nano-scaled structures

Inspired by 1) the hierarchical structures of natural bone at both micro- and nano-scales and 2) the cell-implant interaction that occurs from
micro- to nano-scales, it is essential to craft both micro- and nano-scaled structures on implant surfaces. It needs to answer whether the combinatorial effect of microscaled and nanoscaled topography on osteoblast responses is synergistic or not.

3. The integration of surface topographies and anti-bacterial surface coatings

Aseptic loosening and peri-prosthetic joint infection are two main causes of implant failures. Anti-bacterial coatings such as elements (e.g., silver, zinc, selenium) and antibiotics (e.g., aminoglycosides, glycopeptides) have been used to inhibit the growth of bacterial pathogens on implants. Further studies are needed to identify the suitable anti-bacterial coatings on titanium implant surfaces. The integration of the optimum surface topography that can enhance osteoblast functions and anti-bacterial coatings can be an effective way to achieve rapid and robust osseointegration.

4. Surface topographical modifications of metal implants using additive manufacturing (3D printing)

Personalisation of orthopaedic metal implants has been realised through additive manufacturing, which can construct complex shapes for patient-specific parts. To improve biocompatibility and osseointegration of these implants, further work should concentrate on post-treatment via surface topographical modifications, for instance, acid-etching and formation of nanotubes.

5. The influence of surface topographies on cell signalling pathways and particularly their underlying mechanism
Further studies are needed to understand the cellular mechanisms behind the differentiation and proliferation altered by the topographical patterns, such as specific mechanotransductive and signalling pathways. This information is crucial to develop better orthopaedic implants and enable the translation of research findings to clinical applications.

6. In vivo experiments

Since notable differences between in vitro studies and in vivo results may exist, in vivo testing of the designed surface patterns will be needed to determine the consistency between these results. In vivo studies are therefore needed to validate whether designed surface patterns are beneficial in terms of biofunctionality and longevity of orthopaedic implants.

7. Cell migration dynamics on surface patterns

Cell migration is crucial to bone regeneration, which is a dynamic process. Limited studies have been conducted on assessing the migration characteristics of osteoblasts on patterned surfaces. Therefore, more studies are needed to reveal how topographic cues mediate cell migration dynamics.