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Luiz E. Bertassoni
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Engineering Mineralized and Load Bearing Tissues

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Engineering Mineralized and Load Bearing Tissues

Springer

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Preface: Engineering Mineralized and Load-Bearing Tissues: Progress and Challenges

From cartilage and bones, which enable body growth, calcium storage, and a range of hematopoietic functions, all the way to teeth and ligaments, which are elegantly structured to bear a range of functional loads in the most diverse conditions, mineralized and load-bearing tissues represent a unique category of biological materials that perform a range of critical functions in the human body.

Treatment of diseases and injuries associated with these tissues results in a yearly financial burden of hundreds of billions of dollars worldwide. Tissue engineering has emerged as an exciting alternative for the treatment of these conditions. By bringing together principles of life sciences, biology, engineering, and clinical medicine and dentistry, the regeneration of mineralized and load-bearing tissues has gradually become a clinical reality.

Recent research has shown that the regeneration of truly biomimetic, clinically relevant, and functional load-bearing tissues will only be achieved through a complex combination of novel biomanufacturing methods, smart engineering solutions, supported by solid biological foundations. This book carefully outlines each of these individual aspects to offer a comprehensive overview of recent progresses and challenges toward effective regeneration of functional tissues, including the bone, cartilage, dentin, enamel, cementum, and periodontal ligament.

We present recent progress on the fabrication of load-bearing tissues using emerging biomanufacturing methods. Jeong and Atala provide a comprehensive overview of 3D printing strategies focusing primarily on the fabrication of bone and cartilage tissue constructs. Prof Nikkhah and Maas cover recent developments relative to lithography-based microfabrication of cell-laden hydrogels. In Chap. 3, Araujo et al. offer their insights into the synthesis of bioinspired polymeric nanofibrils for the regeneration of cartilage, bone, and dental structures. Combined, these emerging methods have changed the landscape of regenerative medicine in recent years. For instance, these methods have enabled recent strategies which are primarily focused on the fabrication of 3D tissue constructs, with more clinically relevant dimensions, which is a substantial development from earlier methods relying on thin scaffolds and cells seeded on planar 2D culture substrates.

In the second part of this book, applied strategies for bone, cartilage, and dental regeneration are presented. Clinical and surgical challenges associated with regeneration of oral and maxillofacial tissues are described by Young et al. Recent attempts to engineer pre-vascularized bone scaffolds are

reviewed by Bertassoni and colleagues, while osteointegration of implants via unique healing pathways is discussed by Coelho et al. in Chap. 7. Exciting methods to chemically synthesize cell-laden hydrogels functionalized or conjugated with bone morphogenic peptides are presented by Jabbari and his group. Arguably, these latter methods represent the future of smart polymeric delivery platforms for tissue regeneration, which take advantage of bioinspired growth factors in ECM-mimetic 3D microenvironments for functional tissue engineering. A similar pathway is explored by George and co-workers, who focus their attention on dentin matrix proteins and their role in bone mineralization and regeneration.

Exploring the interface between the mechanics of load-bearing tissues and approaches to regenerate them, Armitage and Oyen offer a unique perspective of the challenges associated with engineering stiff and tough tissues, such as the ligament and bone, while using comparatively soft and brittle scaffold materials, such as hydrogels and ceramics, respectively. Correia, Reis, and Mano present recent strategies for the regeneration of cartilage using multiphasic, multistructured, and hierarchical scaffolds, which appear as a highly effective method for fabrication of multilevel hierarchical tissues, such as the cartilage and bone. In a more focused review, Deghani's group address current challenges and regenerative treatments for injuries of the anterior cruciate ligament.

In a separate section, the regeneration of dental tissues is comprehensively covered by Bartold and colleagues, Tsuji's group, and Prof. Uskokovic, who address recent progress and challenges toward effective cementum and periodontal ligament regeneration, enamel tissue engineering, and, lastly, whole tooth regeneration as a future dental treatment, respectively.

Overall this book brings together researchers from diverse backgrounds, from materials science, engineering, and biology to clinical medicine and dentistry, to discuss various aspects of mineralized and load-bearing tissue engineering. We argue that this collection of manuscripts will represent a unique platform stimulating progress toward the regeneration of functional mineralized and load-bearing tissues on the lab bench and facilitate the translation of these strategies into a clinical reality in the near future.

Portland, OR, USA — Luiz E. Bertassoni
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Engineering Mineralized and Load Bearing Tissues

Scope, Target Audience and Highlights

Mineralized and load-bearing tissues represent some of the most important structural entities in the animal kingdom. They provide shape to all individuals, enabling foremost steps in evolution, such as gait, nutrition, development, and immune response. Diseases related to load-bearing tissues yield an annual economic burden of over $20 billion/year in America, a cost that is expected to quadruple with an increase in the aging population. A large part of such conditions involve load-bearing bone, cartilage, and/or dental structures requiring assisted repair. Tissue engineering has long held great promises as improved treatment options for these conditions. Tremendous progress has been achieved in the last decade; these have included successful strategies in materials sciences, biology, engineering, and, most importantly, the interface among these fields. Here, we aim to address recent developments in engineering of fully functional mineralized and load-bearing tissues. We envision this will represent a valuable reference for researchers in their endeavors to fabricate biomimetic load-bearing tissues. Similarly, by bringing together high-caliber scientists from bone, cartilage, and dental fields – integrating backgrounds in materials sciences, engineering, biology, mechanics, fluidics, etc. – we will provide a unique platform to facilitate the functional regeneration of mineralized and load-bearing tissues.

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Part I
Fabrication Methods and Techniques

3D Printing and Biofabrication for Load Bearing Tissue Engineering

1

Claire G. Jeong and Anthony Atala

Abstract

Cell-based direct biofabrication and 3D bioprinting is becoming a dominant technological platform and is suggested as a new paradigm for twenty-first century tissue engineering. These techniques may be our next step in surpassing the hurdles and limitations of conventional scaffold-based tissue engineering, and may offer the industrial potential of tissue engineered products especially for load bearing tissues. Here we present a topically focused review regarding the fundamental concepts, state of the art, and perspectives of this new technology and field of biofabrication and 3D bioprinting, specifically focused on tissue engineering of load bearing tissues such as bone, cartilage, osteochondral and dental tissue engineering.

Keywords

Biofabrication • 3D printing • Tissue engineering • Bone • Cartilage

1.1 Introduction

There is no doubt that biofabrication and bioprinting are some of the most stimulating technological recent advances in the field of tissue engineering and regenerative medicine, with the ultimate goal of creating 3D multi-cellular functional tissues and organs. This is evidenced by the launch of relatively new peer-reviewed journals *Biofabrication* in 2009 and *3D Printing and Additive Manufacturing* in 2014, and many domestic/international conferences and workshops solely devoted to this aspect of the field. Also, biofabrication and bioprinting are certainly anticipated as a dominant technological platform and leading manufacturing paradigm of the twenty-first century due to their broad spectrum of potential applications and rapidly advancing technologies in tissue engineering and regenerative medicine (Mironov et al. 2009a; Bajaj et al. 2014). Considering the relatively short history of this field, there are approximately

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1800 articles with 'bioprinting' and 3800 articles with 'biofabrication' as of now in a search in Google Scholar representing the rapid growth and development in this field.

The fundamental concept of tissue engineering, first defined in 1988 is to combine different bio-components, such as living cells, biomaterials, and/or biologically active factors, to form tissue engineered constructs to promote the repair and/or regeneration of tissues (Langer and Vacanti 1993). Based on this concept, a myriad studies of tissue engineered constructs by a variety of scaffold fabrication technologies have been reported, and particularly new methods of engineering and fabricating three-dimensional (3D) structured tissues supported and guided by 3D scaffolds have been an important topic that resulted in significant achievements, and has captured further interests due to its development potential. Scaffolds—temporary mechanical and functional supporting structure for cells to produce engineered tissues effectively—are crucial for cell survival, colonization, migration, proliferation and differentiation (Langer and Vacanti 1993; Yang et al. 2001). Both their internal architectural design (i.e. pore shape, pore/strut size, and pore interconnectivity) and physical and material composition and properties (i.e. mechanical strength and degradation kinetics) play a significant role in the success of tissue engineered constructs as they control and maintain the behavior and well-being of the seeded cells (Yang et al. 2001, 2002; Hollister 2005; Hutmacher 2000; Derby 2012). Conventionally, techniques such as solvent casting, particulate leaching, solution casting, freeze drying, phase separation, gas forming, melt molding, fiber bonding and electrospinning are employed to fabricate scaffolds and there are several excellent reviews which summarize and cover these topics in detail (Fig. 1.1) (Hutmacher 2000; Sachlos and Czernuszka 2003; Murugan and Ramakrishna 2006; Billiet et al. 2012). In order to overcome the disadvantages of conventional techniques, such as lack of precise control and consistency over inner pore architecture and spatial distribution, and deficiencies of incorporating cells and proteins in the fabricating process, the development of rapid prototyping (RP) or commonly referred to as solid freeform (SFF) fabrication techniques for 3D scaffolds have enabled the design and manufacturing of scaffolds with finely tunable and scalable internal and external porous structures, fulfilling requirements for specific tissue regeneration (Yang et al. 2001, 2002; Hollister 2005, 2009; Hutmacher 2000). This

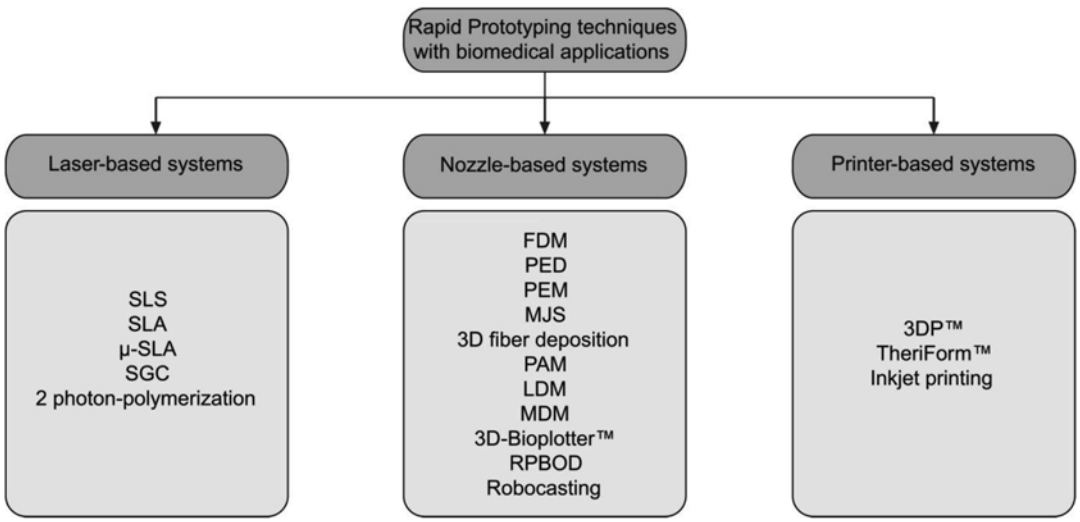

Fig. 1.1 Classification of rapid prototyping (RP) techniques for biofabrication and bioprinting (Billiet et al. well summarized and divided the RP techniques into three sub-categories and this figure was adopted from Billiet et al. 2012)

technique is also now generally referred to as additive manufacturing (AM), as scaffolds are manufactured by adding materials layer by layer. Melchels et al. (2012) nicely summarized and presented the history of additive manufacturing and its application in tissue engineering with major intervention of technologies and consequent scientific findings (Fig. 1.2). Based on this scaffold-based tissue engineering approach, a new approach termed as 'biofabrication and 3D bioprinting' has been developed. These technologies overcome the limitations of conventional scaffold fabrication when engineering relatively large sized tissues or cells and tissues that require complex 3D microenvironments for their regeneration. The success of producing massive tissue by a scaffold-based tissue engineering method alone, however, was often limited due to random and uneven cell seeding, distribution and attachment on the scaffold surface leading to uncontrolled tissue formation. Also, incorporation and administration of controlled growth factors within manufactured scaffolds are also challenging (Albrecht et al. 2006; Nakamura et al. 2010). For this increasing demand in more carefully designed and engineered assembly of biocomponents as a whole in the fabrication process, the terms and field of biofabrication and bioprinting gained its attention and became a niche that researchers and clinicians in tissue engineering and regenerative medicine began to define, employ, and develop.

Mironov et al. (2009a) defined the term, 'biofabrication,' as the production of complex living and non-living biological end products from raw materials which are not limited to living tissues and organs, but must include biologically active components such as living cells, molecules, extracellular matrices, and biomaterials. Whereas, 'bioprinting' or commonly known as 'additive layer-by-layer biomanufacturing' is a more specific technology or a set of techniques, which enables a combination of biological elements (different types of cells in defined positions, supporting matrix or scaffolds, and/or biochemical cues) to make a certain organization or structure to achieve biological functions (Derby 2012; Mironov et al. 2006). Compared to conventional AM techniques, this automated approach of bioprinting delivers several advantages such as feasibility to custom design a desired level of complexity into the scaffold, freely scalable and readily reproducible mass production of tissue engineered constructs,

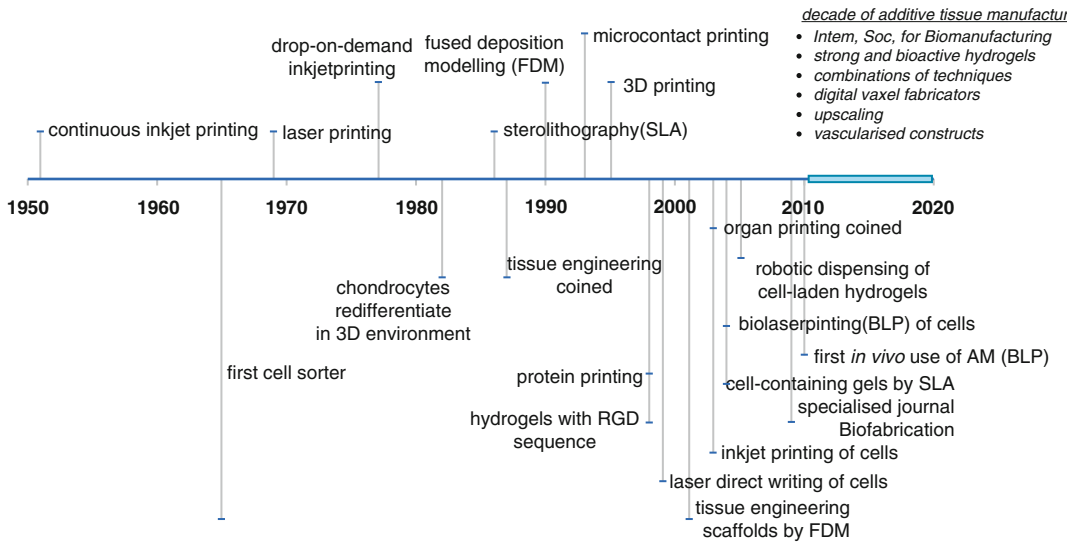

Fig. 1.2 Historical overview of additive manufacturing (AM) techniques in tissue engineering. Melchels et al. presented important landmarks of the introduction of new technologies and relevant major scientific findings in the field of tissue engineering (This figure was adopted from Melchels et al. 2012)

precise 3D positioning and layering of multiple cell sources or cell aggregates, simultaneous or sequential deposition of cells during the fabrication of scaffolds, effective vascularization, and industrial biofabrication of complex human tissues (Jakab et al. 2004; Mironov et al. 2008; Visconti et al. 2010; Stevens et al. 2008). As you can imagine, there- are many topics to cover for a comprehensive review on the biofabrication and bioprinting field, such as diversity of materials or 'biopaper' (i.e. types, uses, and limitations of hydrogels, polymers, and composite materials (Bajaj et al. 2014; Billiet et al. 2012; Melchels et al. 2012; Vaezi et al. 2013; Fedorovich et al. 2007)), techniques (types of conventional biofabrication methods, bioprinting, and photolithography technology (Bajaj et al. 2012, 2014; Derby 2012; Limpanuphap and Derby 2002; Stringer and Derby 2010; Peltola et al. 2008; Li et al. 2009)), 'bioink' (cells and cell aggregates (Boland et al. 2003; Mironov et al. 2009b; Wilson and Boland 2003; Imani et al. 2012)) and different types of tissues and organs reproduced via these techniques. As summarized and presented in Fig. 1.3 (Nakamura et al. 2010), there are many components and technologies that are involved and interlink each other in each step from 'bio-design' to complex tissue and organ engineering via biofabrication/bioprinting and bio-processing. However, in this topical review, we are focused on the state of the art and application of biofabrication and bioprinting in load bearing tissues only, such as bone, cartilage, and others (osteochondral regions, meniscus, and dentin/teeth), thereby we provide a basis for a comprehensive survey of recent progress in biofabricated or bioprinted load bearing tissues and give insights on future potentials and challenges.

1.2 Biofabrication and Bioprinting of Load Bearing Tissue Engineering

Musculoskeletal tissue, load bearing tissues like bone, cartilage, osteochondral regions, and so forth, are under extensive investigation in tissue engineering research. The application of emerging technologies, biofabrication and bioprinting, is no exception and clinicians, researchers and tissue engineers have began to employ these relatively new fabrication methods to create and regenerate load bearing tissues for future clinical applications.

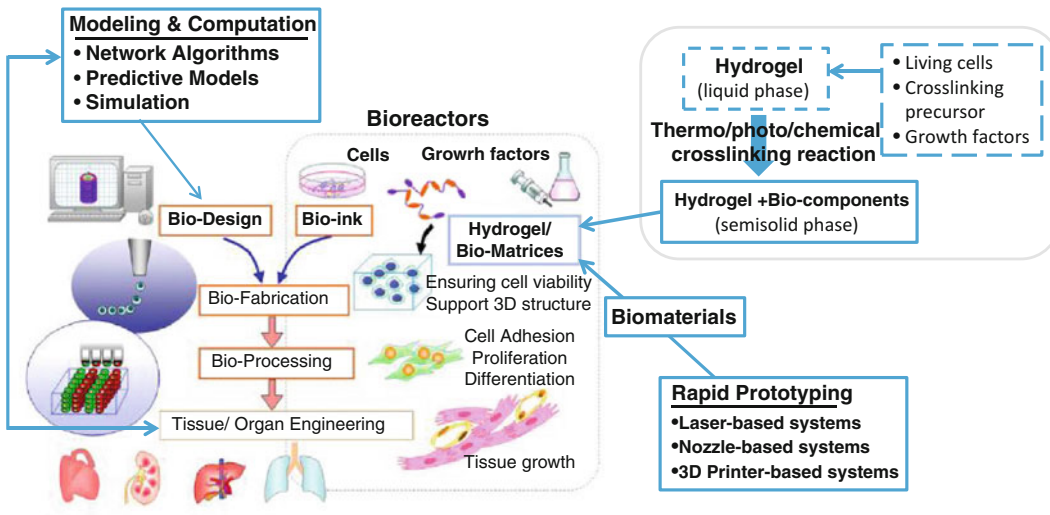

Fig. 1.3 Outlines of tissue and organ engineering with advanced technologies and fabrication processing elements. The combined potential and knowledge and technologies from multidisciplinary fields have opened an exciting route in developing tissue and organ engineering such that biological tissues and solid organs will be eventually manufactured through several engineering approaches with desired properties (This figure was adopted and modified from Nakamura et al. 2010)

1.2.1 Bone

Bone has a remarkable intrinsic property to remodel and regenerate spontaneously and the homeostasis of bone tissue is mediated by several bone cells (osteoblasts, osteoclasts and osteocytes). However, the critically sized bone defects are not subject to this spontaneous regeneration and thus the demands for clinically applicable tissue-engineered bone are increasing. There are allograft bone graft substitutes as treatment options, and several recent reviews (Eppley et al. 2005; Pryor et al. 2009; Laurencin et al. 2006; Nandi et al. 2010) are available for reference, but this review will discuss biofabricated and bioengineering approaches primarily. Before we explore the state of the art for biofabrication and bioprinting of bone tissue engineering (BTE) it is important and essential to define the goal of BTE and the performance criteria for bone regeneration. To treat patients, the success of tissue-engineered bone should be clinically competitive in terms of improved patient outcomes, reduced complication and expenses. More specifically, a scaffold needs to be integrated within the surrounding bone tissue and needs to provide a 3D framework for seeded cells to attach, proliferate, and eventually lead to their extracellular matrix (ECM) formation. Then, the accelerated connection of newly formed tissues to the host vasculature should promote the viability and growth of the seeded cells and promote the quality of the newly formed bone (Rouwkema et al. 2008). Overall, not only the structure but also physical/mechanical properties of tissue engineered bone need to be mimicked and well-matched with those of native surrounding bone.

Scaffolds fabricated by various RP methods have been developed over the past 20 years since stereolithography (SLA) was first introduced by Chuck Hull in 1986 (Bose et al. 2012, 2013). SLA enables scaffolds to be built by using the ability of photopolymerizable liquid polymers (i.e. poly(ethylene glycol) (PEG), poly(propylene fumarate) (PPF)/diethyl fumarate (DEF)) and ultraviolet (UV) light focused on deposited liquid polymers to crosslink and solidify. Once a single layer is completely deposited and solidified, the next layer is deposited until the entire construct is processed through a layer by layer deposition (Yang et al. 2002; Melchels et al. 2010). Several studies (Chang et al. 2003; Cooke et al. 2003; Lee et al. 2007; Kim et al. 2010a, 2011) have shown the feasibility of stereolithographic bone scaffold fabrication and there are thorough reviews (Kim et al. 2011; Duan et al. 2010) solely devoted to this technique. However, SLA techniques were still limited due to a restricted selection of materials and necessary post-processing techniques for bioceramics. The selective laser sintering (SLS) technique has been employed as one of RP methods to overcome such limitations. SLS technique utilizes a laser over a thin layer of polymer powder and the laser raises the temperature of the powders to be melted and fused, thereby manufacturing a layer of polymer to build a 3D scaffold (Yang et al. 2002; Peltola et al. 2008). More variety of biomaterials could been used for bone scaffolds fabricated via SLS, for instance, polycaprolactone (PCL), hydroxyapatite (HA), calcium phosphate (CaP), poly(L-lactic acid) (PLLA), β-tricalcium phosphate (β-TCP), composite polymers/ceramics and so forth others (Stevens et al. 2008; Duan et al. 2010; Goodridge et al. 2006; Shuai et al. 2013; Williams et al. 2005; Xia et al. 2013). For load bearing tissues like bone, low mechanical strength of engineered scaffolds has been a major challenge. As mechanical strength, pore size/scaffold architectures, and native material properties are all interlinked with each other to create unique scaffold properties (Shrivats et al. 2014), it is difficult to make a conclusion on the best material and architectural conditions for bone scaffolds. Recent studies (Lan Levengood et al. 2010; Wagoner Johnson and Herschler 2011) demonstrate that microporous materials seem to promote enhanced bone ingrowth compared to non-microporous materials and compressive strength of 3D scaffolds could range from 2.0 to 3.2 MPa for PCL based scaffolds with 1.75–2 mm pore diameter and 63–79 % porosity (Williams et al. 2005) and 6.4–10.95 MPa for TCP based scaffolds with 500 μm pores and 42 % porosity (Tarafder et al. 2013), which shows that scaffolds built via SLS method present low to high range of properties for human trabecular bone. Also, studies (Tarafder et al. 2013; Karageorgiou and

Kaplan 2005) have shown that pore size and porosity affect enhanced new bone formation. It has been noted that the minimum requirement for pore size is considered to be ~100 μm due to cell size, migration requirements and necessary transport properties. However, pore sizes >300 μm are recommended due to enhanced new bone and capillary formation and the decrease in designed pore size from 1,000 to 500 μm can elicit an increase in cell proliferation (Tarafder et al. 2013). Although SLS methods also have disadvantages compared to SLA, such as a rough surface and lower feature resolution, the feasibility of directly sintering biocompatible materials without any post-processing step and the ability of manufacturing various ranges of mechanically bone-like scaffolds give SLS advantages over SLA (Bose et al. 2013).

Bone printing is recently getting attention and seen as a promise in BTE. As 3D printing technologies in the field of tissue engineering and regenerative medicine are relatively new and recently employed, there are only a few studies (Fedorovich et al. 2007, 2011a; Kim and Kim 2012; De Coppi et al. 2007; Cooper et al. 2010a, b; Phillippi et al. 2008; Temple et al. 2014) directly related to 3D bone printing or bone related cell biopatterning, with the first report published in 2007 (De Coppi et al. 2007). The requirements to print bone first need to be carefully considered and defined such that the bone structure fabricated by 3D bioprinting needs to be both flexible and strong and subject to mineralization and vascularization. Cell viability during and after printing, cell densities and ratios, printability and types of hydrogel materials used for deposition, the feasibility of printing system with interconnected pore architectures, and the stackable properties of both hydrogels and any other scaffold materials all need to be considered (Billiet et al. 2012; Murphy et al. 2013). In fact, to achieve the survival and differentiation of embedded bone progenitor cells in bone printing, the work of encapsulating multiple cells (Fedorovich et al. 2011b) with PCL/β-TCP nanofibers (Kim and Kim 2012), calcium phosphate particles (Trojani et al. 2006), and growth factors such as bone morphogenetic proteins (BMPs) (Cooper et al. 2010a) and fibroblast growth factor-2 (FGF-2) (Campbell et al. 2005; Miller et al. 2006) with patterning in vitro and in vivo are already being done successfully. All these studies certainly have shown and proved the potential and promises for bone printing in BTE yet there are still gaps to fill in to achieve our defined goal of BTE earlier. For example, most of hydrogels or biopaper materials that are used now for printing are purely used as temporary cell carriers lacking any surface modification to promote further cellular activities (attachment, proliferation, differentiation, and migration). The modification of printable hydrogels with osteoinductive or osteoconductive peptides or proteins needs to be explored for better induction and maintenance of osteogenesis, just like how we modify hydrogels when seeding cells in scaffolds built via SFF fabrication techniques (Burdick and Anseth 2002; Guenther et al. 2013; Shin et al. 2005; Zhang et al. 2009; Kang et al. 2011).

Unfortunately as several authors noted (Hollister 2009; Shrivats et al. 2014; O'Keefe and Mao 2011; Hollister and Murphy 2011), although the currently available BTE scaffold technologies and therapeutics including biofabrication and 3D bioprinting have advanced notably over past decades, they have not yet obtained success relative to the defined performance criteria discussed earlier due to technical and translational challenges and barriers. The organ printing approach still has many important issues that need investigation. It will be interesting to see if printed bioactive implants can trigger vascularization and can lead to bone formation in the near future.

1.2.2 Cartilage and Osteochondral Regions

Unlike bone, cartilage shows little tendency for self-repair and regeneration due to its avascular structure and less metabolically active nature with relatively low cell density. Once cartilage is injured, it is highly likely to eventually degenerate. There are three types of cartilage: hyaline, fibrocartilage and elastic cartilage, which are all

composed of chondrocytes and extracellular matrix macromolecules such as collagens and elastin (Fosang and Beier 2011; Triche and Mandelbaum 2013; Ulrich-Vinther et al. 2003; Umlauf et al. 2010). Autologous chondrocyte implantation (ACI) and matrix-assisted autologous chondrocyte transplantation (MACT) techniques have been established, over the last few decades (Filardo et al. 2013; Temenoff and Mikos 2000). Similar to bone scaffolds, various scaffold materials have been employed, and the potential for both naturally derived and synthetic polymers have been assessed, and there are extensive reviews on more complete listings of cartilage scaffold materials (Benders et al. 2013; Balakrishnan and Banerjee 2011; Lu et al. 2001). Unlike bone, little work has been done on the chondrogenic capacity of scaffolds built by the SLA technique, as this technique requires photopolymerizable biomaterials such as PCL and PPF. These biomaterials are generally stiffer than native cartilage. A few groups have attempted to show the feasibility of fabricating scaffolds suitable for cartilage (Schuller-Ravoo et al. 2013; Lee et al. 2008; Linzhong et al. 2010; Elomaa et al. 2011) or osteochondral regions with SLA techniques. Even though constructs in those studies all showed good biocompatibility and proliferation of seeded cells, scaffold geometry on chondrocytes adhesion (Lee et al. 2008), a critical role of controlled accurate internal architectures on mechanical strength matching with articular cartilage (Linzhong et al. 2010) has not been achieved. The feasibility of hydrogel modification for higher cell viability for engineered cartilage and osteochondral sites has been achieved as a proof of concept, but without functional assessment (Seck et al. 2010; Lin et al. 2013). Also, the use of selective laser sintering (SLS) technique was similar to SLA in terms of their application in cartilage tissue engineering, and a few studies directly related to cartilage engineering have been performed (Chen et al. 2011).

Biofabrication and 3D bioprinting seem be the next emerging technologies and popular tools for cartilage scaffold engineering, as evidenced by an increasing number of studies reported with bioprinted cartilage or/and osteochondral constructs. Recently, several research groups have embarked on engineering soft tissues like cartilage and osteochondral regions using biofabrication and bioprinting methods. These methods include inkjet (Phillippi et al. 2008; Arai et al. 2011; Boland et al. 2006; Cui et al. 2012; Nishiyama et al. 2009; Xu et al. 2005) and mechanical extruders (Smith et al. 2007; Jakab et al. 2010). There is extensive literature (Jeong and Hollister 2010; Kemppainen and Hollister 2010; Liao et al. 2007; Ye et al. 2014; Hung et al. 2014; Zhang et al. 2014; Eyrich et al. 2007; Fecek et al. 2008; Martinez-Diaz et al. 2010) available for simple 3D printing of scaffolds and seeding cells on manufactured scaffolds (known as 'two-step procedures (Filardo et al. 2013)'). Studies conducted in vitro and in vivo proved that chondrocytes encapsulated in the hydrogel proliferated and differentiated more effectively into the cartilage than non-printed chondrocytes (Kundu et al. 2013; Xu et al. 2013; Lee et al. 2014; Liu et al. 2010; Fedorovich et al. 2012). Several groups for osteochondral tissue engineering (Fedorovich et al. 2011b, 2012; Shim et al. 2012; Park et al. 2014) presented the possibility of manufacturing viable bioprinted tissues created with multiple encapsulated cells within various hydrogels. Successfully separating dispensed cell sources that can retain their initial location, and can remain viable are benefits of bioprinting, especially when two or more different types of tissues need to be engineered in one construct. Also, the ability to combine different biomaterials (hydrogels or/and polymer-based) in one construct gives greater flexibility to the design of mechanically stable scaffolds for specific tissues like osteochondral regions, or other parts of load bearing tissues that may require different biochemical microenvironments and mechanical properties (Xu et al. 2013; Park et al. 2014). Xu et al. (2013) successfully applied a hybrid inkjet printing/electrospinning system to fabricate viable cartilage tissues by combining electrospinning of PCL fibers and chondrocytes suspended in fibrin-collagen hydrogels with high cell viability, proliferation, and proper ECM formation (evidenced by the deposition of type II collagen and

glycosaminoglycans (GAG)). Gurkan et al. (2014) demonstrated the engineering of anisotropic multiphase 3D biomimetic fibrocartilage models with a potential impact for in vitro drug testing and drug discovery. Further developments include the application of nanoliter droplets containing human mesenchymal stem cells (MSCs), bone morephogenetic protein 2 (BMP-2), and transforming growth factor β1 (TGF-β1). The work by Cohen et al. (2010) presented the in situ repair of chondral and osteochondral defects using in situ additive manufacturing (AM) techniques, all extending the applications of bioprinting in cartilage tissue engineering. Recent work confirms the promising potential of biofabrication and bioprinting technologies in cartilage and osteochondral tissue engineering.

In order to create functional cartilage or osteochondral tissues applicable to the clinical setting, further investigation on the quality of bioprinted tissues, and their comparison to normal tissues and tissues fabricated by other conventional fabrication methods will be needed. Also, in order to have a comprehensive picture, various types of hydrogels or polymer materials need to be explored for bioprinting based approaches.

1.2.3 Dental Tissue Engineering

Scaffold based tissue engineering methods, especially biofabrication and bioprinting, slowly being applied to the field of dentistry and for dentin regeneration (Azari and Nikzad 2009). SLA, SLS and inkjet-based systems (simple 3D printing) are common technologies already used in dentistry, especially for reconstructive surgeries and sub-periosteal dental implant surgery (Azari and Nikzad 2009; Chan et al. 2004; Soares et al. 2013; Bidra et al. 2013; Lima et al. 2014). Azari and Nikzad (2009) reviewed and summarized how each 3D scaffold fabrication method has evolved and employed in dentistry. Compared to other load bearing tissues though, tooth engineering or dental tissue engineering is still at its initial stage of investigation. Optimal cell sources (Bluteau et al. 2008; Keller et al. 2011; Nakahara 2011; Peng et al. 2009; Yen and Sharpe 2008) and biomaterials have been explored (El-Bialy 2012; Hu et al. 2006; Ohara et al. 2010; Sharma et al. 2014; Ueda 2003; Young et al. 2002). Among a few studies based on scaffold tissue engineering techniques Young et al. (2002), Park et al. (2010), Jaramillo et al. (2012), Letic-Gavrilovic et al. (2004), Xu et al. (2008), Zheng et al. (2011), Kim et al. (2010b), Park et al. (2010), and Zheng et al. (2011) demonstrated how biofabricated 3D composite hybrid polymeric scaffolds could be used to form tooth cementum-like tissue, ligament, and/or bone structures. Kim et al. (2010b) incorporated stromal-derived factor-1 (SDF1) and bone morphogenetic protein-7(BMP7) in scaffold microchannels and showed regeneration of tooth-like structures and periodontal integration by cell homing. This approach definitely offers potential for the clinical implementation of customized periodontal scaffolds that may enable regeneration of multi-tissue interfaces required for oral and dental engineering applications. This field of dentistry should benefit greatly from these this rapidly growing and emerging technology.

1.3 Summary and Conclusions

We can easily anticipate that biofabrication and 3D bioprinting are going to play a critical role in the field of tissue engineering as more developed scaffold fabrication technologies will evolve to meet the rigorous and strict demands that native tissues require for their complete repair and regeneration. Several studies noted here already provide sufficient evidence for the feasibility of these techniques which are involved in load bearing tissue engineering and regeneration. The advances in biofabrication techniques and precise control over the scaffold internal and external properties will allow enhanced tissue engineering inspired therapies for load bearing tissues, and hopefully for technologies that can eventually be used for clinical applications.

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2 Microfabrication of Cell-Laden Hydrogels for Engineering Mineralized and Load Bearing Tissues

Chia-Cheng Li, Mahshid Kharaziha, Christine Min, Richard Maas, and Mehdi Nikkhah

Abstract

Microengineering technologies and advanced biomaterials have extensive applications in the field of regenerative medicine. In this chapter, we review the integration of microfabrication techniques and hydrogel-based biomaterials in the field of dental, bone, and cartilage tissue engineering. We primarily discuss the major features that make hydrogels attractive candidates to mimic extracellular matrix (ECM), and we consider the benefits of three-dimensional (3D) culture systems for tissue engineering applications. We then focus on the fundamental principles of microfabrication techniques including photolithography, soft lithography and bioprinting approaches. Lastly, we summarize recent research on microengineering cell-laden hydrogel constructs for dental, bone and cartilage regeneration, and discuss future applications of microfabrication techniques for load-bearing tissue engineering.

Keywords

Load-bearing tissue engineering • Dentistry • Bone • Cartilage • Hydrogel • Microfabrication • Photolithography • Soft lithography • Bioprinting

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Abbreviations

2D	Two dimensional
3D	Three dimensional
BMP	Bone morphogenetic protein
μCP	Microcontact printing
DPSC	Dental pulp stem cell
ECM	Extracellular matrix
GelMA	Gelatin methacrylate
HA	Hydroxyapatite
MAPLE DW	Matrix assisted pulsed laser evaporation direct write
MSC	Mesenchymal stem cell
PCL	Poly-ε-caprolactone
PDL	Periodontal ligament
PDMS	Polydimethylsiloxane
PD-PEGDA	Photodegradable PEG diacrylate
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PLGA	Poly-L-lactate-co-glycolic acid
PVA	Poly(vinyl-alcohol)
RGD	Arg-Gly-Asp
SCAP	Stem cells from apical papilla
SHED	Stem cells from human exfoliated deciduous teeth

2.1 Introduction

Load-bearing tissues, namely bone, cartilage and teeth, serve various physiological functions, including mechanical support, protection, as well as ion homeostasis (Gotfredsen and Walls 2007; Confavreux et al. 2011; Chen et al. 2013). Conditions such as trauma, infection or neoplasms impair the structures and functions of these tissues, and in turn significantly impact the life quality of patients (Gotfredsen and Walls 2007; Confavreux et al. 2011; Marcenes et al. 2013; Jackson et al. 2001). Medical treatments currently available for bone and cartilage reconstruction include grafts or artificial prostheses in addition to stable fixation (Finkemeier 2002). For tooth loss, dental implants or artificial crowns are the major treatment options (Sunnegardh-Gronberg et al. 2012). However, secondary infection, compromised biocompatibility, and the limited durability and accessibility of grafting materials and artificial prostheses remain major concerns (Finkemeier 2002; Puppi et al. 2010). To overcome these limitations, novel approaches that integrate stem cells and tissue engineering may provide valuable treatment alternatives for the regeneration of load-bearing tissues (Langer and Vacanti 1993; Cortesini 2005).

Tissue engineering is an interdisciplinary field that integrates biological sciences and bioengineering techniques to maintain, restore and enhance tissue or organ functions (Langer and Vacanti 1993). Tissue engineering approaches are mainly based on the use of isolated cell substitutes, acellular scaffolding biomaterials to initiate the regeneration process, or cell-laden biomaterials (Khademhosseini et al. 2006). While each approach possesses unique advantages, numerous challenges still exist such as the lack of renewable cell sources, a shortage of suitable biomaterials with enhanced mechanical, chemical, and biological properties, and an inability of in vivo revascularization (Khademhosseini et al. 2006; Langer and Vacanti 1999). The advancement of microfabrication techniques and biomaterial science in the past few years has paved the way to address some of the shortcomings of conventional tissue engineering (Khademhosseini et al. 2006). Microscale technologies were originally developed for fabricating semiconductor and microelectronic devices (Whitesides et al. 2001). Due to a wide range of length scale (i.e., 1–1,000 μm) and high resolution, microscale technologies provide a remarkable ability to facilitate the fabrication of miniaturized cell-laden constructs (Zorlutuna et al. 2012). Moreover, these technologies enable the precise control of the microenvironment, and organized vascularization for delivery of oxygenation and nutrients within engineered tissue constructs (Khademhosseini et al. 2006; Zorlutuna et al. 2012; Nikkhah et al. 2012a). In addition, the integration of microscale technologies with advanced biomaterials (e.g., hydrogels) promotes the development of high-throughput miniaturized assays to determine stem cell fate at single-cell level (Nikkhah et al. 2012a).

To date, microfabrication techniques have been applied for the development of load-bearing tissues (Petersen et al. 2002; Pelaez-Vargas et al. 2011). Using these technologies, it is possible to enhance cellular organization, tissue integration and interfacial strength (Charest et al. 2006; Gallant et al. 2007; Meredith et al. 2007; Kim et al. 2013a). In particular, the interfacial strength in cell-substrate interactions could be increased through deposition and adsorption of extracellular matrix (ECM) proteins on micro- and nanoscale patterned features (Kim et al. 2013b). Furthermore, these technologies facilitate the reciprocal cellular signaling, vascularization, and the delivery of growth factors for load-bearing tissue regeneration via precisely controlled spatial and temporal features of the cellular microenvironment (Kim et al. 2013a; Gray et al. 2003; Chung et al. 2007; Jager et al. 2008). This chapter outlines the applications of microscale technologies and hydrogel-based biomaterials for engineering load-bearing tissues. We first discuss the unique benefits of hydrogels in the development of engineered tissue constructs. Our discussion then focuses on fundamental microfabrication techniques, including photolithography, soft lithography and bioprinting. We finally highlight specific studies that are devoted toward the generation of cell-laden constructs for dental, bone and cartilage regeneration.

2.2 Hydrogels: Artificial Extracellular Matrices

The concept of tissue engineering stems from the ability of dissociated cells to recapitulate in vivo physiological functions under the appropriate settings (Kim and Mooney 1998). Since the ECM is important in tissue regeneration, an artificial ECM is normally used in tissue engineering to create a biomimetic microenvironment and to direct cell/tissue functions (Kim and Mooney 1998; Cohen et al. 1993). To date, numerous attempts have been made to develop synthetic or natural based biomaterials that closely resemble native ECM for tissue engineering applications (Kim and Mooney 1998; Tabata 2009). In this regard, hydrogels have attracted significant attention due to their suitable properties (Kim and Mooney 1998). Hydrogels are polymeric networks that are formed from hydrophilic polymers, and crosslinked to form insoluble gel matrices, which preserve a large amount of water (up to 99 %) (Peppas et al. 2006). The three-dimensional (3D) microenvironment of hydrogels circumvents some of the limitations of traditional two-dimensional (2D) cell culture systems (Petersen et al. 1992; Birgersdotter et al. 2005; Le Beyec et al. 2007). The biomimetic microenvironment within hydrogel constructs allows the diffusion of oxygen, nutrients and waste, as well as the transport of soluble factors (Slaughter et al. 2009). Due to their biocompatible nature, hydrogels have been widely used in regenerative medicine as an artificial ECM that provides cells with an initiating niche and support cell-cell and cell-matrix interactions (Slaughter et al. 2009).

Hydrogels can be fabricated from synthetic or naturally-derived materials (Peppas et al. 2006). Synthetic hydrogels (e.g., polyethylene glycol [PEG], polyglycolic acid [PGA], polyvinyl alcohol [PVA]) have the advantages of reproducible large-scale fabrication as well as tunable and consistent properties, but lack cell-recognizable motifs, such as Arg-Gly-Asp (RGD) (Kim and Mooney 1998). On the other hand, naturally-derived hydrogels (e.g., collagen, silk and hyaluronic acid) are attractive candidates for tissue engineering due to their biocompatibility and tunable biodegradability that support cell-matrix interactions (Peppas et al. 2006; Annabi et al. 2014). Compared to synthetic hydrogels, naturally-derived hydrogels offer a better optimized 3D microenvironment that promotes cell functions (e.g., attachment and proliferation) (Slaughter et al. 2009). However, the concerns of using naturally-derived hydrogels include low mechanical strength, batch-to-batch variance, and potential immunogenicity and contamination (Annabi et al. 2014). To further strengthen the mechanical properties of naturally-derived hydrogels, the incorporation of functional groups (e.g., acrylate) or other composites (e.g., synthetic hydrogels and nanoparticles) have been

studied (Ifkovits and Burdick 2007; Shin et al. 2013; DeKosky et al. 2010). Detailed descriptions on the properties and comparisons of various hydrogels are covered in previously published review articles (Peppas et al. 2006; Annabi et al. 2014).

Mechanical properties are key parameters when designing hydrogels for specific tissue engineering applications. In particular, the mechanical characteristics of hydrogel constructs, such as stiffness and ratio of stress/strain, have been shown to significantly influence cell behaviors (Huebsch et al. 2010; Baker and Chen 2012). Murine mesenchymal stem cells, for instance, differentiated toward an osteogenic fate in 3D RGD-modified hydrogels with stiffness similar to native osteoid matrix, which ranged from 11 to 30 kPa (Huebsch et al. 2010). Similarly, other cell types (e.g., fibroblasts in ligament and tendon) are capable of sensing stress and strain in the surrounding ECM, and respond accordingly by morphology, migration, proliferation and differentiation (Riehl et al. 2012). Beyond serving as scaffolds that support cell adhesion and promote cell-matrix interactions, hydrogels also regulate the spatial distribution of effector soluble molecules (e.g., morphogens, cytokines and growth factors) and gases through diffusive or convective transport as well as sequestration (Baker and Chen 2012). In this regard, techniques, such as microfabrication, have proven instrumental in adjusting the physical features (e.g., geometry and topography) of hydrogel constructs in order to support specific functionalities of multiple cell types within an organized tissue construct (Brock et al. 2003; Albrecht et al. 2006). As a result, the utility of cell-laden hydrogels in the field of regenerative medicine has seen a marked surge.

2.3 Microfabrication Techniques to Engineer Cell-Laden Hydrogels

One notable groundbreaking innovation in the field of tissue engineering is the use of microfabrication technology. So far, microfabrication techniques, including photolithography and soft lithography, have been widely applied for patterning or topographical guidance of cell-laden contructs (Andersson and van den Berg 2004). Tissue engineering has enormously benefited from microfabrication technology in terms of high flexibility, precise control in microenvironment design, efficient performance and cost-savings benefits due to the expediency for high-throughput and faster experiments (Andersson and van den Berg 2004). Below, we summarize the basic concepts and current applications of major microfabrication techniques.

2.3.1 Photolithography

Photolithography is a highly reliable microfabrication technique to manipulate features accurately at micro- and nano-scale (Liu Tsang et al. 2007; Shao and Fu 2014). In conventional photolithography, a photoresist is spin-coated uniformly on a flat substrate followed by exposure with UV light through a pre-fabricated photomask (Tabata 2009). UV light alters the chemical structure of a photoresist, further modifying its solubility in the developer solution and transferring the pattern of the photomask on the flat substrate (Borenstein et al. 2007). Through photolithography, it is possible to precisely pattern biomolecules or cells of interest on the substrate surface by etching or lift-off process in order to control the surface topographies (Andersson and van den Berg 2004; Liu Tsang et al. 2007). However, the major shortcoming of conventional photolithography is the high sensitivity of the procedure. Even the smallest dust particle can distort the spreading of photoresist molecules during the spinning process (Karp et al. 2006). Therefore, it is mandatory to carry out photolithography in a clean room (Karp et al. 2006) via relatively costly equipment (e.g. spin coater, mask aligner and wet benches) (Hwang et al. 2010).

On the other hand, hydrogel photolithography can be used on the bench-top to build 3D cell-laden constructs by the sequential patterning of photocrosslinkable hydrogels (Andersson and

van den Berg 2004; Liu Tsang et al. 2007). Compared to conventional photolithography, hydrogel photolithography is a fast, simple, and a low-cost technique. Photocrosslinkable hydrogels (e.g., gelatin methacrylate [GelMA], photodegradable PEG diacrylate [PD-PEGDA], methacrylated tropoelastin) can be used to manipulate cell behaviors (e.g., cell migration, cell proliferation and cell differentiation) and guide tissue organization (Khademhosseini et al. 2006; Moon et al. 2010a; Annabi et al. 2013). In a study by Nikkhah et al., endothelial cells-encapsulated GelMA constructs were precisely patterned with variable geometrical features using photolithography. The outcome of this study demonstrated that the cells rearranged toward the periphery of the constructs and formed highly organized cord-like structures that expressed endothelial cell markers, CD31 and VE-cadherin (Nikkhah et al. 2012b). This cord-like structure could act as a template during implantation to guide the formation of robust vessels integrated with the host tissue (Nikkhah et al. 2012b; Baranski et al. 2013).

2.3.2 Soft Lithography

Soft lithography (i.e., microcontact printing, microfluidic patterning) and replica molding techniques refer to a set of non-photolithographic approaches to develop 2D and 3D precisely ordered constructs with resolutions up to nanoscale (Whitesides et al. 2001; Yu and Ober 2003). In soft lithography, a prefabricated stamp or mold made of elastomeric polymers, such as polydimethylsiloxane (PDMS), is used to pattern biomolecules. On the other hand, replica molding techniques enable creating microscale features of heat-crosslinkable or photocrosslinkable hydrogels to control the distribution of the biomolecules or cells in a 3D microenvironment (McMillan et al. 1999; Selimovic et al. 2012; Occhetta et al. 2013).

Self-assembled monolayers, peptides and ECM can be efficiently patterned on various types of flat and curved surfaces using microcontact printing (μCP) (James et al. 1998). This technique facilitates the patterning of several molecules on a substrate using different stamps (Bernard et al. 2000), as well as a molecular gradient using stamps composed of arrays of high-resolution patterns (Crozatier et al. 2006). When using μCP, there are certain difficulties for patterning proteins on structurally soft substrates (e.g., hydrogels) (Damljanovic et al. 2005; Burnham et al. 2006; Rape et al. 2011), such as the stability of the biomolecules (Hynd et al. 2007). Therefore, a modified μCP process called "soft protein lithography" has been developed for patterning applications on hydrogel based surfaces (Polio et al. 2012; Turunen et al. 2013).

Microfluidic patterning refers to another set of soft lithography techniques, through which patterns can be created at desired locations of a substrate by restricting the flow within the microchannels formed by contacting a PDMS stamp on the substrate (Vanapalli et al. 2009). This technique was originally developed using capillary flow, but was further extended to pattern proteins and cells on larger channels (e.g., 100 μm) based on pressure-assisted flows. In this approach, using multi-layer PDMS stamps, it is possible to indirectly pattern different cell types at desired locations on a substrate (Chiu et al. 2000) or to generate heterogeneous multi-layer tissue constructs (Bernard et al. 2000; Vanapalli et al. 2009; Kenis et al. 1999; Jeon et al. 2000).

2.3.3 Bioprinting

Bioprinting has been utilized as a powerful tool to develop microscale engineered tissue constructs (Mironov et al. 2008; Moon et al. 2010b; Xu et al. 2010). Although bioprinting falls under the category of conventional microfabrication, the application of this technology to pattern biomolecules and cells holds unique benefits (Mironov et al. 2008). The major advantages of bioprinting include a fast and automated fabrication process and the development of 3D multi-layered constructs comprised of co-cultures of different cell types on a single substrate (Moon et al. 2010b; Mironov et al. 2003). Various types of bioprinting systems, such as inkjet-based

printing (Nakamura et al. 2005), laser printing (Barron et al. 2004; Nahmias et al. 2005), acoustic cell encapsulation (Demirci and Montesano 2007a) and valve-based printing (Demirci and Montesano 2007b; Song et al. 2010), have been used so far for tissue engineering applications. We refer readers to Chap. 1 of this book for more detail (Tasoglu and Demirci 2013).

2.4 Applications of Microfabrication Technology in Regenerative Dentistry

Teeth are highly mineralized organs used for various purposes, including mastication, phonetics and esthetics (Volponi et al. 2010). Although the morphology of teeth varies by species and location within the oral cavity, there is only slight variation in the composition of teeth, which consists of enamel, dentin, pulp, cementum, and periodontal ligament (PDL) (Yen and Sharpe 2008; Rodriguez-Lozano et al. 2012). Tooth loss due to periodontal disease, caries, trauma, or genetic predisposition remains a global health issue, and can significantly affect quality of life (Marcenes et al. 2013). Current treatment options for missing teeth are prosthetic replacements, such as crowns, bridges, dentures, and dental implants. A potentially attractive strategy for tooth replacement is tooth regeneration through the integration of biomimetic scaffolds, stem cells, cocktails of growth factors and micro- or nano-engineering technologies. It has been previously shown that extracted tooth buds from mouse embryos fully developed with correct orientation, size and morphology after transplanting into the diastema region of adult mice, suggesting that adult oral cavity provides a suitable environment for tooth regeneration (Ohazama et al. 2004). Furthermore, multiple cell types with odontogenic potency, such as dental pulp stem cells (DPSCs) (Gronthos et al. 2000) and stem cells from human exfoliated deciduous teeth (SHED) (Miura et al. 2003), have been identified as potential cell sources for tooth regeneration. These findings thus shed light on potential routes for the creation of bioengineered teeth to replace missing teeth in adults, through various combinations of embryonic tooth primordia and cultured progenitor cells (Nakao et al. 2007). Microfabrication may be particularly beneficial for regenerating the highly organized tissues of the tooth and periodontium since the microenvironment can be precisely controlled. In this section, we outline current accomplishments, challenges, and potential applications of microfabrication techniques in regenerative dentistry.

2.4.1 Regeneration of a Bioengineered Tooth

Odontogenesis is a strictly controlled developmental process that involves epithelial-mesenchymal interactions (O'Connell et al. 2012). To generate a whole tooth with its complex and mineralized load-bearing structures, a precisely-designed scaffold that can guide cell assembly and tissue organization is critical (Hacking and Khademhosseini 2009). With the aid of microfabrication, cell-laden hydrogel constructs can be prepared and spatially arranged with customized functional and architectural features (Khademhosseini et al. 2006). The scaffold-based approach typically involves harvesting, expanding, and differentiating the cells in vitro, seeding them onto pre-fabricated scaffolds, and subsequently implanting them in vivo (Fig. 2.1a) (Yen and Sharpe 2008; Yu et al. 2008).

To date, numerous biomaterials have been used in tooth regeneration studies, such as PGA (Duailibi et al. 2008), poly-L-lactate-co-glycolic acid (PLGA) (Duailibi et al. 2008), and collagen sponges (Fig. 2.1b) (Sumita et al. 2006). In one scaffold-based approach, a bioengineered tooth was generated by recombining and seeding dissociated epithelial cells and mesenchymal cells from an isolated embryonic tooth germ into a collagen gel droplet, and essentially reproducing the reciprocal epithelial-mesenchymal interactions in early odontogenesis (Nakao et al. 2007). To achieve the optimal size and morphology of teeth, a pre-fabricated scaffold that anatomically reflects the natural shape and size of a tooth has also been explored. Kim et al., in particular, used a 3D bioprinting technique to create a life-sized

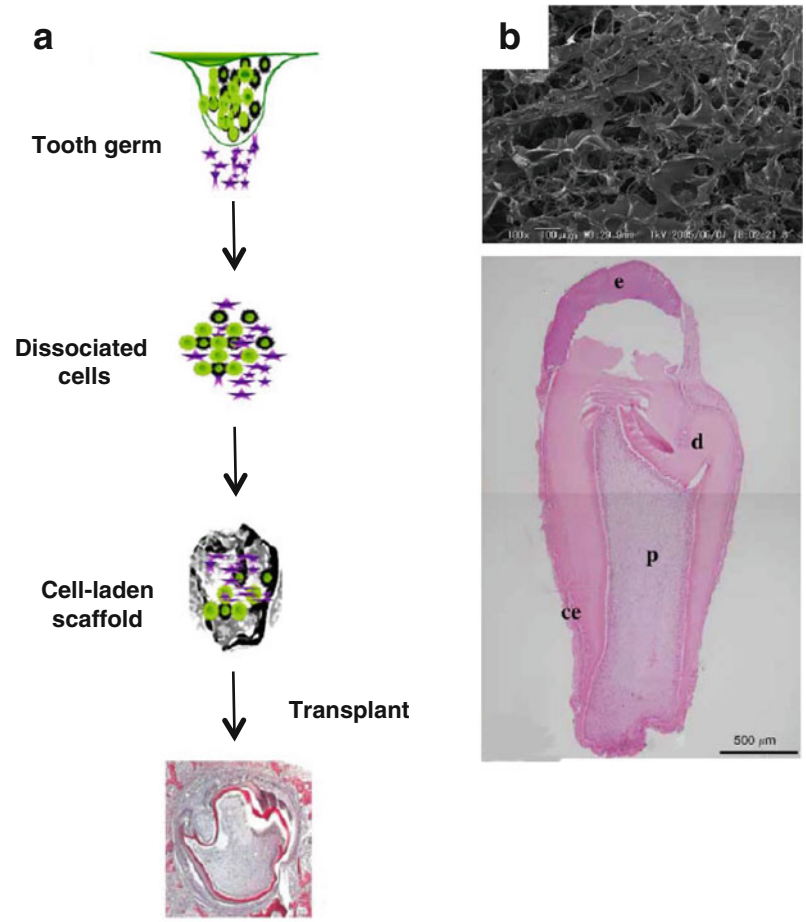

Fig. 2.1 Application of microfabrication technology to regenerative dentistry. (**a**) Schematic diagram of a scaffold-based approach, typically involving harvest of epithelial cells and mesenchymal cells from an embryonic tooth germ, followed by dissociation, recombination, and seeding onto a pre-fabricated tooth-shaped scaffold. Subsequently, the cell-laden construct is transplanted in vivo to generate a bioengineered tooth (Yen and Sharpe 2008) (Adapted from Yen and Sharpe with permission from **Cell and Tissue Research**. Copyright © 2007 Springer). (**b**) Application of microfabrication and novel biomaterials to generate a bioengineered tooth. *Upper panel*: Scanning electronic microscopy of the collagen sponge scaffold (Sumita et al. 2006). *Lower panel*: A bioengineered tooth that imitates anatomic tooth architecture formed 25-week post-transplantation, revealing enamel (*e*), dentin (*d*), cementum (*ce*) and pulp (*p*) (Sumita et al. 2006) (Adapted from Sumita et al. with permission from **Biomaterials**. Copyright © 2006 Elsevier)

tooth scaffold made of poly-ε-caprolactone (PCL) and hydroxyapatite (HA) with 200-μm-diameter interconnecting microchannels (Kim et al. 2010a). Moreover, the infusion of stromal-derived factor-1 (SDF1) and bone morphogenetic protein 7 (BMP7) into the microchannels of the scaffold was shown to recruit significantly more progenitor cells. Taken together, these findings demonstrate the potential of using a scaffold-based approach in regenerative dentistry (Kim et al. 2010b).

It is also important to consider the disadvantages of the scaffold-based approach for tooth regeneration, such as interrupted cell-matrix interactions, compromised biocompatibility, and poor preservation of growth factors within pre-fabricated scaffolds (Yu et al. 2008). However, these issues can be addressed by introducing alternative materials and modes of delivery. Cell-matrix interactions and biocompatibility, for instance, can be improved by using naturally-derived hydrogels, such as collagen and gelatin

(Slaughter et al. 2009). Furthermore, the microencapsulation or binding of critical growth factors to pre-fabricated scaffolds can prevent the unwanted diffusion of ligands (Carrasquillo et al. 2003; Lin et al. 2008), and this technique can potentially be applied for tooth regeneration.

2.4.2 Regeneration of Dental Pulpal Tissues

Regenerative endodontics aims to regenerate the dental pulp, which consists of vital neurovascular tissues. The integration of stem cells, scaffolds, and growth factors provides a promising avenue for revascularization and pulp tissue regeneration (Murray et al. 2007). In a recent study, DPSCs and stem cells from the apical papilla (SCAP) were seeded on a PLGA scaffold, inserted into the root canal spaces of root fragments, which were then implanted subcutaneously into immunocompromised mice. Three to four months after implantation, histological analysis showed pulp-like tissue and vascularization within the root canal spaces, as well as a continuous layer of dentin-like calcified deposition along the canal wall (Huang et al. 2010). While the exogenous application of stem cells is a commonly studied approach, one study suggested that an exogenous source may not be a critical component in regenerative endodontics (Volponi et al. 2010), and that proper vascularization may be sufficient to home progenitor cells into an empty canal for pulp regeneration. Microfabrication techniques have already been used to create vascular networks, and could potentially be used to enhance revascularization of the dental pulp in an organized and efficient manner (Nikkhah et al. 2012b; Morgan et al. 2013). There are, however, no major studies thus far that explore the use of microfabrication techniques for tooth revascularization.

2.4.3 Regeneration of Periodontium

Teeth are supported and anchored by the periodontium, which consists of cementum, periodontal ligament (PDL), gingiva, and alveolar bone. Since tooth loss occurs when these supporting structures are impaired by inflammatory conditions, such as severe periodontal disease, the restoration of these tissues is crucial. While more can be done, microfabrication has already demonstrated useful benefits in various studies of periodontal regeneration. Soft lithography, for instance, has been used to create modified surfaces that encourage periodontium regeneration. Pelaez-Vargas et al. demonstrated that micropatterned silica coatings on dental implant surfaces were biocompatible and notably capable of guiding the adhesion, spreading, and propagation of osteoblast-like cells for periodontal tissues regeneration (Pelaez-Vargas et al. 2011). 3D bioprinting has also been used to design a fiber-based scaffold to facilitate the formation of bone-ligament complexes that mimic the natural anatomy of the periodontium (Park et al. 2014; Ivanovski et al. 2014; Lee et al. 2014). With proper geometrical control, PDL fibers were regenerated in their proper orientation, and anchored in a cementum-like layer on the root surface. A multiphasic scaffold is another approach for regenerating the different components of the periodontium in a cohesive structure, and has only recently been considered (Ivanovski et al. 2014). In one study, 3D bioprinting was used to construct three continuous yet distinct phases: 100-μm microchannels with recombinant human amelogenin for the cementum/dentin interface, 600-μm microchannels with connective tissue growth factor for the PDL, and 300-μm microchannels with bone morphogenetic protein 2 for alveolar bone. The sizes of the microchannels for each phase were specifically chosen based on previous studies in fibro-osseous tissues regeneration. After in vivo implantation of the scaffold, PDL-like collagen fibers were seen inserted into bone-like and cementum-like tissues (Lee et al. 2014). The findings from in vitro and in vivo studies using multiphasic scaffolds, although promising, should be investigated further before large animal and human clinical trials.

Tooth and periodontium possess highly organized and complex structures. In this regard,

microengineering can be particularly useful for creating precisely designed platforms for dental tissue regeneration. Although significant progress has been made thus far in regenerative dentistry, more studies are warranted to eventually offer tooth and periodontium regeneration as a treatment option in a dental practice.

2.5 Applications of Microfabrication Technology in Bone Regeneration

Bone, which contributes to mechanical support and protection of the organism, is a complex mineralized organ containing collagenous fibrous matrix and nanocrystals of carbonated apatite (Weiner and Wagner 1998; Nguyen et al. 2012). In addition, bone plays critical biological roles in our bodies, such as ion homeostasis and hematopoiesis (Confavreux et al. 2011). Current medical management for severe bone damage consists of bone grafts (autografts, allografts, or xenografts) (Finkemeier 2002); however, several limitations exist due to the limited accessibility of graft materials, the morbidity of the donor sites, and potential for transmission of infectious pathogens (Simonds et al. 1992; Dimitriou et al. 2011). To eliminate these complications and to improve clinical outcome, novel biocompatible materials have been investigated for bone tissue engineering (Baler et al. 2014). These materials include: collagen (Geiger et al. 2003), calcium phosphate (Bucholz et al. 1989), ceramics and cements (Dorozhkin 2010), bioglasses (Bohner 2000), bioactive glass ceramics (Kinnunen et al. 2000), and a hybrid of PCL and nanocrystalline silicon-substituted hydroxyapatite (nano-SiHA) (Meseguer-Olmo et al. 2013). Studies have shown that SiHA possesses great bioactive behavior for bone formation (Porter et al. 2003), and that the addition of nanocrystalline ceramic particles can further enhance its biomineralization (Meseguer-Olmo et al. 2013). These nanocrystalline ceramic particles exhibit higher surface areas, and therefore have an enhanced dissolution rate and reactivity in contact with the surrounding tissue fluids (Meseguer-Olmo et al. 2013). In addition to the chemical compositions of the scaffolds, overall architecture of the constructs (e.g., density, pore shape, and pore size) and osteo-inductive biomolecules (e.g., BMP family members) rank among the other important qualities that encourage bone regeneration (Torricelli et al. 1999; Hutmacher 2000).

In addition to biocompatibility, biodegradability and accessibility, an ideal biomaterial for bone tissue engineering should meet other criteria, such as low viscosity for bioinjection and micromolding, and a capacity for incorporating cells or growth factors (Nguyen et al. 2012; Nguyen and Lee 2010). Injectable hydrogels, such as calcium alginate containing nano-HA (Tan et al. 2009) and nano-HA/PEG-PCL-PEG hydrogel nanocomposites (Fu et al. 2009), possess tunable injectability, degradability and setting time, and demonstrate in situ gelation activity. As a result, these biomaterials have been fabricated for bone tissue engineering, and several strategies have been employed to further enhance the calcification and mechanical strength of cell-laden hydrogel constructs. The incorporation of inorganic phases (e.g., calcium phosphates and bioglasses) into hydrogels is a common method to provide nucleation sites and induce physiological biomineralization (Kamitakahara et al. 2008; Gkioni et al. 2010; Rea et al. 2004). Use of a polymeric hydrogel backbone with anionic functional groups (e.g. $PO4^{3-}$, -COOH, and -OH groups), as well as incorporation of growth factors and osteoblast-like cells have also been suggested options to induce mineralization (Gkioni et al. 2010). Moreover, the degradation of hydrogel-based biomaterials allows for the replacement with newly-formed bone and for integration with surrounding native bone, thus increasing overall mechanical stability (Rezwan et al. 2006).

Microfabrication techniques have been utilized to introduce physiochemical cues within the 3D microenvironment (e.g., size, shape, porosity, stiffness, roughness and topography), and to influence the behavior of mesenchymal stem cells (MSCs) (Jiang et al. 2005). In recent studies, using photolithography, photoreactive PVA was

grafted on the polystyrene surfaces to construct micropatterns and provide a biocompatible platform for the long-term culture of MSCs. Bone marrow-derived MSCs were then cultured on these precisely-defined micropatterned PVA surfaces to investigate the effects of surface charge, cell spreading, seeding density and cell-cell interactions on MSC fate determination, including adipogenic, chondrogenic and osteogenic differentiation (Fig. 2.2a) (Wang et al. 2013; Lu et al. 2009; Song et al. 2011; Nedjari et al. 2014). In addition, micropatterning was utilized for studies in a single-cell level to reduce the heterogeneity of MSCs (Chen 2014). The findings from this study suggested that minimal cell-cell interactions, large cell spreading area, and increased contractility favored the osteogenic differentiation of MSCs (Wang et al. 2013; Chen 2014). μCP of biologically relevant ligands within cell-laden hydrogel constructs is another promising approach to achieve spatial control of ligand distribution (Park and Shuler 2003; Corum et al. 2011). The flexibility of pattern designs (e.g., shape and size) allows the micropatterned 3D co-cultures of cells, further facilitating cell proliferation and differentiation (Torisawa et al. 2010). μCP has also been applied to generate the micropatterns of bioactive glass nanoparticles on chitosan membranes, thereby regulating ionic release from these bioactive glass nanoparticles, maintaining the local pH value within the microenvironment, and enhancing biomineralization (Luz et al. 2012).

In addition, 3D bioprinting techniques have been demonstrated to develop cell-laden scaffolds that exhibit anatomically-shaped architecture, porosity and thickness for bone regeneration (Fig. 2.2b) (Meseguer-Olmo et al. 2013; Fedorovich et al. 2007; Murphy and Atala 2014). To achieve zonal distribution of multiple cell types, bioengineers have injected cell-laden hydrogel constructs that are gelled in situ or photopolymerized in layers, recapitulating the structures of native bone tissue (Fedorovich et al. 2007). Matrix assisted pulsed laser evaporation direct write (MAPLE DW) has been utilized for direct writing biomaterials and cells (Fig. 2.2c) (Schiele et al. 2010; Doraiswamy et al. 2007). This technique provides high accuracy in terms of spatial resolution, and improves cell-material integration. Co-deposition of osteoblast-like cells (MG63 cells) and bioceramic scaffold materials using the MAPLE DW strategy demonstrated that MG63 cells retained the viability and the capacity for proliferation, indicating this effective strategy can potentially be employed in cell-laden scaffolds for bone tissue engineering (Doraiswamy et al. 2007). Bottom-up approaches applying assembly of PCL and starch–PCL microfabricated sheets with human bone marrow stem cells allowed precisely imprinting microvasculatures and micropores (Lima et al. 2014).

2.6 Applications of Microfabrication Technology in Cartilage Regeneration

Cartilage tissue creates a nearly frictionless surface for joints to move and slide freely, but it may experience undesirable excessive forces and trauma (Jackson et al. 2001; Kim et al. 2012). The degeneration of articular cartilage and the associated osteoarthritis are one of the most prevalent age-related chronic conditions in the United States, affecting approximately 80 % of people older than 75 years old (Jackson et al. 2001). Disability caused by cartilage damage is an economic burden to the health care system, with a direct medical cost of roughly $15 billion each year (Jackson et al. 2001). Due to the avascularity and low mitotic activity of cartilage, it has a particularly limited capacity for self-healing when damaged (Buckwalter and Mankin 1998). The mainstay of treatment to repair damaged cartilage is still surgical intervention, such as arthroscopic lavage/debridement, autologous chondrocyte implantation, and osteochondral grafting (Kim et al. 2012). These surgical options provide temporary symptom relief and improve joint functions, but fail to fully restore damaged cartilage tissue (Kim et al. 2012). To address this limitation, bioengineering-based alternatives have been proposed to create an appropriate microenvironment and to regenerate cartilage tissue through the incorporation of cells, biochemical factors, and biomaterials (Petersen et al. 2002).

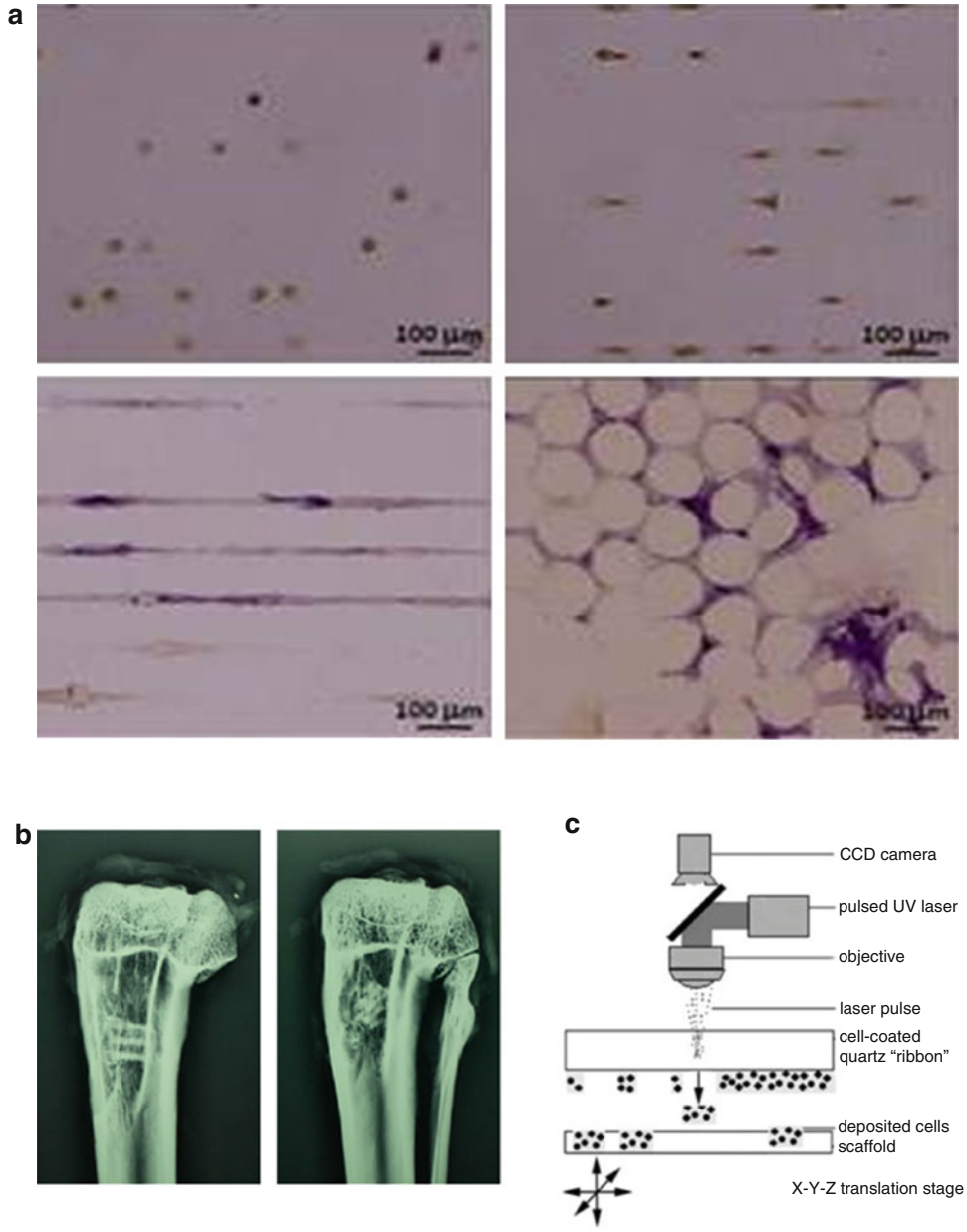

Fig. 2.2 Application of microfabrication technology to bone regeneration. (**a**) MSCs were cultured on the micropatterned surfaces for 2 weeks for osteo-induction. The Alkaline phosphatase (ALP) assay was used to investigate how different surface micropatterns influence osteogenic efficiency. *Purple* and *brown* colors indicate positive and negative staining for ALP, respectively (Wang et al. 2013) (Adapted from Wang et al. with permission from **Journal of Biomedical Materials Research**. Copyright © 2013 Wiley Periodicals, Inc). (**b**) Radiographic analyses of cell-laden construct implantation for bone regeneration. *Left panel*: At outset. *Right panel*: After 4 months (Meseguer-Olmo et al. 2013) (Adapted from Meseguer-Olmo et al. with permission from **Journal of Biomedical Materials Research**. Copyright © 2012 Wiley Periodicals, Inc). (**c**) Schematic diagram of the MAPLE DW process (Doraiswamy et al. 2007) (Adapted from Doraiswamy et al. with permission from **Journal of Biomedical Materials Research**. Copyright © 2006 Wiley Periodicals, Inc)

MSCs are an attractive cell source for cartilage regeneration due to their potential for chondrogenic differentiation under specific culture conditions (e.g., supplementation with transforming growth factor β [TGFβ]) (Pittenger et al. 1999; Diekman et al. 2010; Lai et al. 2013). Moreover, MSCs tend to commit to a chondrogenic fate when encapsulated in micropatterned constructs with high seeding density (Gao et al. 2010). In this regard, hydrogels can be applied as either cell-laden constructs to promote cartilage regeneration or cell-free implants to replace damaged cartilage (Spiller et al. 2011; Scaglione et al. 2014). Naturally-derived hydrogels, such as hyaluronic acid (Spiller et al. 2011) and elastin-like polypeptides (Mauck et al. 2000), are particularly appealing candidates due to their compositional similarity to cartilage ECM (Cushing and Anseth 2007). Furthermore, these naturally-derived hydrogels are able to enhance chondrogenic differentiation and proliferation (Spiller et al. 2011). However, these types of hydrogels are mechanically weak, and thus have limited use as cell-free implants for cartilage replacement (Spiller et al. 2011). Therefore, hybrid hydrogels consisting of natural and synthetic polymers have been suggested to strengthen the compressive and wear properties of constructs (Neves et al. 2011; Nguyen et al. 2011; Liao et al. 2013). In a study by Yasuda et al., double-network hydrogels of poly (2-acrylamido-2-methylpropane sulfonic acid) and poly(N,N-dimethyl acrylamide) were developed to imitate the collagen and glycosaminoglycan components of cartilage. These hydrogels exhibit low friction coefficients, and the compressive moduli are similar to articular cartilage (Yasuda et al. 2005). As the ECM of hyaline cartilage is a fiber-reinforced composite material, various kinds of composite hydrogels have been developed to mimic the mechanical and physical characteristics of native cartilage (Marijnissen et al. 2002; Chen et al. 2003; Ameer et al. 2002; Slivka et al. 2001). Slivka et al. developed PLGA hydrogels reinforced with polyglycolic acid fibers with mechanical properties in the range of native cartilage as a function of materials ratio (Slivka et al. 2001).

Since chondrocytes lose their phenotype and become fibroblast-like cells when cultured in vitro on traditional 2D cell culture substrates (Freed et al. 1999), it is important to control cell-cell and cell-ECM interactions, and to maintain the chondrogenic features of the cell-laden constructs (Petersen et al. 2002). With micropatterning techniques, it is possible to develop a well-defined substrate that can guide the chondrogenic differentiation of progenitor cells. Surface-patterned scaffolds that were prefabricated to support chondrogenesis demonstrated the capacity to promote adhesion, restrict spreading, maintain chondrocytic phenotypes and support the deposition of cartilage ECM (e.g., aggregan) (Petersen et al. 2002). In another study employing PEG hydrogels and photolithography, 2D microarrays of cell-adhesive circular domains ($\phi = 100$ μm) surrounded by PEG-coated cytophobic areas were constructed to promote the aggregation and spheroid formation of chondrocytes (Otsuka et al. 2012). This approach demonstrated its capacity to stimulate aggrecan production, and to maintain the chondrocytic spheroids for more than a month (Otsuka et al. 2012).

In addition to micropatterning techniques, 3D cell-laden biomimetic microengineered constructs can be used to imitate the architectural and mechanical characteristics of target organs or tissues (e.g., cartilage) (Klein et al. 2009). Articular cartilage exhibits zonal maturation with variations in cell phenotype, matrix organization, composition and mechanical properties along the depth of the cartilage plate. Multi-layered photocrosslinkable hydrogels can be used to recreate the biomimetic zonal maturation of articular cartilage (Nguyen et al. 2011; Sharma et al. 2007). Photodegradable PEG-based hydrogels were applied to encapsulate stem cells, and post-gelation control of the constructs was demonstrated to introduce dynamic temporo-spatial changes and to affect cell migration and chondrogenic differentiation (Kloxin et al. 2009). In another study by Xu et al., a hybrid bioprinting/electrospinning approach was utilized to develop layer-by-layer chondrocyte-laden fibrin/collagen hydrogel constructs for cartilage tissue engineering. Compared to the conventional bioprinting

method, the proposed hybrid approach enhanced mechanical properties of the constructs, maintained cell viability, and induced the deposition of cartilage ECM both in vitro and in vivo (Xu et al. 2013). Further refinement of this hybrid technique to produce oriented fibers is envisioned to guide chondrocyte growth and to construct functional cartilage tissues.

2.7 Conclusion and Future Perspectives

During the past decade, significant progress in microfabrication and biomaterial science has been made in developing complex biomimetic transplantable constructs that can guide cell growth and differentiation as well as tissue organization. However, challenges still remain, such as achieving the precise control of 3D cell-laden constructs, dynamic changes in microenvironment, and in the enhancement of revascularization. The development of improved scaffolds with customized physical characteristics is critical, and microfabrication with higher resolution is likely to prove important. Innovative and optimized microfabrication techniques are essential for enriching specific biological functions, such as cell seeding and vascularization, as well as for facilitating the natural healing process in vivo. Beyond advances in bioengineering, it is also attractive to incorporate biochemical cues within 3D cell-laden constructs. A thorough understanding of the underlying biological mechanisms for these load-bearing organs development is thus a necessary pre-requisite. The integration of biological insights and bioengineering technologies will help to significantly advance the field of regenerative medicine.

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Electrospinning of Bioinspired Polymer Scaffolds

3

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Abstract

Electrospinning is a technique used in the production of polymer nanofibre meshes. The use of biodegradable and biocompatible polymers to produce nanofibres that closely mimic the extracellular matrix (ECM) of different tissues has opened a wide range of possibilities for the application of electrospinning in Tissue Engineering. It is believed that nanofeatures (such as voids and surface cues) present in nanofibre mesh scaffolds, combined with the chemical composition of the fibres, can stimulate cell attachment, growth and differentiation. Despite the widespread use of electrospun nanofibres in tissue engineering, the present chapter will focus on the advances made in the utilisation of these materials in bone, cartilage and tooth related applications. Several aspects will be taken into consideration, namely the choice of polymers, the surface modification of the nanofibres in order to achieve mineralisation, and also the biological application of such materials.

Keywords

Electrospinning • Scaffolds • Tissue engineering • Bone regeneration • Biofabrication • Nanofibers

3.1 Introduction

Electrospinning as it is known today was initially mentioned in 1934 (Anton 1934). The technique consists of the application of high voltages to a droplet of a polymer solution passing through a metallic needle under constant flow (Fig. 3.1). At a specific electric field, the solution is continuously ejected from the tip of the needle towards a collector. The elongation of the polymer chains

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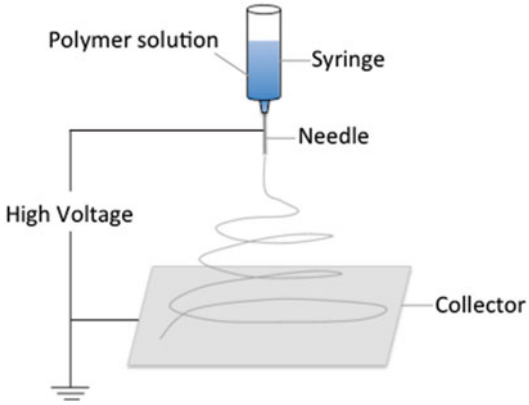

Fig. 3.1 Electrospinning setup

during this process leads to the deposition of randomly aligned thin fibre meshes on the collector. Several factors have to be taken into account when elaborating a project to obtain electrospun polymer nanofibre meshes. For instance, polymer nature and molecular weight, solvent, solution concentration, applied voltage and distance between the tip of the needle and the collector surface are known to strongly affect the diameter of the fibres produced (Martins et al. 2007).

Initially, electrospun fibres found their application in the fabrication of textile threads (Anton 1944; Lee and Obendorf 2007; Zhang et al. 2004) and filtration membranes (Gopal et al. 2006; Qin and Wang 2006; Ramakrishna et al. 2010). Although some patents have been published, only a few groups were developing research on electrospinning during most of the second half of the twentieth century. This scenario changed considerably in the 1990s with the advent of Tissue Engineering as a new field of research. Substantial attention has been given to electrospun nanofibre meshes as tissue engineering requires the production of biomaterials that can mimic the structure of tissues and organs. Polymer electrospun fibres are architecturally similar to the collagen nanofibrils found in most of the extracellular matrices (ECMs) of tissues (Min et al. 2004a).

Studies suggest that cell adhesion and proliferation can be regulated by both architecture and topography of biomaterials (Flemming et al. 1999; Green et al. 1994; Recum et al. 1996; Badami et al. 2006). Comparatively to structures containing smooth surfaces, electrospun nanofibre meshes have been reported to be more efficient than non-fibrous biomaterials in promoting proliferation, spreading and orientation of cells when used as scaffolds for tissue engineering (Badami et al. 2006). Such behaviour is attributed to topographical cues and distribution of the fibres in the meshes. In addition, the structure of the electrospun fibres, along with the high porosities of the meshes, meet the requirements expected in an ideal scaffold (Li et al. 2002). Therefore, there is a crescent number of publications on the use of electrospun fibres in the regeneration of tissues, for example cartilage (Moroni et al. 2008; Thorvaldsson et al. 2008), nerve (Ghasemi-Mobarakeh et al. 2008; Prabhakaran et al. 2009) and muscle (Choi et al. 2008; Mo et al. 2004; Riboldi et al. 2005), and also organs, such as skin (Kumbar et al. 2008; Zhou et al. 2008; Zhu et al. 2008), heart (Amoroso et al. 2012; Van Lieshout et al. 2006; Zong et al. 2005) and bladder (Ajalloueian et al. 2014a; Stankus et al. 2008). Despite the widespread use of electrospun fibres in tissue engineering, the present chapter will highlight the recent advances in the development of electrospun-based materials with potential application in the treatment of cartilage and mineralised tissues, such as bone and teeth. These areas of research have presented enormous advances in the past few years with the development of electrospun-based bioactive composites and also with the introduction of straightforward procedures to modify the surfaces of electrospun fibres. Therefore, the polymers used, as well as their surface and bulk modification to increase bioactivity, will be described. The biological assessment of the fibres will also be discussed in order to provide the reader with a complete insight on the utilisation of these materials in cartilage, bone and dental related applications.

3.2 Polymers

While it is not the objective of this chapter to describe in detail the properties of the biodegradable polymers used in the production of electrospun fibres, it is important to understand the

differences between natural and synthetic polymers. Synthetic biodegradable polymers were more commonly used within the biomedical engineering field, as it was easier to replicate their chemical structure and their mechanical properties than with natural ones. Nowadays, the development of newer ECM-derived gels with well controlled physical and mechanical characteristics, along with enhanced biological properties, has helped recent wide-spreading of natural polymers in this field. Polyesters such as poly(caprolactone) (PCL), poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are amongst the most common synthetic polymers used for biomedical purposes. These polymers also possess high solubility in organic volatile solvents, such as chloroform and dichloromethane, which facilitates the production of fibres during electrospinning. Different synthetic polymers exhibit different structural, mechanical and degradation properties (Khan et al. 2008). A high molecular weight results in slower degradation and improved mechanical strength (Braunecker et al. 2004). However, synthetic polymers present some drawbacks, such as high cost, absence of bioactivity (Hsu et al. 2004) and release of acid residues during degradation (Bueno and Glowacki 2009). Polymers of natural origin allow the design of biomaterial systems that function at the molecular level and often help minimise the risk of chronic inflammation and rejection. The main advantages of the natural polymers are their chemical versatility, low immunogenic potential and, in some cases, the ubiquitous nature of their sources. Additionally, these polymers have natural bioactive motifs, such as RGDs, which tend to enhance cell activity. Natural biodegradable polymers are obtained either from animal or vegetable sources. Chitosan, hyaluronic acid and collagen are just a few examples of natural polymers. However, the relatively poor mechanical properties of those polymers require their combination with more mechanically resistant materials, such as calcium phosphates, depending on the application envisioned. As for electrospinning, natural polymers are generally soluble in non-volatile aqueous solutions, which may require adaptation in the electrospinning process, such as increase in tip-to-collector distance, in order to produce fibres. Table 3.1 summarises the most common natural and synthetic polymers used in the production of electrospun fibres for tissue engineering-related applications. The solvents used in the preparation of the polymer solutions and the range of distribution of the fibre diameter are also presented.

Electrospun fibre meshes of polymer blends have also been produced. For instance, the effects of the blending ratio of PLA and PCL on fibre morphology have been studied (Lu et al. 2012). Results showed that fibres containing lower quantities of PCL had smoother surfaces. Phase separation was observed within the fibre and attributed to the immiscibility between PLA and PCL. The choice of solvents was also observed to play an important role on fibre morphology. In another study, blends of PCL and poly(glycolic acid) (PGA), with varied PGA/PCL ratios were produced (Aghdam et al. 2012). The increase in fibre diameter with increasing amounts of PGA was observed. Contrary to the PLA/PCL blends, the PGA/PCL one possessed a high degree of miscibility as demonstrated by thermal analysis. Blends containing mixtures of natural and synthetic polymers (Choi et al. 2008; Ajalloueian et al. 2014b; Jeong et al. 2013; Yin et al. 2013; Chakrapani et al. 2012; Daranarong et al. 2014; Jing et al. 2014; Kolbuk et al. 2013; Lee et al. 2012; Duan et al. 2006; Schnell et al. 2007; Venugopal et al. 2006) as well as between natural polymers (He et al. 2013; Lai et al. 2014; Zhang and Mo 2013; Chen et al. 2008, 2010; Yeo et al. 2008; Zhong et al. 2005) have been obtained.

3.3 Composites and Hybrid Materials

Materials used in the regeneration of mineralised tissues are expected to present bioactivity. In other words, the formation of a chemical bonding between the material surfaces and the surrounding tissue is desired and considered a crucial factor during tissue regeneration. It is observed that, in the lack of such an interaction, the material implanted can either induce acute inflammation

Table 3.1 Polymers and solvents most commonly used in the preparation of electrospun nanofibres

Polymer	Solvent	Fibre diameter (nm)
Chitosan[a]	Acetic acid (Geng et al. 2005; Homayoni et al. 2009; De Vrieze et al. 2007), HFP (Min et al. 2004b), TFA/DCM (Sangsanoh and Supaphol 2006)	100–290 (Geng et al. 2005), 89–191 (Homayoni et al. 2009), 90–480 (Min et al. 2004b), 120–140 (Sangsanoh and Supaphol 2006), 25–115 (De Vrieze et al. 2007)
Collagen[a]	HFP (Matthews et al. 2002; Rho et al. 2006; Zhong et al. 2006; Powell et al. 2008)	250 (Matthews et al. 2002), 100–1,200 (Rho et al. 2006), 250 (Zhong et al. 2006), 130–4,500 (Powell et al. 2008)
Gelatin[a]	TFE (Huang et al. 2004; Zhang et al. 2006), formic acid (Ki et al. 2005), acetic acid/ethyl acetate/distilled water (Sisson et al. 2010)	100–340 (Huang et al. 2004), 200–300 (Zhang et al. 2006), 70–170 (Ki et al. 2005), 70–150 and 490–710 (Sisson et al. 2010)
Hyaluronic acid[a]	DMF (Yao et al. 2013), DMF (with salt aqueous solutions) (Brenner et al. 2013), water/formic acid/DMF (Liu et al. 2011), ammonium hydroxide/DMF (Brenner et al. 2012)	33–113 (Yao et al. 2013), 88–110 (Brenner et al. 2013), 30–50 (Liu et al. 2011), 32 (Brenner et al. 2012)
PCL[b]	Chloroform (Yoshimoto et al. 2003), methylene chloride/DMF (Lee et al. 2003), chloroform/DMF (Araujo et al. 2008)	20–5,000 (Yoshimoto et al. 2003), 200–5,500 (Lee et al. 2003), 250–2,500 (Araujo et al. 2008)
PGA[b]	HFP (Boland et al. 2001, 2004	110–1,190 (Boland et al. 2001), 220–880 (Boland et al. 2004)
PLA[b]	HFP (Badami et al. 2006), DCM/DMF (70:30) (Yang et al. 2005; Xu et al. 2004)	246 (Badami et al. 2006), 150–3,000 (Yang et al. 2005), 235–3,500 (Xu et al. 2004)
PLGA[b]	Tetrahydrofuran/DMF (1:1) (Li et al. 2002; Xin et al. 2007; Zhao et al. 2008), HFP (Kim et al. 2010)	500–800 (Li et al. 2002), 550–970 (Xin et al. 2007), 100–200 and 400–1,000 (Zhao et al. 2008), 290–770 (Kim et al. 2010)
Silk fibroin[a]	Formic acid (Min et al. 2004a; Zhang et al. 2012a; Cestari et al. 2014; Cho et al. 2012), hexafluoroacetone (Ohgo et al. 2003), TFA (Andiappan et al. 2013)	30–120 (Min et al. 2004a), 100–1,000 (Ohgo et al. 2003), 50–300 (Zhang et al. 2012a), 160–250 (approximate) (Cestari et al. 2014), 600–800 (Andiappan et al. 2013), 100–800 (Cho et al. 2012)

PCL poly(ε-caprolactone), *PGA* poly(glycolic acid), *PLA* poly(lactic acid), *PLGA* poly(lactic-co-glycolic acid), *HFP* 1,1,1,3,3,3-hexafluoro-2-propanol, *TFA* trifluoroacetic acid, *DCM* dichloromethane, *TFE* 2,2,2-trifluorothanol, *DMF* N,N-dimethylformamide

[a]Natural polymer

[b]Synthetic polymer

or be coated with collagen fibrils, thus losing its capacity to support cell attachment and growth. Bioactive substances, such as bioactive glasses and calcium orthophosphates, are generally combined with the polymer fibres to form bioactive composites. Composites may be obtained by either mixing or coating the fibres with the bioactive substances. In the latter case, chemical surface modification is generally required.

3.3.1 Electrospun Fibre Reinforcement with Bioactive Substances

Several papers describe the production of electrospun fibres reinforced with hydroxyapatite (HA). Li et al. (2006) developed a new method that combines silk and hydroxyapatite nanoparticles (nHAP) in the production of electrospun

nanofibre composites. In that approach, nHAP was suspended in silk fibroin/polyethylene oxide (PEO) aqueous blending solutions. PEO works as a fibre-forming additive that increases solution electrospinnability. The formation of nHAP aggregates was minimised by addition of phosphate buffer to the polymer solution. Although the presence of nHAP has been confirmed by FTIR, no influence of the nanoparticles on fibre diameter and morphology, as well as on the mechanical properties of the meshes, has been discussed. In a similar approach, nanofibres of chitosan containing nHAP have been produced (Zhang et al. 2008). Contrary to the procedure developed by Li et al. (2006), nHAP was initially co-precipitated with chitosan (CS) rather than simply added to the polymer solution. The composite resulting from co-precipitation was mixed, along with PEO, with an acetic acid/dimethyl sulfoxide (DMSO) solution to dissolve the polymer. The resulting suspension was then used in the production of electrospun fibres. The molecular weight of PEO appeared to have great influence on the production of the fibre meshes. In fact, the production of electrospun fibre meshes free of beads was only achieved when ultra-high molecular weight PEO was used. PLA nanofibres have also been successfully reinforced with nHAP particles. Kim et al. (2006a) produced electrospun PLA fibres containing nHAP obtained via a sol-gel method. The nanoparticles were dispersed in the PLA solution before electrospinning. Hyroxysteric acid, an amphiphilic surfactant, was introduced to improve the interaction between hydrophilic nHAP and hydrophobic polymer. Ceramic/polymer nanofibre mesh composites containing nHAP particles uniformly distributed in the PLA matrix were obtained. Other bioactive compounds, such as β-tricalcium phosphate (β – TCP) (Kim and Kim 2012, 2014; Baykan et al. 2014; Patlolla and Arinzeh 2014; Zhang et al. 2012b; Bianco et al. 2011), bioactive glass (Silva et al. 2013; Lin et al. 2012; Allo et al. 2010; Kim et al. 2008) and amorphous calcium phosphates (ACPs) (Ma et al. 2011; Buschmann et al. 2012) have also been proposed in the reinforcement of electrospun polymer fibres. Table 3.2 shows different polymer electrospun fibres and their respective bioactive fillers used in the production of composites. The particle sizes of the reinforcement agents, as well as the distribution of fibre diameters are presented.

Table 3.2 Reinforcement agent used in the preparation of electrospun-based composites

Polymer	Reinforcement agent (average size/size distribution in nm)	Average diameter/diameter distribution of the reinforced electrospun fibres (nm)
Chitosan	HA (<200) (Liverani et al. 2014)	<350 (Liverani et al. 2014)
Collagen	HA (32) (Ji et al. 2012), HA (15) (Stanishevsky et al. 2008), HA (5) (Teng et al. 2008)	250–350 (Ji et al. 2012), 500 (Stanishevsky et al. 2008), 60 (Teng et al. 2008)
Gelatin	β-TCP (200) (Zhang et al. 2012b), bioactive glass (Gao et al. 2013)	192 (Gao et al. 2013)
PCL	β-TCP (<25,000) (Baykan et al. 2014), β-TCP (500) (Bianco et al. 2011), β-TCP/HA (100) (Patlolla and Arinzeh 2014), bioactive glass (Lin et al. 2012; Allo et al. 2010)	800–1,000 (Baykan et al. 2014), 1,000–2,000 (Bianco et al. 2011), 2,500 (Patlolla and Arinzeh 2014), 200–700 (Lin et al. 2012), 220–420 (Allo et al. 2010)
PLA	HA (35) (Kim et al. 2006a), ACP (20–80) (Ma et al. 2011), bioactive glass	1,000–2,000 (Kim et al. 2006a), 200–400 (Ma et al. 2011)
PLGA	ACP (22) (Buschmann et al. 2012), HA (<200) (Krucinska et al. 2014; Lee et al. 2010), HA (267) (Lao et al. 2011)	<7,000 (Buschmann et al. 2012), 460 (Krucinska et al. 2014), 600–800 (Lee et al. 2010), 494–537 (Lao et al. 2011)
Silk fibroin	HA (nano) (Kim et al. 2014a), HA (30–60) (Sheikh et al. 2013)	480 (Kim et al. 2014a), <273 (Sheikh et al. 2013)

PCL poly(ε-caprolactone), *PGA* poly(glycolic acid), *PLA* poly(lactic acid), *PLGA* poly(lactic-co-glycolic acid), *HA* hydroxyapatite, *β-TCP* β-tricalcium phosphate, *ACP* amorphous calcium phosphate

3.3.2 Surface Mineralisation of Electrospun Nanofibres

Polymer electrospun nanofibres can also be coated with a bioactive HA layer. The HA deposition occurs spontaneously when bioactive materials are immersed in solutions that are supersaturated with respect to HA, such as human blood plasma (Kokubo 1991; Gamble and Harvard Medical S 1941) and the simulated body fluid (SBF) (Kokubo et al. 1990; Kokubo and Takadama 2006). SBF is a solution with ionic concentration very similar to that of blood plasma (Kokubo et al. 1990). SBF is normally used to either test the bioactivity of a material, i.e. to verify if a material induces the spontaneous precipitation of HA, or to biomimetically coat known bioactive materials with an apatite layer similar in composition to the inorganic phase of bone. Although the efficiency of the SBF approach in predicting the bioactivity of materials is controversial (Bohner and Lemaitre 2009), mainly due to the limitations in the SBF composition (e.g. lack of proteins known to mediate apatite deposition in vivo), this method is still widely used due to its simplicity and ease of preparation.

Due to their hydrophobic nature, most polymer electrospun fibres need to have their surfaces modified in order to allow HA deposition. Overall, surface modification strategies aim to "activate" specific functional groups, such as hydroxyl and carboxyl groups, on the electrospun polymer fibres. These groups will increase polymer hydrophilicity and facilitate their interaction with ions in solution, thus contributing to the in situ deposition of HA crystals. The functional groups can be exposed by either chemical treatment using, for example, alkaline solutions, or by plasma treatment. In that context, Araujo et al. (2008, 2010) treated the surfaces of PCL nanofibre meshes with diluted sodium hydroxide solutions. The fibres were subsequently immersed in solutions containing calcium and phosphate ions, for calcium phosphate nucleation, and then soaked in 1.5× SBF (SBF containing 1.5 times the ionic concentration of blood plasma) to form bone-like HA layer. The mechanism of HA precipitation initially involved the interaction of calcium ions with carboxyl groups on the fibre surfaces. Subsequent interaction with phosphate ions in solution resulted in the formation of ACP that functioned as nuclei for the HA growth. The HA layer was observed to homogeneously coat the fibre surfaces while keeping fibre morphology and mesh porosity. The main advantage of such a method was the fast coating of the fibres, which was observed to occur after just a few hours of immersion in SBF. In addition, the thickness of the inorganic coating could be controlled by the time of immersion in 1.5× SBF. Coated fibres presented increased hydrophilicity, which may facilitate ion exchange and interaction with surrounded tissue after implantation. Figure 3.2 shows scanning electron microscopy (SEM) images of PCL electrospun fibres coated with HA according to the protocol developed by Araujo et al. (2008). Yang et al. (2008) described the coating of electrospun PCL fibres with HA after argon plasma treatment. Fibre mineralisation was observed to occur within 2 h of immersion in 10× SBF (containing 10 times the ionic concentration of blood plasma). Although samples were homogeneously coated in a brief space of time, supersaturated solutions like 10× SBF solutions may require careful handling in order to prevent spontaneous precipitation, which may affect their ionic strength and thus the efficiency of coating. PLA electrospun fibres have also been coated with HA after plasma treatment (Seyedjafari et al. 2010). Contrary to the procedure described previously for PCL, oxygen was chosen for plasma formation rather than argon. The higher oxidant capacity of the gas drastically increased fibres hydrophilicity due to the formation of high densities of anionic groups on their surfaces. The HA coating resulted from the overnight immersion of the plasma treated fibres in nHAP aqueous suspension. Despite the HA particles could be observed by SEM, this procedure is not as efficient as the SBF mineralisation process as nHAP particles may detach from the fibre surfaces due to inhomogeneous coating.

The role of functional groups in promoting apatite deposition has been observed. In an interesting study, Cui et al. (2010) investigated the

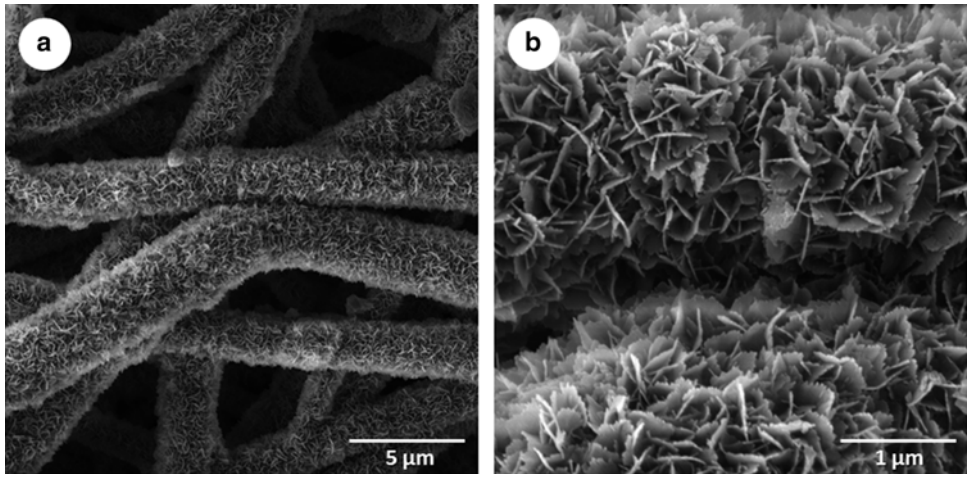

Fig. 3.2 SEM images of HA-coated poly(ε-caprolactone) electrospun fibres (**a**) and detail of the HA layer (**b**)

apatite nucleation capacity of PLA electrospun fibres functionalised with carboxyl, hydroxyl and amino groups, and their mixtures. Chemical groups were introduced in the polymer chain through specific chemical reactions. Electrospun modified-PLA fibres were soaked in SBF at 37 °C for different time periods. HA nucleation seemed to be favoured by higher contents of carboxyl groups, as well as by different ratios of hydroxyl/carboxyl and amino/hydroxyl/carboxyl groups with predominant amounts of carboxyl groups. As discussed previously, the mechanism for HA deposition was attributed to electrostatic interactions between the calcium and phosphate ions in the SBF solution and the ionically charged functional groups on the PLA fibres.

All of the composites described above improved osteoblast growth and viability during biological studies in vitro. The influence of HA on cell behaviour will be discussed in more details in Sect. 3.7.

3.4 Ceramics

The production of electrospun nanofibre meshes composed of ceramic materials, such as bioactive glasses and HA, has been proposed (Asgharnia and Alizadeh 2013; Kim et al. 2006b; Gao et al. 2011; Dai and Shivkumar 2007a; Hong et al. 2010; Xia et al. 2007). In general, a polymer solution containing ceramic precursors is used as carrier for the formation of the ceramic fibres. After the electrospinning process, the polymer is removed from the electrospun fibres by thermal treatment. Such a procedure was initially proposed by Kim et al. (2006b) in the production of bioactive glass nanofibres. Bioactive glasses were originally developed in the 1970s (Hench and Paschall 1973; Ducheyne et al. 1979; Hench et al. 1977) and are currently widely used in bone regeneration due to their bioactivity and capacity to induce cell attachment and proliferation. In comparison to other glass structures, nanoscaled electrospun bioactive glasses may present higher bioactivity, thanks to their elevated surface area to volume ratio, in addition to displaying a better interfacial adhesion when used as reinforcement of resins in the preparation of different materials, such as dental composites (Li et al. 2014a). A sol-gel approach was initially used in the production of glass nanofibres through electrospinning (Kim et al. 2006b). In that procedure, the sol mixture containing the glass precursors was dispersed in polyvinylbutyral (PVB) solution in ethanol prior to electrospinning. PVB/sol electrospun composites were obtained and the polymer removed by heat-treatment at 700 °C. The sol concentration played an important role in controlling fibre diameters, which decreased from 630 to 84 nm as de sol concentration dropped from 1 to 0.25 M. The electrospun glass fibres

were observed to be bioactive, as confirmed by the deposition of a bone-like apatite layer after immersion in SBF. Similar results were obtained by other authors (Asgharnia and Alizadeh 2013; Gao et al. 2011; Xia et al. 2007).

Recently, Hong et al. (2010) proposed that the bioactivity of bioactive glass nanofibres could be increased through the introduction of nanopores to their structures. The pores resulted from the burn out of the carrying polymer (PEO) and could have their diameters predicted by controlling the shrinkage of the fibres during heat treatment. This approach suggests an elegant and straightforward way to increase fibre surface areas, besides allowing for a more intimate contact between the fibre surfaces and ions in solution, therefore improving bioactivity. This would be of ultimate importance in the control of fibre degradation and also in the adsorption of higher quantities of biologically active substances for drug delivery applications. More recently, the production of bioactive glass fibres by electrospinning of pure sol-gel solutions, i.e. without a supporting polymer, has been reported (Scarber et al. 2013). This approach brings the advantage of being less time consuming then the conventional method, as there is no need for thermal treatments to remove a polymer phase.

HA electrospun fibres have also been produced using a polymer binder system. Wu et al. (2004) mixed HA precursors with a polymer additive to produce an electrospun composite. The composite was comprised of large fibres (around 30 μm) with smooth surfaces. The type of the polymer used as carrier was not mentioned in the publication, making it difficult to determine whether any interaction between the polymer and HA precursors takes place. The polymer was removed from the composite by heat treatment. The remaining HA fibres were crystalline and possessed a rough surface resulting from the complete removal of the polymer. Thinner HA fibres, with fibre diameters in the range 200–800 nm, were produced by electrospinning a mixture of polyvinyl alcohol (PVA) and a calcium phosphate based sol, followed by calcination (Dai and Shivkumar 2007b). As observed in the glass fibres, polymers with lower molecular weights were able to produce fibres with smaller diameters.

Although ceramic-based fibres have been successfully produced, details about their structural stability are scarce. Therefore, in-depth investigation on the mechanical performance of these fibres is still to be performed.

3.5 Designs for 3D Structure Generation

The production of electrospun nanofibre meshes with the appropriate architecture for tissue engineering applications has been of a great challenge. For instance, meshes produced through conventional electrospinning have 2D configuration with pseudo pores as large as 30 μm. This poses a limitation in the regeneration of 3D mineralised tissues as it requires the utilisation of 3D scaffolds with interconnected pores, which have to be sufficiently large to allow the infiltration and effective distribution of cells. In fact, efforts have been made to convert nanofibres into 3D scaffolds. In an initial attempt, electrospun fibres were combined with 3D scaffolds produced by different techniques, such as rapid prototyping (Moroni et al. 2008; Martins et al. 2009) and compression moulding (Tuzlakoglu et al. 2005). Schneider et al. (2008a) described the production of a 3D electrospun composite composed of PLGA and ACP. The composite produced had a cotton wool-like appearance and was proposed for non-load bearing bone defects. Although the authors stated that the 3D scaffolds were produced by manually unravelling the fibres, no additional detail was given. Obata et al. (2013) described in detail the production of cotton wool-like fibres of PLA reinforced with siloxane-doped vaterite. The wool-like structure was obtained by blowing air towards electrospun fibres during deposition, which caused the polymer to rapidly evaporate and also pushed the fibres against a small container used as the wool collector. Recently, the production of cotton wool-like bioactive glasses by electrospinning inorganic sol-gel solutions has been proposed (Poologasundarampillai et al. 2014). In that approach, a rotating drum was placed at 10 cm

from the capillary. The production of the 3D structure was attributed to the presence of Ca^{2+} ions, which are supposed to increase the charge density of the sol-gel solution with the formation of multiple jets during the electrospinning process. Ethanol bath-assisted deposition has also been used for the production of electrospun 3D scaffolds (Kim and Kim 2014; Kim et al. 2014b; Yokoyama et al. 2009). In this process, an ethanol bath containing a conductive plate immersed in it is used as collector. The low surface tension of the alcohol allows the electrospun fibres to slowly settle down and arrange themselves in a 3D array (Aghdam et al. 2012; Yokoyama et al. 2009; Hong and Kim 2011). The main disadvantage of this method is related to the pore size that is considerably small (<10 μm) to permit cell infiltration. Kim et al. (2014b), in an attempt to overcome such a limitation, used laser to create pores as large as 380 μm, which, according to the literature, are adequate to support cell attachment and proliferation (Murphy et al. 2010; Roosa et al. 2010). Other methods used to produce 3D electrospun scaffolds through a disc spinneret (needleless electrospinning) (Li et al. 2014b) and electrostatic repulsion (Cai et al. 2013) can also be found in the literature.

3.6 Load-Bearing Structures

The outstanding mechanical properties found in some load-bearing tissues are attributed to specific arrangements of collagen nanofibres. For instance, bone is composed of collagen fibrils organised in concentric lamellae sheets aligned in a 3D fashion (Deng et al. 2011) while menisci (constituted of fibrocartilaginous wedges) is built in compact network of collagen fibres circumferentially aligned (Setton et al. 1999; Petersen and Tillmann 1998). Although 3D structures have been developed, the production of mechanically stable electrospun scaffolds able to support the physical loads after implantation is still a challenge. Recently, Meng et al. (2011) developed a simple and effective method to produce load bearing electrospun scaffolds that combined electrospinning technique and polymer blending strategy. In that approach, nonwoven electrospun 2D fibre meshes of the PLGA/poly[(glycine ethyl glycinato)$_1$(phenylphenoxy)$_1$phosphazene] (PPHOS) blend were rolled up in a concentric way to replicate the bone laminated structure. The resulted material was highly porous (~87 %) and possessed tensile strength comparable to that of the trabecular bone.

The influence of fibre alignment in the mechanical properties of electrospun fibre-based fibrocartilaginous meniscus replacement has been proposed (Baker and Mauck 2007). In that study, both aligned and nonaligned 2D PCL fibre meshes were seeded with chondrocytes. Results showed that, while having a comparable amount of ECM, aligned fibres presented a considerable increase in modulus values when compared with their nonaligned counterparts over the 10-week study. In a similar study, Nerurkar et al. (2011) investigated the role of collagen and glycosaminoglycan (GAG) in tensile and compressive properties of fibrocartilage constructs made of aligned 2D PCL electrospun fibres. It was observed that collagen molecules controlled the tensile response of the constructs while GAG seemed to influence compressive properties. In fact, GAG removal through enzymatic digestion caused considerable stiffening in tension.

Although the reinforcement of electrospun fibres with bioactive particles in both 2D and 3D arrangements has contributed to improve the mechanical properties of the fibres, the results obtained make them more suitable for non-load bearing applications, such as in some treatments of oral and maxillofacial defects (Frohbergh et al. 2012; Schneider et al. 2009). For example, attempts have been made to mimic the structure and properties of periosteum, the outer layer of bone, by reinforcing chitosan electrospun fibres with nHA (Frohbergh et al. 2012). It was hypothesised that the composite obtained would reproduce the mineralised composition and non-weight bearing properties of bone ECM. Mechanical characterisation revealed that the composite, after crosslinking with genipin, presented Young's modulus of 142 MPa, which, according to the authors, is comparable to the value expected for periosteum. Compressive

tests have been performed on 3D wool-like scaffolds made of electrospun bioactive glasses (Poologasundarampillai et al. 2014). Results showed that the stress was related to the density of the scaffolds and that the materials behaved like regular wool when compressed, presenting also very little recovery on unloading. These properties suggest the use of such a material as a bone graft substitute in the application of dental implants. The wool-like scaffolds are thought to initially fill up the cavity of the bone defect. Once bone regeneration around the scaffold has occurred, a tooth implant can be inserted into the new bone. The high flexibility of the fibres, combined with their high porosity, bioactivity and ease of packing, is expected to accelerate healing time and also decrease the time required for implant placement.

3.7 Electrospinning and Tissue Engineering

While some load bearing tissues, such as bone, present good healing capability, many others, for example cartilage, dentin and enamel, either do not have that capacity or present poor ability to heal (Badylak et al. 2011). This may result in significant pain and disability after injury. Return of function can be achieved by surgical intervention, however, the healing response is generally scar-mediated rather than regenerative (Langer and Vacanti 1993). The good healing capacity of bone is limited to small portions of the damaged tissue and the development of suitable replacements that can help regenerate large defects is still a challenge.

Aligned electrospun fibres have been used to guide cells during tissue regeneration (Lyu et al. 2013), while electrospun fibre-based hybrid composites have been tailored to better suit hard tissue application purposes (Vasita and Katti 2006). In addition, fibres with a core-shell structure have been designed to incorporate and release therapeutic drugs during tissue healing and regeneration (Tiwari et al. 2010; Wang et al. 2010).

The next sections will present the recent advances in the use of electrospun nanofibres for the treatment of cartilage (osteochondral defects) and mineralised tissues for tissue engineering purposes, as well as in drug delivery systems.

3.7.1 Bone

Different studies have suggested the utilisation of electrospun nanofibres in bone-related applications. Li et al. (2002) described the production of PLGA electrospun fibres with an architecture resembling that of bone tissue. In vitro biological studies revealed that the fibre orientation guided cellular growth and enabled cells to maintain their phenotypic shape, while supporting cell attachment and proliferation. In a similar study, Yoshimoto et al. (2003) produced a microporous biodegradable scaffold composed of non-woven electrospun PCL nanofibres. The scaffold was seeded with rat neonatal bone marrow mesenchymal stem cells (bMSCs) and dynamically cultured for up to 4 weeks. Scanning electron micrographs showed that the scaffold surfaces were covered with cell multilayers. The production of ECM was evidenced by the clear presence of mineralisation and detection of type I collagen.

Badami et al. (2006) studied the effect of fibre diameter on cell (osteoprogenitor cells) behaviour using diblock copolymers of PLA and poly(ethylene glycol)poly(lactic acid) to produce fibres with mean diameters of 0.14–2.1 μm. Cell density on fibre surfaces after 7 and 14 days of culture increased with fibre diameter and was comparable with or exceeded the one of the controls. Also, cells cultured on electrospun fibres of 2.1 μm diameter proliferated more than those cultured on smooth surfaces.

The effect of additional nanoroughness on electrospun nanofibre membranes of poly-D,L-lactide (PDLLA) was also investigated. Moderate surface roughness, induced in the form of beads, promoted initial adhesion, spreading, and proliferation of mesenchymal stem cells (MSCs) as well as their osteogenic differentiation. The inclusion of these beads had no negative effect on cell behaviour (Aniket et al. 2014).

There have been significant efforts to tailor composite/hybrid combinations that mimic bone

architecture and surface. The materials produced are designed with appropriate mechanical properties so that they can sustain loading forces as well as provide a favourable environment for cell recruitment and tissue mineralisation. As stated previously, the most common inorganic constituent of these composites is nHAP, which is normally dispersed in biodegradable polymers (Frohbergh et al. 2012; Shin et al. 2012). The incorporation of nHAP into polymeric materials reduces their hydrophobicity, therefore increasing cell adhesion and growth. In addition, the incorporation of HA greatly increases the surface roughness of electrospun fibres, creating a nanotextured surface onto which cells preferentially adhere (Lao et al. 2011).

Many studies have reported the development of bioceramic/polymer composite fibres to enhance the scaffold mechanical properties, while simultaneously adding the bioactivity of bioceramics. Biomimetic nanofibre-based composites of nano-hydroxyapatite/chitosan (nHAP/CS) were prepared through the combination of a co-precipitation method and electrospinning. Cell studies with human foetal osteoblasts for a period of 15 days revealed that composites significantly increased bone formation pathways when compared with electrospun CS scaffolds alone (Zhang et al. 2008). On another example, electrospun PLA scaffolds coated with nHAP supported attachment and proliferation of human cord blood-derived stromal cells, while also inducing significant increase in the in vitro expression of osteogenic markers and mineralisation compared with cells seeded on uncoated nanofibres (Seyedjafari et al. 2010).

Recently, biological studies on 3D electrospun nanofibre based composites have been performed. Schneider et al. (2008b) evaluated the viability of MSCs seeded onto a cotton wool-like electrospun nanocomposite of PLGA/amorphous tricalcium phosphate (ATCP). Cells had no morphological changes and the in vitro proliferation was unaffected when compared with pure PLGA scaffolds. The osteogenic differentiation in the wool-like nanocomposite, as expressed by ALP activity and osteocalcin content, was comparable with pure PLGA scaffolds. In an in vivo study using New Zealand White rabbit, the same research team evaluated the performance of the cotton wool-like nanocomposite of PLGA/ATCP (60:40) (Schneider et al. 2009). Empty defects served as negative controls while porous bovine-derived mineral (Bio-Oss®) were the positive controls. Histology and microcomputed tomography unveiled a newly formed bone structure on PLGA/ATCP nanocomposites that resembled spongy bone, whereas a solid cortical bone was observed on Bio-Oss®-treated defects. After 4 weeks of implantation, the area fraction of newly formed bone on ATCP-containing fibres was significantly higher than those of pure PLGA.

Cell studies were also performed on electrospun wool-like bioactive glasses (Poologasundarampillai et al. 2014). Fibres were seeded with MC3T3-E1 preosteoblast cells and demonstrated to have no adverse cytotoxic effect, allowing cells to attach and spread throughout the material. This study, although lacking more prolonged time points and in vivo validation, confirmed bioactive glass electrospun wool-like scaffolds suitability for non-load bearing, complex shaped bone defects.

The application of electrospun nanofibres in bone tissue engineering evolves continuously. Particular effort is currently made to improve cellular interaction in a 3D fashion while allowing for vascularisation and also mineralisation of the scaffold implanted (Novotna et al. 2014; Jamshidi Adegani et al. 2014; Tetteh et al. 2014). Considerable improvement is expected in the near future with the in vivo validation of the outcomes.

3.7.2 Osteochondral

Osteochondral regeneration combines the principles of bone and cartilage tissue engineering. For instance, osteochondral tissue engineering can be considered a typical case of the emerging field of ‘interfacial tissue engineering’, i.e. specialised areas that intimately connect tissues with different biomechanical characteristics (Moffat et al. 2009; Wang et al. 2004).

It has been widely reported in the field of tissue engineering that electrospun nanofibres play an important role enhancing the regeneration of bone tissue, while collagen does the same for cartilage. Recent studies have assessed the effect of the combination of collagen and PLA electrospun fibres, in the form of a bi-layer scaffold, on osteochondral regeneration (Zhang et al. 2013). This scaffold was cultured with MSCs and applied in a rabbit osteochondral defect model. Results demonstrated that the scaffold induced better cartilage formation and overall functional repair of the defects with subchondral bone emergence. Yunos et al. (2013) designed bilayered constructs that combined electrospun poly-DL-Lactide (PDLLA) fibres and Bioglass®-derived scaffolds proposed to act as substitutes for osteochondral tissue. Chondrocyte cells (ATDC5) were able to attach, proliferate and migrate into the fibrous network, confirming the potential of the bilayered scaffolds for osteochondral tissue engineered replacements.

An interesting study by Cui et al. (2013), developed porous poly(D-lactide)/PCL nanofibre scaffolds by electrospinning. The scaffolds were prepared by impregnating a thermoresponsive chitosan solution onto the porous electrospun scaffold, and applied in vivo in rat osteochondral defects. After 6 weeks of implantation, results revealed that both scaffolds, with and without chitosan, were able to sustain new bone formation. Authors concluded that the incorporation of chitosan within the porous scaffolds did not affect tissue ingrowth. However, the scaffolds presented no evidence of cartilage formation after 15 weeks, indicating that either cells or growth factors, or even their combination, will be necessary to support effective osteochondral tissue regeneration.

3.7.3 Tooth

Tissue regeneration is in high demand in dental related applications due to defects resulting from postcancer surgery, trauma, periodontal disease and congenital malformations. Current pulp capping materials, generally made of phosphate cements and resins, may not be able to induce new tissue formation as they are lack of chemical stability/functionality for cell loading (Olsson et al. 2006). Tissue engineering approaches offer the potential for the development of temporary tooth substitutes as an alternative to the irreversible repair methods (e.g. dental restorations or oral implants). Successful regeneration of affected tissues is necessary to reconstruct facial support, allow for mastication, and coordinate with sensory organs (eyes, nose, ears, and mouth) (Li et al. 2014c). Many of the currently applied approaches, such as scaffold-based tooth regeneration, try to mimic as closely as possible the natural process of tooth regeneration, recreating tooth-like structures.

Although electrospun scaffolds are still lack of in vivo validation for immature permanent teeth pulpless models (both human and animal), recent studies have reported their great potential in regenerative approaches. These bioactive scaffolds have also been suggested for regeneration of dentin and pulp tissues, as well as root canal disinfection, with continuous developments in their production and possible applications (Albuquerque et al. 2014).

Yang et al. (2010) explored the behaviour of electrospun PCL/gelatin scaffolds, with or without the addition of nHAP, seeded with dental pulp stem cells (DPSCs). Results showed that DPSCs were able to adhere, proliferate, and undergo differentiation towards odontoblastic lineage. Nude mice subcutaneous model was used and results showed scaffolds were surrounded by a thin fibrous tissue capsule, and there was evidence of hard tissue formation, although no signs of tissue ingrowth were present. Authors concluded that odontoblastic differentiation of DPSCs was enhanced by the incorporation of nHAP in the nanofibres. Nevertheless, this study failed to reveal any dentin-pulp-like structure formation.

Kim et al. (2013) investigated the odontogenic differentiation behaviour of human DPSCs when seeded onto mineralised polycaprolactone (PCL) nanofibrous scaffolds. The study revealed that mineralised scaffolds promoted the proliferation of cells, formation of mineralised nodules and

expression of odontoblastic genes, namely dentin sialophosphoprotein and dentin matrix protein-1. Authors reported this to be an attractive scaffold for dentin tissue engineering purposes, considering DPSCs were able to grow and undergo odontogenic differentiation.

Although not related to osteodental regeneration, electrospun fibres have also been used to improve the bioactivity and biocompatibility of implant materials. Recently, a biodegradable hybrid (PCL/PLA) electrospun membrane was developed to coat titanium (Ti) surfaces (Abdalhay et al. 2012). Polymer-treated Ti samples were compared to untreated Ti samples, when seeded with MC3T3-E1 cells. Former samples presented superior cellular behaviour than the latter ones. Moreover, polymer-coated Ti samples also evidenced corrosion resistance properties.

3.8 Drug Delivery Systems

Drug delivery strategies using nanofibre meshes normally involve the suspension of the drug in the polymer solution that will be electrospun. Due to their importance in regulating osteoblast behaviour, the development of effective strategies to deliver osteogenic cues, such as bone morphogenetic proteins (BMPs) fibroblast growth factors (FGFs), transforming growth factors (TGFs) and insulin-like growth factors (IGFs) in a sustained manner remains an area of intense research focus (Yoon and Kim 2011).

As for BMPs, particular attention should be given to the incorporation process into the nanofibres since these proteins are very susceptible to denaturation under processing conditions involving high temperatures, low pH, and organic solvents. Kaplan's group has explored the delivery of BMP-2 within a silk fibroin-based nanofibre scaffold for bone regeneration (Li et al. 2006). The efficacy of BMP-2 incorporated in the nanofibre matrix was demonstrated by the superior osteogenic response of human MSCs, namely cellular calcification. Casper et al. (2005) used heparin to achieve higher affinity for growth factors, including FGF. A sustained release of basic FGF could be maintained for up to 14 days when poly(ethylene glycol) (PEG) nanofibres containing heparin were used.

In a recent study, Srouji et al. (2011) coaxially electrospun BMP-2 in an aqueous core solution of PEO, coated with PCL blended with PEG. The blending process induced pore formation in the shell of the nanofibres produced, thus enabling the release of proteins. When implanted in a calvarial defect model, the core-shell nanofibres incorporating BMPs promoted human MSCs induction into an osteoblastic lineage and the treated defects exhibited significant increase in bone formation.

Coaxial electrospun nanofibres composed of poly(l-lactide-co-caprolactone) and collagen were used as carriers to release BMP-2 and dexamethasone, a potent glucocorticoid that induces osteoblastic differentiation, in a sustained manner (Su et al. 2012). Human MSCs seeded on these nanofibres exhibited increased expression of osteoblastic markers compared to blended electrospun fibres alone. In a different study, Martins et al. (2010) incorporated amorphous dexamethasone into electrospun PCL nanofibres at different concentrations, in a single-step process. The authors were able to maintain a sustained release of dexamethasone over a period of 15 days. Human bone marrow mesenchymal stem cells (hBMSCs) were cultured on 15 wt.% DEX-loaded PCL scaffolds, and the osteogenic activity of the loaded growth factor was corroborated by increasing levels of ALP and mineralised matrix deposition.

Nie et al. (2008) developed PLGA/HA composite electrospun fibrous scaffolds loaded with BMP-2. The sustained release of the protein over a period of 2–8 weeks was achieved and accelerated with increasing levels of HA on the scaffolds. MSCs seeded onto the scaffolds showed that the presence of HA decreases cytotoxicity and promotes cell attachment.

Erisken et al. (2011) developed a delivery system within the two side of a PCL nanofibrous scaffold via the application of twin-screw extrusion process and electrospinning method, thus generating different insulin and beta-glycerophosphate gradients. Human adipose-derived stromal cells were cultured on the PCL

mesh for up to 8 weeks, revealing that cells would differentiate towards either chondrogenic or osteogenic lineages according to their proximity to insulin-rich or beta-glycerophosphate-rich locations, respectively.

In order to enhance the therapeutic efficacy of electrospun nanofibres, some chemical drugs, such as antimicrobial agents, have also been introduced in addition to biological molecules. For periodontal purposes, for instance, a mixture of polymers (PLA and poly(ethylene-co-vinyl acetate)) was loaded with tetracycline hydrochloride. Changes in the composition controlled the release of the incorporated tetracycline within 10–12 h (el Kenawy et al. 2002). The particular characteristics of the polymers used have an obvious influence on the encapsulation process.

In a recent study by Bottino et al. (2013), Metronidazole or Ciprofloxacin was mixed with a polydioxanone polymer solution at 5 and 25 wt.% and electrospun processed into fibres. Human DPSCs were used to assess cytotoxicity of both material and drugs, and *Porphyromonas gingivalis* (*Pg*and) and *Enterococcus faecalis* (*Ef*) were used to evaluate the antibacterial properties of the constructs. Results evidenced growth inhibition of the bacteria in the presence of the antibiotic drug. This study showed that antibiotic-containing electrospun scaffolds could be used in endodontic regenerative approaches to mitigate the effects of different bacteria present locally.

3.9 Future Perspectives

Although great progress has been observed in the design and application of electrospun fibres in tissue engineering, there is still place for improvement. Enhancement of the load-bearing properties in 3D electrospun fibrous scaffolds is possibly the main challenge electrospinning technique faces in the near future. That can possibly be achieved through the precise control of the fibre architectures. For instance, the production of aligned electrospun fibres that intimately interact with bioactive nanoparticles in a 3D fashion could originate scaffolds that closely mimic bone structure and, therefore, its outstanding mechanical properties. Those scaffolds would also have to contain pores sufficiently large to permit cell infiltration and tissue ingrowth, as a satisfactory model that allows blood vessels formation and tissue regeneration in vivo is still desired.

The use of electrospun fibres as careers for bioactive molecules in drug delivery approaches, although evolving exponentially in the past few years, is still recent. Only few studies have demonstrated the in vivo viability of those delivery systems, particularly regarding bone and tooth trials, which constitute challenging fields of research. A better understanding of the release kinetics of therapeutic biofactors through the combination of theoretical and empirical modelling is still necessary.

Continuous advances in using electrospun structures as tissue-engineered substitutes are expected in the forthcoming future. The major contribution in that field may originate from the synergy between electrospinning technique and emerging technologies that will allow for a better control of the fibre deposition and also of the mesh architectures.

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Part II

Applied Strategies for Tissue Engineering: Bone and Cartilage

Bone Tissue Engineering Challenges in Oral & Maxillofacial Surgery

4

Brandon T. Smith, Jonathan Shum, Mark Wong, Antonios G. Mikos, and Simon Young

Abstract

Over the past decades, there has been a substantial amount of innovation and research into tissue engineering and regenerative approaches for the craniofacial region. This highly complex area presents many unique challenges for tissue engineers. Recent research indicates that various forms of implantable biodegradable scaffolds may play a beneficial role in the clinical treatment of craniofacial pathological conditions. Additionally, the direct delivery of bioactive molecules may further increase de novo bone formation. While these strategies offer an exciting glimpse into potential future treatments, there are several challenges that still must be overcome. In this chapter, we will highlight both current surgical approaches for craniofacial reconstruction and recent advances within the field of bone tissue engineering. The clinical challenges and limitations of these strategies will help contextualize and inform future craniofacial tissue engineering strategies.

Keywords

Bone regeneration • Tissue engineering • Maxilofacial reconstruction • Bone scaffolds • Stem cells • Growth factor

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4.1 Introduction

Tissue engineering was defined by Langer and Vacanti in 1993 (Langer and Vacanti 1993), as a new approach which "applies the principles of biology and engineering to the development of functional substitutes for damaged tissue". In contrast to the traditional biomaterials approach at that time, this nascent field integrated knowledge across a diverse set of disciplines with the goal of inducing regeneration of damaged tissues rather than performing replacement with inert parts.

More than two decades later, the growth of the tissue engineering and regenerative medicine (TERM) field continues unabated. At the turn of the twenty-first century, about 360 original TERM articles were being published yearly, while over 4,000 original TERM articles were published in 2010 alone (Fisher and Mauck 2013).

This heightened interest in TERM has led to virtually all tissues in the body (and even whole organs) having been investigated by TERM researchers across the globe. The focus of this chapter however, is to highlight current bone tissue engineering strategies within the craniofacial complex. Of additional importance from a translational standpoint, we will also describe the specific challenges of applying bone tissue engineering strategies to this anatomical region, and briefly discuss current methods of surgical reconstruction.

4.2 Challenges for Bone Tissue Engineering in the Craniofacial Complex

Generally speaking, bone displays a high capacity for regeneration following injury. This fact, aided by continued advancements in surgical technique and bone fixation hardware, has resulted in progressive improvement in treatment outcomes of complex craniofacial bony defects.

Nonetheless, situations arise in which either the sheer quantity of bone required for reconstruction exceeds the bone's intrinsic ability to regenerate, or the host tissue's regenerative ability has been compromised (or both). The former situation may be encountered following resection of large pathological lesions or the result of avulsive injuries due to high-energy trauma. On the opposite end of the spectrum, seemingly small traumatic insults inflicted on the dentoalveolar ridge may progressively worsen into large, non-healing lesions of the jaws in conditions such as osteoradionecrosis (ORN) or medication-related osteonecrosis of the jaws (MRONJ), where radiation or certain medications adversely affect bone healing (Almazrooa and Woo 2009). The result is that bone reconstruction in these situations may require the use of a grafting material (whether it be autogenous, allogeneic, xenogeneic, or synthetic in origin) or microvascular free tissue transfer, as described later in this chapter.

It is interesting to note that despite the extensive TERM research focused on bone over the last few decades, many clinicians still consider autologous sources as the "gold standard", and more than 50 % of the three million musculoskeletal procedures performed annually in the USA use autologous or allogeneic sources of bone graft (Jahangir et al. 2008). Tissue engineering strategies combining the use of a scaffold with bioactive factors and/or living cells hold promise as viable alternatives, but the only FDA-approved systems currently available involve the release of supraphysiologic doses of recombinant bone morphogenetic proteins (either rhBMP-2 or -7) from simple collagen matrices (Jahangir et al. 2008).

The cost of commercializing a tissue engineering bone substitute may be partly responsible, but this also underscores the challenge of developing a successful tissue engineering solution for bone regeneration in the oral and maxillofacial environment.

The demand for such materials certainly exists. In addition to the enormous amount of bone grafting procedures performed annually as mentioned above, it has been estimated that greater than 85 % of the US population requires repair or replacement of a craniofacial structure (Scheller and Krebsbach 2009). While this appraisal includes numerous tissues in addition

to bone (i.e. tooth, temporomandibular joint, salivary gland, mucosa), it illustrates not only the sheer diversity of structures within the craniofacial complex, but also their vulnerability to damage.

Indeed, there are a host of etiologies that contribute to the need for tissue replacement in the oral cavity and surrounding structures bounded by the lower jaw inferiorly and the cranial base superiorly (i.e. the oral and maxillofacial region). Our discussion is centered on the skeletal elements which form the underlying framework of this area, but it should be noted that a complex overlying neurovascular network, skin and subcutaneous tissues, salivary glands, and dental structures are all densely localized within this small area. The result is that complex composite tissue defects can result from high-energy trauma, congenital deformities, and disease.

A wide spectrum of disease processes can affect the lower (mandible) and upper (maxilla) jaws. Commonly encountered pathological lesions of the jaws include benign cysts and malignant tumors whose growth and ensuing treatment may result in the loss of bone, cartilage, and overlying soft tissues. Traumatic wounds resulting from high-velocity blunt forces or projectiles may also result in significant tissue loss, while congenital facial clefts (most commonly in the form of cleft lip and/or palate) can involve several structures such as the upper lip, maxilla, and palate, resulting in facial deformities.

In contrast to such entities that may be spatially and/or temporally discrete, tissue loss can also occur due to non-physiological loading conditions or autoimmune disease. Alveolar bone resorption following the loss of teeth and degenerative disease of the temporomandibular joint (TMJ) are examples of bone loss resulting from long-term abnormal mechanical loading forces. Similarly destructive loss of TMJ components can also occur in autoimmune diseases which affect the synovium, such as rheumatoid or psoriatic arthritis. Aside from the functional and esthetic complications of these diseases such as difficulty with mastication and the loss of vertical facial height, more debilitating consequences may also arise such as pathological fracture of the atrophic mandible (Van Sickels and Cunningham 2010).

Regardless of the surgical technique or material used for reconstruction, careful consideration of the etiology is of utmost importance. Assuming the causative event has been resolved (i.e. definitive removal of a pathological lesion, a one-time traumatic insult, or a congenital defect), even complex defects can be well characterized and treated accordingly. However, unpredictable results using biological constructs can occur if the source problem continues unabated. For example, in the face of untreated autoimmune disease or persistent non-physiologic loading, even a "perfectly integrated" biologic TMJ construct would be doomed to failure, regardless of its ability to recapitulate the form and function of the original TMJ anatomic components.

Bone tissue engineering strategies in the oral and maxillofacial region must also account for potential exposure of the implanted material to the external environment. At the time of the grafting procedure (and for a short time thereafter, until an epithelial barrier has been re-established), materials used for nasal, orbital, auricular, or mandibular/maxillary reconstruction may directly communicate with the nasal and oral cavities, sinuses, and the outside environment. A specific concern includes a significant presence of pathological organisms within a moist environment. This imposes a potential risk for graft site infection and decreased predictability of the bone grafting procedure. The design of the biologic tissue engineered construct should therefore consider several factors such as the use of antimicrobials (either systemic or locally-delivered), optimized surface characteristics (to minimize potential bacterial colonization and subsequent biofilm formation), and bioactive factor dose/release kinetics (to account for the diluting effects of body fluids at the time of implantation).

Lastly, special consideration should be given to the form and function required of the bone reconstruction material, depending on the location of the defect within the oral and maxillofacial complex. From a functional standpoint, the

minimal mechanical load imposed on a material used to reconstruct the orbital floor is substantially less than the compressive, shear, and tensile forces experienced by materials used to reconstruct a large jaw defect (which may also require further rehabilitation using loaded dental endosseous implants) or temporomandibular joint. Esthetic considerations are important as well, with the need for accurate reconstruction of normal bony contours that are symmetric with the contralateral side. The visually prominent position of the face, in combination with the thinness of the overlying soft tissue envelope imposes a high standard on both the bone tissue engineer and surgeon, to accurately reproduce normal facial morphology in their reconstructive efforts.

4.3 Current Methods of Maxillofacial Reconstruction

Reconstruction in the head and neck has undergone an evolution in surgical options and corresponds to the development of techniques and technological advances. A variety of reconstructions are practiced and are a reflection of experience, and resources available to the treating surgeon. Generally, these methods can be categorized into one of two categories: non-vascularized or vascularized reconstructions (Table 4.1). The defining feature between the two is the presence of a blood supply for the transplanted tissue at the time of placement. For most clinical scenarios, the ideal reconstruction will be defined by the characteristics of the defect to be restored, in addition to patient factors such as overall health and viability of donor sites.

The inciting cause of the defect will also play a significant role in the decision process, as most if not all defects of the head and neck will fall under traumatic defects or result from the treatment of pathology. Within the subgroup of pathology, we further delineate between benign and malignant related defects to consider the effects caused by previous or adjunct therapy. For example, prior surgery can alter the anatomic landscape to make subsequent surgical therapy difficult for accurate identification of vital structures. Radiation can further complicate a surgical site through the effects of radiation-induced fibrosis on soft and hard tissue and sclerosis of blood vessels that can compromise the ability to consider a microvascular reconstruction. Chemotherapy alone has limited effects on soft tissue; however when used in conjunction with radiation can exacerbate the consequences of radiation. With all reconstruction methods, we strive to establish form and function through a reliable method that replaces like tissue with like tissue, while minimizing morbidity.

Table 4.1 Review of reconstruction modalities

Type of reconstruction	Soft tissue replacement	Hard tissue replacement	Composite replacement
Hardware	N/A	Reconstruction plate	N/A
Non vascularized	– Skin grafts – split/full thickness – Allogeneic skin or dermal substitutes	– Allogenic,- demineralized freeze dried bone graft – Xenogeneic – bovine bone – Autogenous- cortico-cancellous bone graft e.g. iliac crest	N/A
Vascularized	– Regional flaps e.g. pectoralis major flap-local flaps e.g. temporalis muscle flaps, buccal fat flap – Microvascular free flaps i.e. radial forearm, anterior lateral thigh, lateral arm	– Local regional flaps – Free tissue transfer e.g. fibula, scapula	– Multi component free flaps e.g. fibula, scapula

4.4 Mandible Reconstruction

The mandible is a unique structure of the head and neck as it serves multiple functions in mastication and speech and provides a significant component of the structure of the face. It is an arch-shaped bone that supports the lower dentition and is under constant function with regular activities such as mastication. Defects in the mandibular region may be isolated to the bone or soft tissue, or occur as composite defects that involve a combination of tissue types within the lower face.

Non-vascularized reconstructions are generally reserved for isolated tissue type defects such as the loss of a segment of mandible with minimal soft tissue loss. In regards to patient host factors, the presence of radiation injury to the defect site, or prior surgery can compromise the success of a non-vascularized reconstruction. Common sources for non-vascularized grafts are of autogenous or allogenic origin. In regards to autogenous grafts, the anterior and posterior iliac crest serves as a preferred location for harvest in the form of both cortical, and/or cancellous bone for the replacement of bone tissue. The anterior iliac crest can yield 40–50 ml of particulate bone and uncompressed marrow or an average corticocancellous block of 5×3 cm (Waite 1998). The posterior iliac crest can produce 100–130 ml of corticocancellous bone or an average 5×5 cm size block of bone (Waite 1998). Other sites commonly used for autogenous bone harvest include the tibial plateau, rib, and calvarium.

It is generally believed that the ideal bone graft should be osteogenic, osteoconductive, and osteoinductive. The use of autogenous bone graft is preferred as it satisfies these requirements; furthermore, allogenic bone grafts are commonly reserved for small defects or are used in conjunction with autogenous grafts to provide bulk and osteoconductive and osteoinductive augmentation. Demineralized bone graft material is commonly used in this manner. Components within cortical bone such as bone morphogenic protein (BMP) exhibit osteoinductive properties to facilitate bone growth in non-vascularized grafts.

The harvest of an iliac crest bone graft is straightforward, and can provide a reliable amount of bone grafting material with minimal morbidity at the donor site. The disadvantages associated with non-vascularized grafts are the tendency for definitive reconstruction to occur in a two-step process. The success of a non-vascularized bone graft largely depends on the vascularity of the recipient site and the quantity of osteoblasts present in the graft material or through recruitment by mediators such as BMP. Bone formation is completed through a process of creeping substitution whereby the matrix of the donor bone graft acts as a framework for new osteoblastic bone deposition to occur for up to 6 months after grafting. The disadvantages of non-vascularized reconstruction are based on the reduced success rates when salivary contamination occurs, and can result in a graft failure rate of at least 50 % (Lawson et al. 1982). The success rate approaches 100 % when reconstruction is delayed and an extraoral approach is utilized to avoid oral cavity contamination. Subsequently, large defect reconstructions are staged to ensure the defect dimension is maintained (usually with a reconstruction plate), and the soft tissue envelope has healed prior to a definitive bony reconstruction at least 3 months later (Lawson et al. 1982). Furthermore, the length of the bony reconstruction has also been demonstrated to affect failure rates with non-vascularized grafts of 6 cm or less failing 17 % of the time, compared with a failure rate of 75 % in grafts more than 12 cm in length (Foster et al. 1999).

Adjunctive soft tissue reconstruction to seal the oral cavity has relied on local regional tissue flaps. These soft tissue maneuvers provide vascularized tissue which can be transferred from a local or regional area to provide coverage, and/or bulk to support the healing of large soft tissue wounds. The classic example is the pectoralis major pedicled regional flap, which is based off of the pectoral branch from the thoracoacromial artery. It can provide significant muscle, fascia and skin from the chest wall to cover defects ranging up to the midface.

Another means of reconstruction of the mandible includes the placement of an alloplastic material such as a titanium reconstruction plate to establish the form of the lost segment. Current use of reconstruction plates are to secure vascularized and non-vascularized bone grafts to the defect site, in addition to maintaining the space of a defect in preparation for secondary definitive reconstruction. This method of mandibular reconstruction using titanium plates was examined by Kim et al. demonstrating a significant failure rate of 52 % when anterior mandible defects were reconstructed with a titanium plate alone (Kim and Donoff 1992). A decrease in hardware failure is noted in lateral mandible reconstructions, having a 7.7–12.5 % failure rate. Dehiscence of the soft tissue overlying a mandibular reconstruction plate was approximately 17 % (Kim and Donoff 1992). Numerous studies also reported similar findings, with most suggesting risk factors for failure to include a history of prior radiation treatment. The average retention time for mandibular hardware to function has been reported to be 8–10 months (Kellman and Gullane 1987).

The development of microvascular reconstruction has led to improvements in head and neck reconstruction by providing an alternative that can restore form and function when composite defects are encountered. Unlike non-vascularized tissue grafts, or hardware reconstruction, a composite microvascular reconstruction is not dependent on adjacent tissue for success. Unfortunately, composite defects are more common due to the indiscriminate nature of traumatic avulsive defects and malignant pathologic processes. Free tissue transfer can accommodate for these complex defects in addition to the difficulties associated with previously radiated tissue, or prior surgery. The potential locations for bony reconstruction are several; whereas, non-vascularized bone harvest sites are limited and less versatile than the options that are commonly used today for mandibular reconstruction.

The fibula osteocutaneous free flap is considered to be the workhorse reconstruction for the head and neck due to the length and quality of bone that can be harvested from the lower leg. Average length for a fibula is 20–26 cm, and the orientation of the vascular pedicle allows for the concurrent harvest of adjacent tissue types such as muscle, skin and fascia. The fibula harvest site can readily be screened for its suitability with clinical examination of the dorsalis pedis, and the posterior tibilais pulse. Preoperative imaging in the form of CT angiogram, MR angiogram, or Doppler ultrasound is commonly performed to ensure there exists adequate vessels that supply the foot. Within the lower leg there are three groups of vessels that branch off from the popliteal artery to travel down the leg. The lack of patent vessels can alert the reconstructive surgeon of the increased risk of peri-operative complications, and would preclude the use of that extremity for fear of comprise to the foot and also of increased chance for free flap failure.

Recent advances in virtual pre-surgical planning have allowed surgeons to collaborate with engineers in designing surgical guides, and cutting splints to facilitate the reconstructive surgery. A case example of virtual planning is illustrated in Figs. 4.1, 4.2, 4.3, 4.4, and 4.5.

Through pre-operative imaging of the defect site (Fig. 4.2) and the harvest site, bony reconstructions can be designed to fit the defect (Fig. 4.3). In terms of the fibula free flap, the straight bone of the fibula can be manipulated by osteotomies at certain points along the bone and at varying angles to allow for the contouring of the fibula to form a variety of patterns that would reconstruct the mandibular form and ultimately function. This patient suffered an avulsive tissue injury to the anterior mandible, floor of mouth and chin. Through the process of virtual planning (Figs. 4.2 and 4.3), the outline of the lower jaw was reconstructed, a cutting guide was produced, and the fibula was truncated to reestablish mandibular form and to provide a foundation for dental implants (Figs. 4.4 and 4.5).

Additional harvest locations for bony free flap reconstruction include the scapula and iliac crest. The scapula vasculature is dependent on the subscapular system. The circumflex scapular artery and vein commonly reach diameters that ranges from 2 to 6 mm. This system of vessels is less

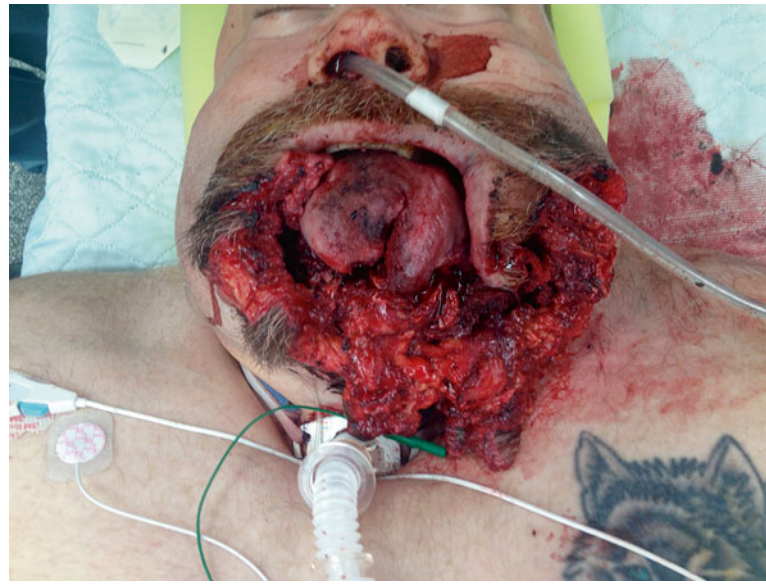

Fig. 4.1 Patient with an avulsive injury to the anterior mandible, chin and floor of mouth

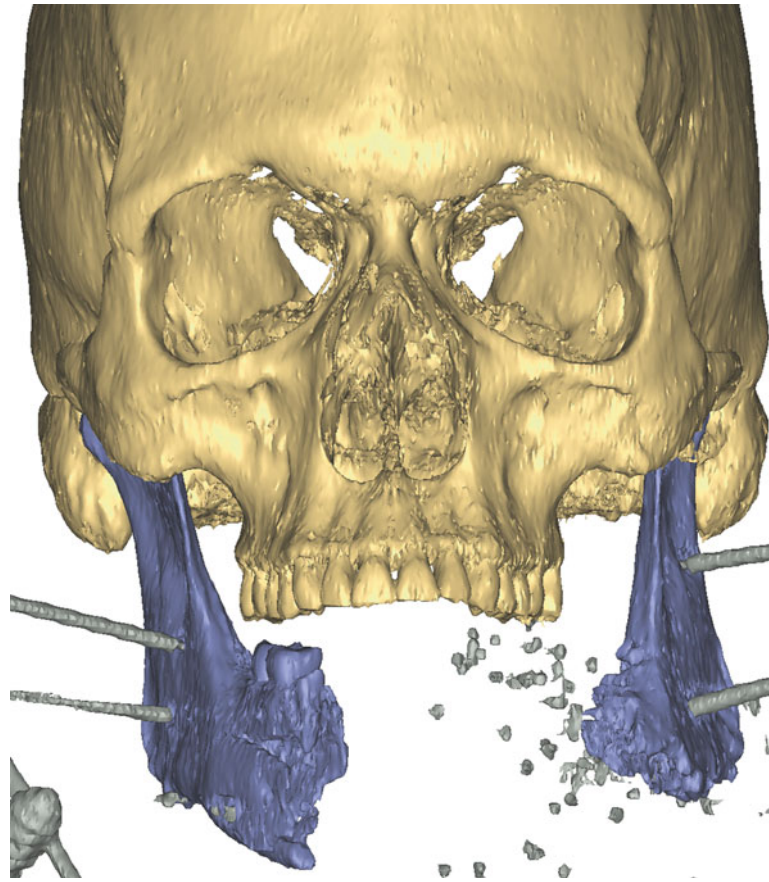

Fig. 4.2 3-D rendering of the patient's avulsive defect following stabilization with external fixation pins. Preparation for free flap reconstructions

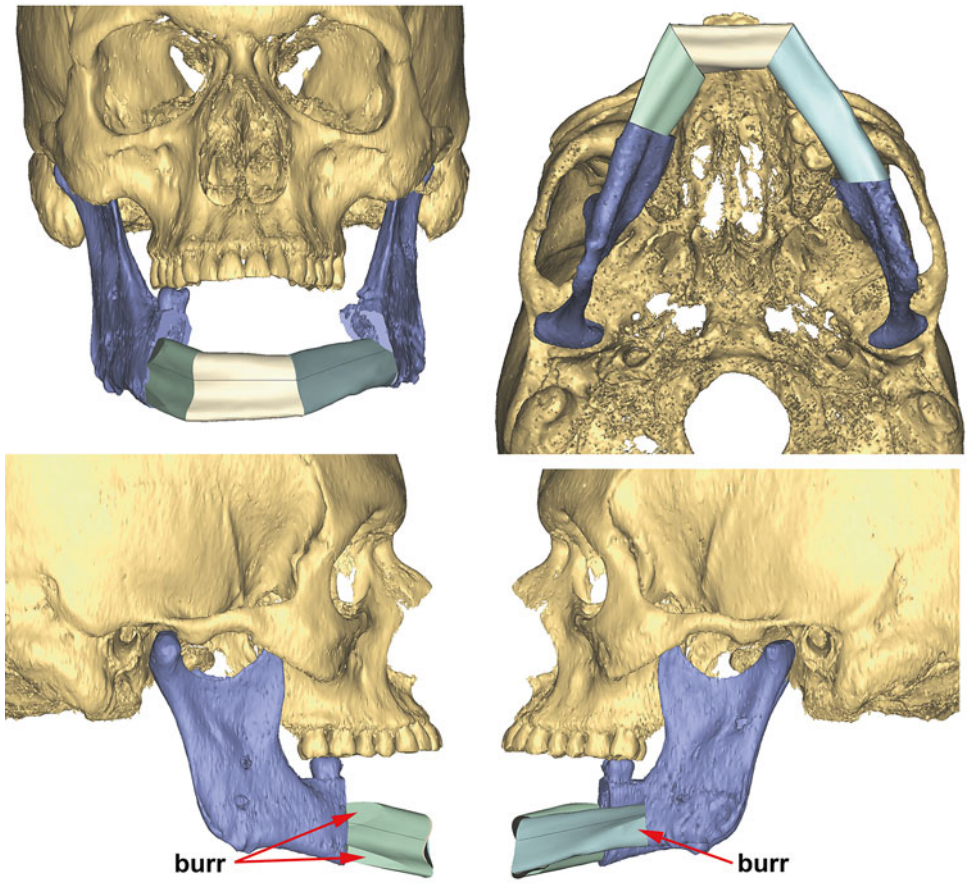

Fig. 4.3 3-D rendering of planned reconstruction with fibula osteocutaneous free flap. *Top left*: Anterior view, *Top right*: Inferior view, *Bottom left*: Right lateral view, *Bottom right*: Left lateral view

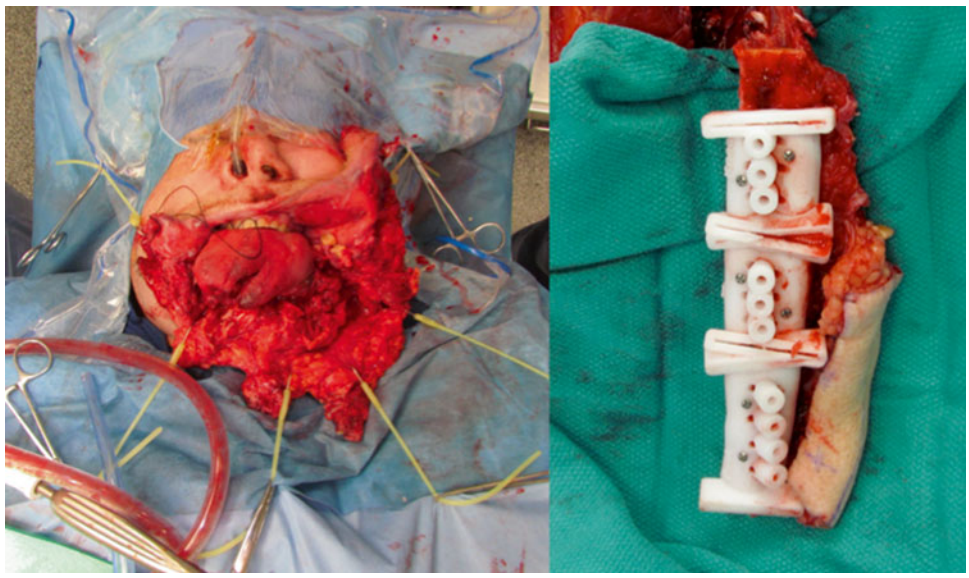

Fig. 4.4 *Left*: defect of the mandible was completely exposed to prepare for inset of fibula free flap. *Right*: fibula with cutting guide still attached to blood supply in lower leg. Note the skin paddle along the right side of fibular construct

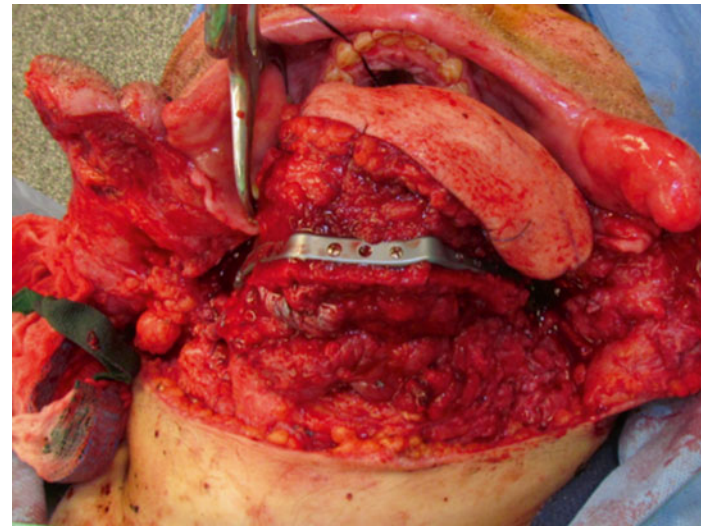

Fig. 4.5 Fibula construct inset to reconstruct mandibular defect. Note the mandibular hardware that has fixated the three piece fibula on to the hardware plate

likely to develop manifestations of peripheral vascular disease when compared with extremity free flaps. The scapula is advantageous due to the multiple skin paddles that it can support in conjunction with the bone, making this an ideal reconstruction for the maxilla and/or through and through defects of the lower jaw. Linear sections of bone can be harvested from the lateral border of the scapula up to 8 cm. In terms of mandibular reconstruction, the scapula flap is limited by the size and volume of bone available. The average scapula is insufficient in bone thickness to predictably accommodate endosseous implants. Furthermore, a significant disadvantage of the scapula flap relates to the difficulty in simultaneous harvest with recipient site preparation due to patient positioning in lateral decubitus.

The deep circumflex iliac artery flap (DCIA) is an excellent choice for maxillofacial reconstruction as it combines both an excellent quality and quantity of bone for reconstruction. The vascular pedicle is reliable with average diameters of 2–3 mm. The donor site is also amenable to primary closure as this flap can be harvested with only the associated muscles for soft tissue coverage. Similar to the fibula flap, the ability to perform simultaneous two-team surgery is possible with the DCIA. A distinct challenge associated with this donor site is the potential for a weakened abdominal wall and resultant hernia formation. Regardless of its challenges, the DCIA is considered to be a likely alternative to the fibula flap for bony reconstructions in the maxillofacial skeleton.

4.5 Maxillary Reconstruction

Defects of the midface are distinct from mandibular reconstruction as vital structures of the orbit and globe lie superiorly, while along the medial aspect the nasal cavity and sinus cavity compose a significant portion of the maxilla. The lateral aspect of the maxilla abuts with the zygoma to provide structure to define facial form. The midface contains the structural pillars and buttresses for daily activities such as mastication and speech. The forces created by the mandible are accepted by the midface and distributed through three buttresses which include: the zygomaticomaxillary buttress laterally, pterygomaxillary buttress posteriorly and the nasomaxillary buttress anteriorly. In terms of reconstruction these buttresses should be reestablished to ensure proper accommodation of occlusal forces.

Maxillary defects have been well described and multiple classifications have been developed to assist in treatment algorithms. Table 4.2 briefly

Table 4.2 Brown classification for maxillectomy defects

	Surgical component (vertical)		Dental component (horizontal)
Class 1	– Defect involving alveolar complex without oro-antral communication	a	– Isolated palatal defect, no involvement of dentoalveolar structures
Class 2	– Defect involving alveolar complex and violation of sinus cavity, but without extension to the orbit	b	– Less than or equal to ½ unilateral
Class 3	– Defect involving alveolar complex and violation of sinus cavity with extension through the orbital rim	c	– Less than or equal to ½ bilateral or transverse anterior
Class 4	– Maxillectomy with orbital exenteration	d	– Greater than ½ maxillectomy
Class 5	– Orbitomaxillary defect		
Class 6	– Nasomaxillary defect		

The increase in classification and lettering refers to the increasing complexity of the maxillary and dentoalveolar defect (Brown and Shaw 2010)

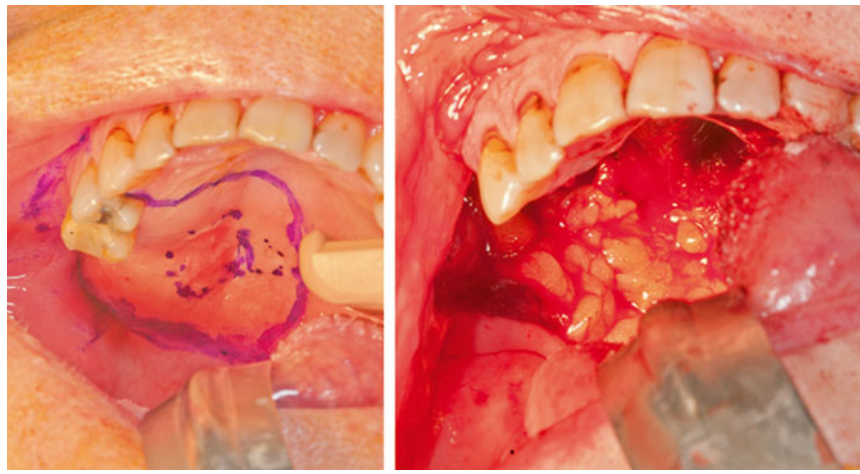

Fig. 4.6 *Left* – Preoperative view of planned partial maxillectomy. *Right* post-operative view of a Brown Class 2b defect after partial maxillectomy. Note the buccal fat pad was transposed to reconstruct the posterior portion of the defect

outlines the Brown classification system for categorizing maxillectomy defects. Reconstruction options are dependent on the extent of tissue loss in terms of volume vs. surface area required for restoration of form and the loss of structural units such as the orbital floor, zygoma and anterior maxilla for support of the globe and facial form, respectively. The patient's prognosis and future plans for further reconstruction should be considered in choosing a definitive reconstruction. For example, patients who undergo total maxillectomy and orbital exenteration for malignant pathology may require a large soft tissue flap for obturation of the defect and the likelihood of dental rehabilitation is low, thereby forgoing a bony reconstruction.

Due to the unique nature of the maxillectomy defect, several options address these features well. The surgical obturator is capable of restoring all sizes of maxillary defects (Figs. 4.6 and 4.7). Prosthetic appliances have excellent aesthetics as well as function, depending on the extent of defect being restored. With dentoalveolar defects, the obturator has great esthetics and retention is excellent when remaining healthy teeth are used for anchoring the prosthesis in place. The effectiveness of an obturator can be compromised with larger defects and when areas of retention

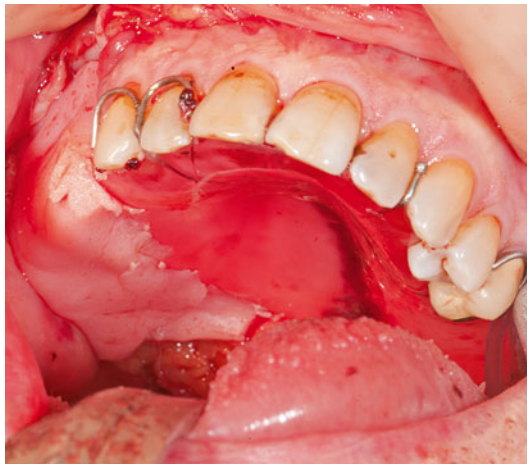

Fig. 4.7 Post operative view with maxillary obturator to seal oral cavity from antral cavity

are not available. Although these devices incorporate teeth and are fitted to separate the oral cavity from the nasal and antral cavities, the appliance is still prone to challenges such as hypernasal speech, regurgitation of fluids into the nasal cavity, the requirement for meticulous hygiene, and the need for repeated adjustments, all of which can decrease its overall effectiveness as a reconstructive treatment.

Non-vascularized bone grafting is indicated in maxillary reconstruction when the defect is isolated to the dentoalveolar process. It is a common procedure for demineralized bone to be used as a bone graft to restore tooth extraction sockets, sinus lifts and to facilitate bony preservation for endosseous implant placement. Manifestations of facial clefting are also repaired in the manner of non-vascularized bone grafting techniques such as autogenous bone harvests from iliac crest or from the tibia.

Vascularized free flap transfer is more likely to succeed in this environment due to the ability to survive independently on the vascular pedicle, as opposed to the surrounding tissue bed. Commonly used for maxillary reconstruction are the radial forearm free flap and anterior lateral thigh flap for reconstructions that intend to solely obturate the defect whereas bony reconstructions are generally completed with the fibula and scapula free flap reconstructions to obturate the defect in addition to providing a foundation from which endosseous implants may be placed for future dental rehabilitation.

Recent advances in reconstructive surgery have demonstrated progress in the field of facial allograft transplantation. While the first operation was performed relatively recently (i.e. 2005), preliminary results are promising with the majority of facial transplants having a successful outcome based on the lack of tissue rejection and patient's return to satisfactory function. All facial allograft transplantations that have been completed are for significant deformities caused by traumatic injuries, burns or congenital defects. In all cases, patients had sustained approximately 25 % pre-operative facial disfigurement (Gordon et al. 2011). There exists no true guidelines on facial allograft transplantation. However, there is also, no defined limitation on the extent of reconstruction. Performing the first comprehensive facial transplantation in 2012, Rodriguez et al. reconstructed a patient with a prior traumatic injury to his face by transplanting the skin of the face, maxilla, zygoma, mandible, tongue and all associated blood vessels and nerves as a single composite tissue transfer from a donor (Dorafshar et al. 2013). Using techniques and principles from facial trauma repair, orthognathic surgery, and microvascular surgery this operation and many like it have successfully reconstructed a number of patients. The 2 year follow up of this patient has demonstrated a reintegration into society, employment, function of his oral cavity with a diet, and also re-innervation of muscles of facial expression in the transplanted face. Current data is promising, however long term data has yet to be collected.

Challenges that face the future of facial transplantation lie in the ability to manage the adverse outcomes associated with chronic immunosuppression, the identification of suitable donors, and the justification of the procedure in regards to the risk–benefit and cost-benefit perspective. Traditional reconstructive techniques do not provide as esthetic a result, and at times function is also severely limited due to the nature of the defect. However, the risk associated with life long immunosuppression may warrant a drastic

attempt to reconstruct an otherwise healthy individual. Regardless, facial transplantation has been proven to be technically feasible and the results sustainable while on immunosuppression. As the surgical technique and management becomes defined, it is likely this modality of reconstruction will soon become another tier on the reconstructive ladder.

4.6 The "Ideal" Material for Craniofacial Reconstructions

The goal of a tissue-engineered scaffold is to create a local three-dimensional environment that encourages cell and tissue regeneration. The ideal material for bone tissue engineering should mimic the natural biomechanical properties of native bone. The scaffold must promote bone cells to adhere, proliferate and generate their natural extracellular matrix. By definition, a material that contains these properties is osteoconductive. In addition, the scaffold should stimulate the bone healing process by recruiting immature cells and facilitating the differentiation of preosteoblasts, rendering the scaffold osteoinductive (Albrektsson and Johansson 2001).

The craniofacial region is a complex mixture of bone, skin, muscle, cartilage, adipose tissue and other support tissues. As a result, there is a wide regional variation of mechanical properties within the craniofacial complex (Kretlow et al. 2009). The scaffold is the source of the construct's preliminary mechanical stability, and it should mimic the mechanical properties of the host environment. Given the complex nature of this region, the ideal scaffold should have spatially varying mechanical properties in order to best imitate the surrounding tissue. The diffusion of oxygen and other nutrients is critical to the survival of cells. As a result, a construct must contain an interconnected pore structure that contains pores that are a minimum of 100 μm in diameter (Bose et al. 2012). Finally, the scaffold should be biodegradable. That is, it should be designed to slowly resorb to allow for new tissue growth as the damaged tissue begins to heal.

To summarize, it is essential that the tissue-engineered scaffold provide a three-dimensional framework that encourages cellular growth and regeneration of the native tissue. There are a multitude of materials currently being investigated for these qualities in bone tissue engineering. The following section is only a snapshot of the current field and will focus on selected materials that are frequently used or have encouraging potential. A complete investigation into each type of material can be found in detailed studies and reviews elsewhere (Kretlow et al. 2007, 2009; Klouda and Mikos 2008; Hutmacher 2000; Babensee et al. 1998; Gunatillake and Adhikari 2003).

4.7 Scaffold Materials

The biomaterial selected for the bone tissue-engineered scaffold is a critical factor in the success of the device. As previously described, it must provide an environment that encourages cellular regrowth. Since there is a wide variation in the local cellular environment, the designer must pick a material that is designed best for that specific situation. Currently, oral and maxillofacial surgery utilizes a number of biomaterials that are approved for clinical applications. These materials can be broken down into two broad categories, the bioceramics and the biopolymers.

4.7.1 Calcium/Phosphate-Based Bioactive Ceramics

The most widely used bioactive ceramic products are mainly composed of calcium phosphate (CaP). Due to its tremendous osteoconductivity and bone regenerative capacity, this biomaterial has demonstrated a large amount of success with craniofacial bone defect repair and regeneration (Xu et al. 2006). Injectable CaP cements are an attractive option due to their ease of application and ability to be molded after injection (Liu et al. 2006). However, since they are a ceramic-based biomaterial, the degradation is limited and the biomaterial is brittle (Friedman et al. 1998). The resorption of CaP bioactive ceramics can be

significantly improved by the creation of macropores. When macropores are introduced into the CaP bulk, a significant amount of new bone formation occurs as the CaP degrades (Del Real et al. 2003). Several types of biomaterials have been utilized in order to increase the porosity of CaP constructs. One method is to introduce synthetic biodegradable polymer microspheres. Due to their long clinical history, (poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres have been incorporated into CaP constructs. The degradation of PLGA microparticles serves as a porgen, thus creating a porous CaP scaffold (Habraken et al. 2006; Ruhé et al. 2005, 2006). Additionally, the acidic environment created by the PLGA degradation favors the generation of the brushite bioactive phase. This CaP phase has drawn considerable attention due to an increased resorption rate compared to hydroxyapatite (Cama 2014). Natural polymers have also been applied for the fabrication of microparticles to accelerate CaP contruct degradation. Of the natural polymers, gelatin is especially notable due to the lack of an immune response. In addition, gelatin is completely resorbed under physiological conditions (Habraken et al. 2008). CaP-based scaffolds holds great promise in craniofacial tissue regeneration especially as recent 3D printing technology is emerging where customized defect constructs presenting various degrees of porosity and phase composition to tailor degradation kinetics may be more easily constructed (Bose and Tarafder 2012).

4.7.2 Polymer-Based Scaffolds

Polymeric-based materials utilized for bone tissue engineering scaffolds can either be naturally occurring or synthetic. Several natural polymers, such as collagen, gelatin and hyaluronic acid, are approved by the FDA for use in clinical settings and are commonly used in oral and maxillofacial surgery. The degradation of natural polymers relies on enzymes present within the host tissue, such as collagenase. Due to this, the degradation kinetics are hard to predict and will vary depending upon the activity of that individual's enzymes. Synthetic polymers that are biocompatible and biodegradable have several advantageous properties when compared to scaffolds composed of natural polymers. Since these polymers are synthesized in highly controlled reaction environments, their average molecular weight and size distribution can be varied. Thus, the designer has the ability to generate a material with reproducible and predictable characteristics, such as degradation kinetics and mechanical properties. As a result, the polymers can be tailored to match specific design criteria. Unlike natural polymers, degradation of synthetic polymers is by simple hydrolysis (Thomson et al. 1995). This eliminates the individual variation that is seen with enzymatic-dependent degradation of natural polymers.

There are numerous families of synthetic polymers that have been investigated for their application in craniofacial tissue engineering but the linear aliphatic polyester family, poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and copolymers (PLGA), are utilized extensively in the clinical environment (Zhang et al. 2014a). The degradation of these polymers is by hydrolysis of the ester bonds. The end result is either the production of lactic acid or glycolic acid. These end products can be cleared by normal metabolic processes and cleared as carbon dioxide. However, if the degradation rate exceeds the rate of clearance, a drop in local pH may occur that can result in cellular damage and potentially tissue necrosis.

Another important aliphatic polyester is poly(ε-caprolactone) (PCL) that has been investigated extensively due to some of its unique properties. Compared to other aliphatic polyesters, PCL can form biocompatible blends and copolymers with a wide range of other polymers. The degradation time for the homopolymer is approximately 2–3 years (Gunatillake and Adhikari 2003). However, generating a copolymer with other lactones decreases the degradation time. The copolymer and its concentration can be varied allowing the degradation rate to be precisely controlled. A polymer that has been developed and studied mainly for bone tissue engineering is poly(propylene fumarate) (PPF), an unsaturated linear polyester. Networks of PPF with tailored mechanical properties can be generated by using

a cross-linking agent or by photoinitiation with ultraviolent light (Timmer et al. 2003; Fisher et al. 2003a, b).

In summary, there is an extensive collection of biocompatible materials that has been studied for their use in bone tissue engineering. This is a brief overview of the major classes of biomaterials and their potential in tissue engineering applications.

4.8 Bioactive Factors

In order for craniofacial bone regeneration to occur, there must be cellular growth, differentiation and proliferation. These processes are highly regulated by the cell and must be initiated by specific bioactive molecules. There are several types of bioactive molecules that serve specialized roles in various physiological processes. The various families of these soluble molecules have previously been described (Singh et al. 2013) and are summarized below.

Growth factors are peptides that communicate cell signals by binding specialized cellular receptors located on a target cell. Once the molecule is bound to its cellular receptor, intracellular cascades are initiated that act to alter various cellular processes. The cellular response elicited depends upon the growth factor, the cell type and the bound receptor (Delany and Canalis 1997). In addition, a single type of growth factor can act on several different types of cells and generate an array of responses. Several growth factors have been investigated for their in vivo potential at regenerating bones of the oral and maxillofacial complex.

4.8.1 Bone Morphogenetic Protein (BMP)

BMPs are a family of proteins which play a pivotal role in embryological development, skeletal osteogenesis, bone maturation and homeostasis within bone (Cheng et al. 2003; Jansen et al. 2005; Schliephake 2002; Sikavitsas et al. 2001). Since Marshall Urist's initial discovery in 1965, over 20 different isoforms have been investigated for their osteoinductive potential (Porter et al. 2009). In order to induce a response from the target cell, BMP must bind to a transmembrane serine/threonine protein kinase. These receptors are classified into type I and type II receptors (Rosen and Wozney 2002). BMP-2, BMP-4 and BMP-7 are the only isoforms that have demonstrated in vitro success at stimulating de novo bone formation (Vo et al. 2012). BMP's osteogenic capacity is due to its ability to stimulate mesenchymal stem cells to differentiate towards an osteoblastic phenotype (Kretlow and Mikos 2007). Only a small amount of BMP is needed for a sizeable generation of bone however delivery of this bioactive molecule must be highly controlled, since overstimulation by BMP-2 has substantial side effects such as bone overgrowth and disorganization (Lee et al. 2014a). Several biomaterials have been developed to control and sustain the delivery of BMP-2 (Patel et al. 2008a).

BMP-2's osteoinductive potential has shown a large amount of success in both ectopic and orthotopic bone healing and in long bone fracture healing in rats, rabbits, and canines (Patel et al. 2008b; Nair et al. 2011). The success of these in vitro studies catalyzed several clinical investigations utilizing recombinant human BMP-2 (rhBMP-2). In 2002, the Food and Drug Administration approved rhBMP-2 for use in spinal fusion. While the use of rhBMP-2 in lumbar spinal fusion reduces the reoperation rate, operating time, and donor site morbidity, there is inconclusive evidence on whether BMPs are better than allografting alone (Resnick and Bozic 2013). Despite the unclear utility of BMP in lumbar spinal fusion, the use of BMP has also been the focus of several clinical investigations for oral and maxillofacial reconstruction (Jung et al. 2003; Moghadam et al. 2001; Carstens et al. 2005; Boyne et al. 2005).

Currently, the use of rhBMP-2 is approved for intraoral clinical applications and also for spinal fusion. There currently are several clinical reports regarding the use of rhBMP-2 for alveolar ridge augmentation, peri-implant bone regeneration, maxillary sinus floor augmentation, cranial vault reconstruction, mandibular reconstruction fol-

lowing tumor resection, and distraction-assisted alveolar cleft repair (Spicer et al. 2014; Tevlin et al. 2014). A review by Schliephake has examined the clinical efficacy of using BMPs in craniofacial regeneration (Schliephake 2015). This systematic review identified 47 different clinical cases where BMPs were utilized to enhance craniofacial tissue regeneration. The review notes that there is an 87.2 % success rate for primary reconstruction of the mandible due to benign bone pathologies and up to 94.6 % of alveolar cleft cases undergo successful bone regeneration (Schliephake 2015). Additionally, the review highlights the importance of an intact periosteum for successful regeneration with BMPs. While these clinical studies reveal the clinical potential of rhBMP-2 for large defects, there is need for further investigation of small to medium craniofacial defects.

Recently, BMP-2 has demonstrated preclinical success in the repair of peri-implant defects in canines. Jung et al. (2014) examined if loading a synthetic bone substitute (SBS) or polyethylene glycol hydrogel with recombinant human BMP-2 was more effective at regenerating bone for a peri-implant defect. The study revealed a statistically significant difference in the percentage of newly formed bone between the rhBMP-2 loaded materials and the control site at 8 weeks (10.75 % for SBS and 13.59 % for the hydrogel vs. 1.75 %). However, no statistical difference was seen in new bone formation between the SBS and hydrogel. This demonstrated that rhBMP-2 could enhance bone regeneration in critical size peri-implant defects irrespective of the carrier material.

4.8.2 Platelet Derived Growth Factor (PDGF)

Platelet derived growth factor (PDGF) is a polypeptide dimer with a molecular mass of 30 kDa (Bilezikian et al. 2002). It exists as three isoforms, two homodimers (PDGF-AA and PDGF-BB) and one heterodimer (PDGF-AB). Originally, PDGF-BB and PDGF-AB were isolated from platelets; however, further investigation has successfully isolated PDGF-AA from bone tissue. PDGF-BB and PDGF-AB are systemically circulating within platelet granules while quiescent osteoblastic cells produce PDGF-AA (Schliephake 2002). It was demonstrated in 1989 that rat osteoblastic tissue increases DNA synthesis and mitosis activity in response to PDGF. Secondary to this effect, osteoblastic cells were noted to have an increased rate of collagen synthesis. However, PDGF increased the rate at which collagen was degraded (Canalis et al. 1989). Numerous studies have since sought to gain a better understanding of this bioactive molecule and its possibilities for craniofacial tissue regeneration. Much like the research into BMPs has been focused around BMP-2, PDGF-BB is the only isoform evaluated in clinical cases. Interestingly, the majority of clinical literature examines the utility of rhPDGF for periodontal regeneration. A review by Kaigler et al. covers several case series where β-TCP was impregnated with rhPDGF for periodontal and peri-implant regeneration, highlighting the potential advantage rhPDGF can give to clinicians in treating challenging periodontal lesions (Kaigler et al. 2011). However, Kaigler notes the need for further investigation with larger clinical trials (Kaigler et al. 2011).

The clinical investigation into recombinant human growth factors has been largely centered on BMP-2 and PDGF-BB. The following sections will highlight other potent growth factors that have limited clinical data but have demonstrated a promising potential in preclinical investigations.

4.8.3 Transforming Growth Factor-Beta (TGF-β)

The transforming growth factor-beta (TGF-β) family is composed of several isoforms that are critical for regulating a wide range of processes such as cell differentiation, resolution of the inflammatory response and wound healing (Luginbuehl et al. 2004). TGF-β's activity is mediated by a transmembrane heterodimer receptor. Upon binding to TGF-β, the receptor

activates an intracellular protein cascade that upregulates target gene expression. Three isoforms, TGF-β1, TGF-β2, and TGF-β3, have been successfully isolated from bone and platelets (Holland and Mikos 2003). Of the three isoforms, TGF-β1 has been shown to induce new bone formation in non-human primates if in proximity to bone. However, unlike BMP-2, TGF-β1 is unable to induce bone formation at ectopic locations (Bonewald and Mundy 1990). Specific to oral and maxillofacial regeneration, TGF-β1 modulates bone cell metabolism and induces neovascularization (Schliephake 2002). In vivo, the extracellular matrix stores TGF-β1 and upon enzymatic cleavage of its disulfide bonds, TGF-β1 is released and can act upon other cells (Yamamoto et al. 2000). Thus biodegradable carriers for this growth factor would closely mimic the natural release of TGF-β1.

Lee et al. (2014b), recently examined the effect of combining rhBMP2, vascular endothelial growth factor (VEGF), and rhTGF-β1 with CaP carrier on bone regeneration in a critical-sized cranial defect rodent model. Although at 24 weeks there was a statistically significant difference in the percentage of newly formed bone with the rhBMP-2 and rhTGF-β1 treated defects versus the CaP cement alone (38.8 % for rhBMP-2 alone, 31 % for rhTGF-β1/rhBMP-2 and 12.7 % for the CaP cement control), the addition of rhTGF-β1 did not significantly increase new bone formation over rhBMP-2 alone. Yet, Simmons et al. (2004) investigation in dual growth factor delivery revealed that the addition of TGF-β1 and BMP-2 significantly improved the regeneration of bone compared to only BMP-2. The differing results serves to highlight the importance to further study the role TGF- β1s in craniofacial tissue engineering.

4.8.4 Fibroblast Growth Factor (FGF)

This family of growth factors is composed of polypeptides that are capable of binding heparin. These bioactive molecules mediate a diverse array of physiological activities ranging from promoting cellular division, angiogenesis, would healing and cellular differentiation (Kempen et al. 2010). The importance of FGF for bone formation is exemplified by achondroplasia. In this condition, a single activating point mutation in the FGF receptor 3 (FGFR3) results in a diminished capacity to perform endochondral ossification (Thisse and Thisse 2005). Within this family, FGF-2 is the most widely studied and utilized for bone regeneration purposes. FGF-2 does not have the ability to regenerate bone alone. However, it plays an important role in normal bone repair. FGF-2 has been used in various orthopedic settings (Kempen et al. 2010).

4.8.5 Insulin-Like Growth Factor (IGF)

This group of growth factors exists as a single chain of 70 amino acids and exhibits a similar structure to that of pro-insulin (Schliephake 2002). Two isoforms, IGF-I and IGF-II, have been isolated from mammalian tissue. However, human osteoblastic cells mainly synthesize IGF-II (Conover and Rosen 2002). The physiological activity is mediated through the IGF-I receptor, which is a tyrosine kinase receptor. This receptor has the strongest affinity for IGF-I. Both of these polypeptides play an integral role in the proliferation of osteoblasts and chondrocytes (Sikavitsas et al. 2001). Interestingly, BMP-2 induces increased synthesis of IGF-I and IGF-II in rodent osteoblastic tissue. Additionally, it may serve to increase osteoblastic cells within the osteon (Conover and Rosen 2002). While little is known about human IGF-II regulation, Knutsen et al. showed that in osteosarcoma cells an increased level of BMP-7 significantly increased the production of IGF-II and alkaline phosphatase (Knutsen et al. 1995). As previously mentioned, the mechanical properties of the tissue are critical for bone regeneration. To this point, increases in mechanical stress resulted in increased in vitro osteoblastic tissue proliferation secondary to increased levels of IGF-II (Fitzsimmons et al. 1992).

The development of recombinant human IGF-I has sparked several preclinical and clinical models based on its clinical utility. However, few studies have examined the effect of IGF-I alone.

In one study, the efficacy of IGF-I to induce bone regeneration in critical size calvarial defects was investigated. IGF-I was systemically administered and resulted in total reconstitution of the calvarial defect (Thaller et al. 1998). Despite the potential demonstrated by this study, a significant portion of the literature explores the dual effect of IGF-I and PDGF. A review by Alvarez et al. covers several preclinical animal studies where IGF-I and PDGF were used in the regeneration of periodontal tissue. This review notes that the dual delivery of IGF-I and PDGF significantly improved de novo bone formation in canine animal models (Alvarez et al. 2012). However, the amount of pre-clinical literature concerning this growth factor remains small.

The use of growth factors to enhance or accelerate bone regeneration has the potential to enhance the current treatment of several pathological conditions. Due to this, a large amount of research has explored the use of bioactive molecules for bone and craniofacial regeneration. Although our knowledge of bone biology has revealed that there are numerous growth factors critical to bone regeneration, only a handful have been applied in the clinical setting. Additionally, when growth factors are utilized for human studies, concentrations far exceeding their natural levels are needed to achieve similar osteoinductive results as seen in animal models. The supraphysiological amount of exogenous growth factors may have detrimental implications. Currently, BMP is increasing our knowledge into the potential adverse events associated with growth factors (Epstein 2013; Argintar et al. 2011; Chan et al. 2014; Dimitriou et al. 2011). In addition, these supraphysiological concentrations may prove too costly when compared to alternative tissue engineering techniques. However, as technology for the production of biologically active recombinant human proteins improves, the cost will decrease.

4.9 Gene Delivery

The drawbacks associated with the use of exogenous growth factors delivery have sparked interest into the use of gene therapy to stimulate the endogenous synthesis of bioactive molecules. In this technique, exogenous genes for a specific soluble factor are delivered to a target cell population. Successfully transfected cells will constitutively synthesize and secrete the target protein into their local environment. This methodology potentially reduces the current diffusion and degradation limitations associated with exogenous growth factor delivery (Fischer et al. 2011). In order for gene therapy to successfully transfect a cell, the desired gene must be able to integrate into the target cell's genome, the target cell must be capable of transcribing the coding gene sequence into mRNA, and finally the target cell must be able to translate the mRNA into a physiological active protein. Oral and maxillofacial tissue regeneration has been successfully performed by transferring DNA utilizing viral (Hu 2014) and non-viral vectors (Wegman et al. 2013).

In order for viruses to be infectious they must successfully deliver their genetic material to the target cell. Tissue engineering employs viral vectors for their inherently efficient delivery techniques. One of the major disadvantages to this method is the public's misconceived notion that all viruses are dangerous (Scheller and Krebsbach 2009). While the viral proteins can trigger an immune response, several techniques have been developed to minimize this reaction (Fischer et al. 2011). The ideal tissue engineering viral vector has yet to be identified or developed. Several viruses have been utilized in craniofacial tissue engineering. The most commonly applied viral vectors are retroviruses, adenoviruses, adeno-associated virus, and lentivirus (Scheller et al. 2012). Constructs loaded with retroviruses integrate well into the target cell's genome and generate a weak immunological response. However, few studies have investigated the use of retroviruses due to the potential of activating the transcription of an oncogene (Fischer et al. 2011).

Adenoviral vectors can efficiently transfect mitotically active and quiescent cells. These vectors are suited for the short-term delivery of genetic information. In addition, they can be produced in high titers making them more cost-effective. The biggest drawback is the robust

immunological response the host generates against this vector. In addition, most recipients have previously been exposed to adenovirus and thus would render the viral delivery ineffective. Despite these drawbacks, adenovirus-mediated BMP-2 synthesis to regenerate craniofacial defects has been reported in the literature. Sun et al. (2013) described the use of adenovirus-induced BMP-2 production to repair mandibular defects in rabbits. In this study, periosteal-derived cells were successfully transfected with adenovirus coding for the rhBMP-2 gene. Bone and cartilage tissue were measured at 4, 8, and 12 weeks. At all three time points, there was a statistically significant increase in the percentage of new bone formation for the adenovirus-modified defect as compared to the defect with a tissue engineered bone graft (30.2 % for the adenovirus-modified vs. 23.4 % for the unmodified tissue engineered bone at 12 weeks). Although viral vectors have shown promising potential in vivo, the concern over their current drawbacks has guided the discovery of non-viral based vectors.

Despite a great diversity in techniques, the majority of non-viral gene therapy is performed with DNA plasmids (Wegman et al. 2013). DNA plasmids are small, circular double-stranded DNA that can easily be fabricated within bacteria. These immunogenic structures are able to transfect target cells with much larger genes. While DNA plasmids address many of the potential problems associated with viral vectors, their in vivo transfection efficiency is reduced. Additionally, the DNA plasmid must successfully cross the cell membrane without altering the coded information. Several approaches, such as lipoplexes and polyplexes, assist the entry into the target cell and prevent enzymatic degradation of the DNA plasmid. Specifically, lipoplexes are a modality for the delivery of genetic information by non-viral vectors that have been applied to tissue engineering. Lipoplexes promote cDNA uptake by stimulating endocytosis (Wegman et al. 2013). These carriers have successfully been used to stimulate the development of bone-like cells (Oliveira et al. 2009). However, this modality can destabilize cell membranes if local concentrations are sufficiently high. Another promising non-viral gene therapy modality is to utilize a biodegradable tissue engineering scaffold to transfect the target cell population.

In order for a biodegradable polymer to be used, it must be positively charged, thus neutralizing the negatively charged cDNA and encouraging endocytosis (Scheller et al. 2012). The utilization of biodegradable scaffolds to transfect cells by non-viral gene therapy has been reported in the literature. Loozen et al. (2013) utilized a porous bioprinted biodegradable scaffold as a gene delivery system. The rhBMP-2 cDNA plasmid was successfully integrated into the target cells and induced cellular differentiation towards the osteoblastic lineage. In addition, the transfected cells showed a statistically significant increase in BMP-2 production after 14 days compared to the non-transfected cells despite no in vivo bone formation after 6 weeks. This highlights that bioprinted constructs may possibly serve as a gene delivery system, but there is still need for further investigation into this technology.

4.10 Mesenchymal and Adipose Derived Stem Cells

All the techniques discussed thus far have not employed the use of cells within the scaffold. Instead, enhancement of the local environment encourages native cells to populate the scaffolds and promote tissue regeneration. The success of these strategies is largely dependent upon the tissue's regenerative capacity. Some pathological insults or clinical treatments may hinder the defect's healing capacity. In order to circumvent this constraint, several craniofacial tissue engineering strategies utilize cell-seeded scaffolds.

Mesenchymal stem cells (MSCs) are well known for their intrinsic ability to differentiate into several tissue types such as bone, cartilage, muscle and adipose (Caplan 1991). They are relatively easy to expand in culture and can be harvested from a number of autologous locations without significant donor site complications (Risbud and Shapiro 2005). Due to these advantageous properties, numerous in vitro studies have investigated the application of MSCs for bone and cartilage tissue engineering (Castano-Izquierdo et al. 2007; Kim et al. 2007; Gomes

et al. 2006; Park et al. 2007). Specific to oral and maxillofacial tissue engineering, MSCs have been shown to enhance de novo bone formation and healing when utilized with synthetic and ceramic scaffolds (Shang et al. 2001; Levi et al. 2012). A review by Zhang et al. (2014b) covers the current clinical application and impact of MSCs in craniofacial tissue engineering. This manuscript demonstrates the important role MSCs may 1 day play in craniofacial regeneration. However, the review stresses the need to better understand the mechanism MSCs play in defect regeneration and the potential long-term adverse effects.

The successful identification of mesenchymal stem cells within adipose tissue has led to an abundance of research into adipose derived stem cells (ASCs). ASCs have not been widely investigated within oral and maxillofacial regeneration but several clinical studies have demonstrated the clinical potential of ASCs in treating traumatic and congenital craniofacial defects. To highlight a few of these clinical cases, autologous ASCs were applied with autologous fibrin glue to a repair a 120 cm^2 calvarial defect (Lendeckel and Jödicke 2004). At 3 months, CT evaluation revealed marked ossification of the defect. In another study, an adolescent boy with Treacher Collins syndrome was treated with a combination of ASCs, BMP-2, bone allograft and periosteum (Taylor 2010). At 4 months, CT scan reveled complete bilateral reconstruction of the patient's zygomas with no ectopic bone formation.

4.11 Future Challenges for Craniofacial Tissue Engineering

Over the past two decades there has been a substantial amount of progress in tissue engineering. Currently there are numerous laboratory methods to regenerate oral and maxillofacial tissue. In addition, recent advances in tissue engineering strategies and techniques have demonstrated an increased efficacy. However, several hurdles must be overcome in order for these laboratory fabrication modalities to be translated into clinically relevant product. Some of these issues that future investigations must aim to understand is how a suitable population of cells can be identified and harvested that fulfill the physiological role of the native tissue, how exogenously or endogenously supplied growth factors can best support cellular differentiation and reproduction, and the role the microvasculature plays in tissue regeneration. As our ability to simulate physiological microenvironments increases, it is essential that our understanding of adverse events, such as infection, must improve. Scaffolds with antimicrobial drugs demonstrate the importance of the collaborative effort that must exits between the clinicians and the engineers. Thus, the future of craniofacial tissue engineering depends on the ability of the clinicians and the engineers to communicate together.

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Engineering Pre-vascularized Scaffolds for Bone Regeneration

5

Giada D.G. Barabaschi, Vijayan Manoharan, Qing Li, and Luiz E. Bertassoni

Abstract

Survival of functional tissue constructs of clinically relevant size depends on the formation of an organized and uniformly distributed network of blood vessels and capillaries. The lack of such vasculature leads to spatio-temporal gradients in oxygen, nutrients and accumulation of waste products inside engineered tissue constructs resulting in negative biological events at the core of the scaffold. Unavailability of a well-defined vasculature also results in ineffective integration of scaffolds to the host vasculature upon implantation. Arguably, one of the greatest challenges in engineering clinically relevant bone substitutes, therefore, has been the development of vascularized bone scaffolds. Various approaches ranging from peptide and growth factor functionalized biomaterials to hyper-porous scaffolds have been proposed to address this problem with reasonable success. An emerging alternative to address this challenge has been the fabrication of pre-vascularized scaffolds by taking advantage of bio-manufacturing techniques, such as soft- and photo-lithography or 3D bio-printing, and cell-based approaches, where functional capillaries are engineered in cell-laden scaffolds prior to implantation. These strategies seek to engineer pre-vascularized tissues in vitro, allowing for improved

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anastomosis with the host vasculature upon implantation, while also improving cell viability and tissue development in vitro. This book chapter provides an overview of recent methods to engineer pre-vascularized scaffolds for bone regeneration. We first review the development of functional blood capillaries in bony structures and discuss controlled delivery of growth factors, co-culture systems, and on-chip studies to engineer vascularized cell-laden biomaterials. Lastly, we review recent studies using microfabrication techniques and 3D printing to engineer pre-vascularized scaffolds for bone tissue engineering.

Keywords
Vascularization • Bone regeneration • Bone scaffolds • Angiogenesis • Microfabrication • Bioprinting • Tissue engineering

5.1 Introduction

Treatment of bone related diseases and injuries results in a significant financial burden for health care systems of countries worldwide. In the United States alone, an estimate of approximately $796.3 billion is spent yearly for the treatment of bone related diseases (Reports of the Surgeon General 2004). This expenditure is expected to increases drastically with the increasing population age. For instance, the number of hip fractures in the world is predicted to increase from 1.7 million in 1990 to a staggering 6.3 million by 2050 (Johnell 1997; Praemer et al. 1992; Cheung 2005). Autologous bone grafts, allogeneic scaffolds or synthetic and avascular materials, including polymers and metals, are the current materials of choice for treatment of bone loss or repair (Goldberg and Stevenson 1987). Despite the use of various biomaterials and different treatment strategies, only approximately 30 % of patients treated with bone replacements regain function without requiring further surgical intervention (Woolf and Pfleger 2003). The current gold standard of treatment for bone regeneration is the use of autologous bone grafts, which is surgically removed from healthy structures in a patient's body and then re-implanted in the compromised areas (Reports of the Surgeon General 2004). However, these techniques have largely been recognized to face serious limitations, such as the need for invasive surgical procedures, difficult post-operative care, high cost, and unpredictable outcomes (Goldberg and Stevenson 1987). Tissue engineering has long held great promises as improved treatment options for these conditions. However, successful outcomes using tissue engineering approaches for bone regeneration remain far from ideal in the clinical setting. Hence, the development of improved strategies to regenerate bone remains a major clinical need.

Long bones are made of a hard external cortical layer constituting approximately 80 % of the total bone mass and a highly porous internal trabecular layer (Curray 2006; Clarke 2008) (Fig. 5.1). The external layer is highly dense with a mechanical strength of 130–190 MPa and a porosity of only about 20 % (Zimmermann et al. 2011; Koester et al. 2008; Karageorgiou and Kaplan 2005). On the other hand, trabecular bone has a strength of only about 10 % of the cortical layer (Hernandez and Keaveny 2006). In spite of their structural differences, both layers in the bone structure rely on a vast and intricate vasculature for their homeostasis (Kanczler and Oreffo 2008; Brandi and Collin-Osdoby 2006). In mature bone, the existing vasculature plays an important role in providing the necessary signaling factors, hormones and metabolites needed to recruit circulating cells, such as haematopoietic cells to the bone marrow (Brandi and Collin-Osdoby 2006; Schmid et al. 1997). Moreover, both in engineered tissue constructs and in the body, cells must be sufficiently close

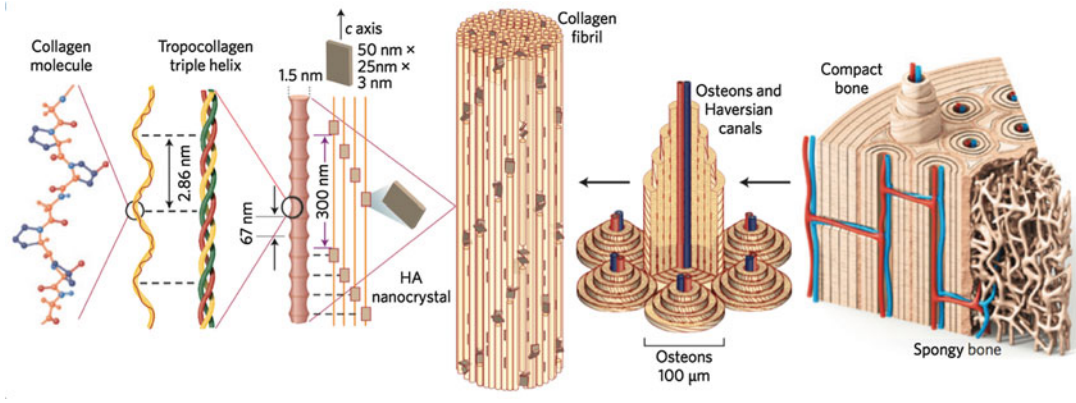

Fig. 5.1 Hierarchical organization of vascularized bone, from single collagen molecules to compact and spongy bone (Reproduced from Wegst et al. with permission. Copyright 2015 Nature publishing group)

to oxygen and nutrient supplies (Jain et al. 2005; Laschke et al. 2009; Sasagawa et al. 2010) to prevent the formation of necrotic spots (Radisic et al. 2004). The structural arrangement of osteons, the functional units of cortical bone (Wegst et al. 2015), provides the necessary physical space for the blood vessels in the highly dense cortical layer by means of central haversian canals (Portal-Nunez et al. 2012; Risau 1997). Hence, approaches for engineering bone need to be customized to comprehensively facilitate biomechanical integrity, remodeling and metabolic activity as regulated by an existing vasculature.

There are a number of strategies that have been studied towards the formation of microvascular networks in tissue engineering constructs. A large body of work on bone regeneration has been devoted to understanding the formation of microvascular beds that appear after osteoinductive and osteoconductive scaffolds are implanted in the body (Mercado-Pagan et al. 2015; Liu et al. 2013, 2015; Zhang et al. 2012). These strategies rely on host-capillary invasion mediated by angiogenesis and vasculogenesis on tissue constructs *post-implantation*. Unfortunately, the slow rate of host-capillary migration (Clark and Clark 1939) in the implanted scaffolds limits the usefulness of these methods to engineer fully vascularized bone. An exciting alterative to overcome these limitations has been the fabrication of biomimetic microvascular networks in-vitro and *prior to implantation*.

The process of engineering vascularized tissues in-vitro generally relies either on (1) cell-based strategies or (2) controlled manufacturing of microchannel networks in biomaterials: (1) Cell-based approaches involve primarily endothelial cells, which form self-organized capillary beds embedded within engineered tissue constructs (Black et al. 1998; Chen et al. 2012; Chiu et al. 2012; Elbjeirami and West 2006; Leslie-Barbick et al. 2009, 2011a, b; Peters et al. 2002). (2) Biomanufacturing approaches for engineering vascularized tissues use precise micro-scale fabrication methods, such as soft- and photo-lithography or 3D printing, to create microchannel networks that function as templates for subsequent population of endothelial cells (Mercado-Pagan et al. 2015; Liu et al. 2015, 2013; Chiu et al. 2012; Bae et al. 2012; Baranski et al. 2013; Bertassoni et al. 2014a; Lee et al. 2014; Miller et al. 2012; Obregon et al. 2015), thus forming vascular networks within engineered tissue constructs.

In this book chapter we provide an overview of the mechanisms involved in bone vascularization and angiogenesis. We discuss existing and emerging strategies to engineer pre-vascularized tissues, including cellular- and biofabrication-based approaches, particularly in the scope of bone and load bearing tissue engineering. We

argue that a thorough understanding of the mechanisms underlying the natural formation of vascular tissues in the body, combined with an overview of existing methods and challenges related to fabrication of pre-vascularized scaffolds, may provide the fundamental knowledge required to develop effective approaches to engineer vascularized tissues.

5.2 Vasculogenesis and Angiogenesis

In order to better understand the existing strategies to engineer vascularized bone and mineralized tissues, it is relevant to discuss the processes that guide and control formation of blood vessels and capillaries in the human body. Blood vessels are formed through two key and often complementing processes: vasculogenesis and angiogenesis (Risau 1997; Folkman and Shing 1992; Vailhe et al. 2001) (Fig. 5.2). Vasculogenesis is the origination of new blood vessels from angioblastic progenitor cells (APCs) or endothelial progenitor cells (EPCs). APCs are recruited during embryonic and fetal growth, whereas EPCs are seen in post-natal vasculogenesis comprising wound and fracture healing, myocardial infarction, ischemia, atherosclerosis and tumor growth (Folkman and Shing 1992; Real et al. 2008; Invernici et al. 2008). Conversely, angiogenesis utilizes sprouting or intussusception to generate microvessels from already existing blood vessels and capillaries (Real et al. 2008). Driven by pro-angiogenic factors, sprouting is characterized by matrix remodeling to allow for the endothelial cells to branch out and proliferate. Subsequently, the migrating cells re-assemble to produce lumen and eventually mature into a functional endothelium (Folkman and Shing 1992; Invernici et al. 2008; Ribatti and Crivellato 2012). These systemic sequence of events is divided under two distinct phases of sprouting angiogenesis: growth and stabilization (Ribatti and Crivellato 2012).

In the initial growth phase, vasodilation of existing vessels causes an increase in permeability

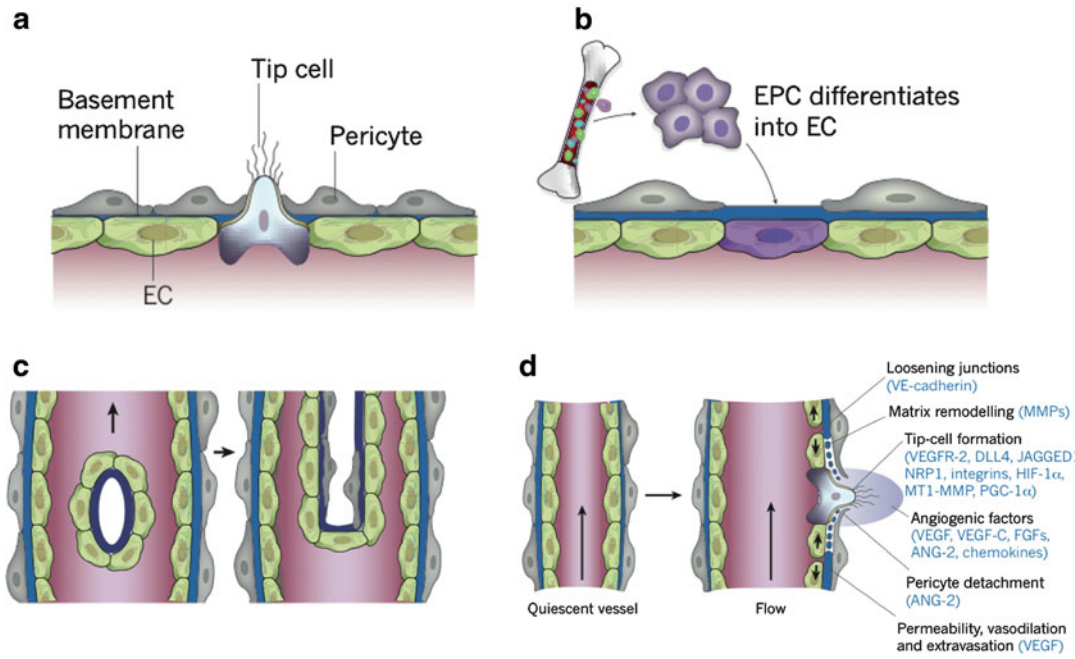

Fig. 5.2 Different steps involved in vasculogenesis and angiogenesis, and influence of different growth factors in formation of new vessels. (**a**) Sprouting angiogenesis. (**b**) Vasculogenesis. (**c**) Intussusception. (**d**) Selection of tip cell (Reproduced from Carmeliet and Jain with permission. Copyright 2011 Nature publishing group)

and degradation of the basement membrane, enabling the migration of endothelial cells towards the tissue mass, expressing angiogenic growth factors, and inducing proliferation and tubulogenesis. The migration of endothelial cells is mainly facilitated by the VEGF receptor 2 (VEGFR2), which aligns the cells with respect to a gradient in the growth factor, VEGF A (Lamalice et al. 2007). During the stabilization phase, endothelial cells cease to proliferate and a basement membrane starts to form around the newly derived capillary (Bergers and Song 2005). Further, this phase is completed by the recruitment of pericytes around the neocapillary (Bergers and Song 2005). Alternatively, intussusception is the phenomenon of vascular network expansion through transcapillary pillars, which ultimately bridges opposite capillary walls. The formation of transcapillary bridges is followed by endothelium reorganization, invasion of myofibroblasts and pericytes leading to vascular network remodeling. Intussusception occurs at a relatively rapid pace and with lower proliferation compared to sprouting (LeBlanc et al. 2012).

During bone formation, more specifically, signaling molecules secreted by the bone endothelium help to recruit circulating cells to coordinate skeletal development (Brandi and Collin-Osdoby 2006). During this process, two well-defined and distinct set of events that are orchestrated by the bone vasculature take place, namely endochondral and intramembranous ossification (Kanczler and Oreffo 2008; Hu et al. 2013). Endochondral ossification occurs in regions occupied by cartilage, where the transition from cartilage to bone depends on the level of vascularization on the particular site of ossification (Gerber and Ferrara 2000). During embryonic bone growth, blood vessels originate from the perochondrium and invaginate into the cartilage by the development of immature vascular networks that enter cartilage canals already formed in the expanding cortical bone (Burkus et al. 1993). After birth, canals formed for blood vessels develop together with maturation of endothelium monolayers that invade cartilage growth plates (Burkus et al. 1993). Lastly, during adulthood, angiogenesis becomes a more dynamic process, which is either up- or down-regulated depending on the remodeling needs of a particular site due to aging, disease, or injury. Intramembranous ossification, on the other hand, is characterized by growth of microcapillary networks into the mesenchymal zone, triggering the recruitment and differentiation MSCs into mature osteoblasts. During this process osteoblasts secrete bone matrix surrounding blood vessels forming the trabeculae. As the trabeculae increase in size and density, they coalesce forming grooves around existing blood capillaries. This interconnected and poorly organized immature bone structure is later replaced by a more organized, stronger, lamellar bone, which emerges as a consequence of continuous deposition of bone matrix from the surface of the haversian canals inwards, towards existing blood vessels (Rodan and Raisz 1996).

5.3 Cellular Approaches to Engineer Vascular Networks

Cellular approaches to engineer vascularized tissues are characterized by the spontaneous organization of cells to form architecturally and functionally relevant vascular networks, by secreting their own extra cellular matrix and remodeling the microenvironment without external intervention (Bae et al. 2012). A variety of pre-vascularization approaches have exploited the inherent propensity of endothelial cells to form capillary-like structures under specific conditions.

A number of studies focused on understanding the relationship of osteoprogenitor and endothelial cells have shed light onto the importance of co-culturing different cell types to mimic the formation of vascularized bone. Several other studies have demonstrated that primitive microvascular structures are formed when endothelial cells are cultured under specific conditions inside hydrogels that mimic the extracellular matrix (ECM) 3D microenvironment, particularly in the presence of growth factors such as VEGF (Folkman and Haudenschild 1980). An early study by Wang et al. for instance, demonstrated

that the presence of endothelial cells near osteoblast-like cells enhance the alkaline phosphatase activity of the bone-derived cells (Wang et al. 1997). It has also been well documented that endothelial cells cultured with smooth muscle progenitor cells can form primitive microvascular networks that anastomose with the host vasculature upon implantation (Black et al. 1998; Sato et al. 1987; Berthod et al. 2006; Supp et al. 2002; Vernon and Sage 1999; Kubota et al. 1988; Montesano et al. 1983).

A key study attempting to control the formation of vascularized bone by taking advantage of the cross-communication between endothelial and bone progenitor cells was published by Tsigkou et al. In this work, the authors showed that human mesenchymal stem cells (hMSCs) co-cultured with endothelial cells (ECs) in a fibronectin-containing collagen gels functioned as a source of perivascular cells, hence promoting the formation of a mature and stable vasculature both in vitro and in vivo over time (Tsigkou et al. 2010). The endothelial cells formed tube-like structures and stable networks 4–7 days after implantation, whereas anastomosis occurred after 11 days and mineralization was present after 4 weeks. The authors demonstrated that hMSCs were essential for stable vasculature development, primarily due to their potential to differentiate towards a perivascular lineage expressing smooth muscle cell (SMC) phenotype. Interestingly, a delayed differentiation of hMSCs towards the perivascular phenotype, due to the lack of TGF-β in the media, formed more stable and extensive microvascular networks.

In a similar report, Ying et al. encapsulated human blood-derived endothelial colony-forming cells (ECFCs) and bone marrow derived mesenchymal stem cells (MSCs) in a methacrylated gelatin hydrogel (GelMA) (Chen et al. 2012). The presence of MSCs not only induced the self-assembly of ECFCs into tubular structures, but also improved their survival rate. However, this effect was not observed in a mono-culture system. The self-assembly induced the vacuoles of ECFCs to coalesce and form capillary like structures with a lumen, also expressing intercellular CD-31 markers. At the same time, the study demonstrated that MSCs provided coverage of the capillary like structures, expressing α-smooth muscle actin (α-SMA) and presenting pericyte-like phenotype. It is noteworthy that this effect was highly dependent on the crosslinking degree and the stiffness of the hydrogel, which could be easily controlled by polymerization with UV light, or the degree of methacrylation of the polymer. Once implanted, these capillary structures also showed a strong presence of erythrocytes within their lumen only after 6 days. A more recent and clinically relevant approach utilized human pluripotent stem cells (hPSCs) to induce co-differentiation of this cell population into early vascular cells that could mature into a bilayer of endothelial cells and pericytes. Interestingly, the hPSCs-derived primitive networks that formed inside 3D collagen gels went on to self organize and form more stable networks that survived implantation, integrated with the host vasculature, and established functional blood flow over time (Kusuma et al. 2013).

5.3.1 Growth Factor Delivery

In vivo formation of a vascular plexus is widely known to be controlled by the association of a cascade of factors and kinetics, spatially and temporally designed to achieve angiogenesis and tubulogenesis. Thus, controlled use of pro-angiogenic growth factors (Vailhe et al. 2001; Jansen et al. 2005; Simmons et al. 2004), like vascular endothelial growth factors (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), in cell-loaded scaffolds has been extensively studied (Bae et al. 2012; Jansen et al. 2005; Simmons et al. 2004; Nguyen et al. 2012). A number of comprehensive reviews have been published on this topic, and we encourage the reader to refer to the cited literature for a more throughout understanding of the complexity of existing methods for growth factor delivery in bone vascularization. The delivery of growth factors using biomaterials for vascular tissue engineering has been studied using primarily one of the tow methods: physical entrapment or chemical immobilization. A few

noteworthy examples of these strategies will be described below.

5.3.1.1 Physical Entrapment of Growth Factors

Physical entrapment of growth factors consists of embedding specific biomolecules, generally in polymeric matrices that allow for controlled diffusion into the surrounding matrices. Such process is usually performed prior to inducing gelation or solidification of the delivery vehicle (Mehta et al. 2012), provided that it is not harmful for the biological activity of the growth factor. Hydrogels are commonly used for these applications due to their controllable porosity, mesh size, degradation rates, and other physical properties relevant for controlled diffusion (Slaughter et al. 2009; Annabi et al. 2014). Additionally, growth factor release can be controlled by regulating the rate of swelling of the gel matrix, the mechanism of degradation, the interactions holding the polymer together, and other factors.

Several studies have shown that determining the optimal rate of release of VEGF can be critical for ensuring the therapeutic effect of the growth factor in inducing angiogenesis and vasculogenesis (Street et al. 2002). For this reason, an important challenge in the field of vascularized bone regeneration has been to deliver angiogenic growth factors, such as VEGF and PDGF, in combination with osteoinductive growth factors, such as BMPs in a controlled fashion. Polymeric micro- and nano-particles exhibiting different physical characteristics and embedded with different growth factors have been extensively researched to address this challenge. Richardson et al. (2001), for instance, studied the effects of the combination of both VEGF and PDGF in a degradable PEG-based hydrogel scaffold. Growth factors were embedded either by mixing lyophilized VEGF directly with poly(lactide-co-glycolide) pre-polymer before constructing the scaffolds, or by encapsulating PDGF in poly(lactide-co-glycolide) microparticles that were subsequently embedded in the VEGF-containing scaffolds. Results showed that release of these two factors alone did not allow for a stable and mature formation of vascular networks, while their combination had a more successful outcome. This results not only confirmed the role of spatial and temporally controlled biological organization in vascular formation, but also provided a powerful tool for therapeutic strategies. Controlled release of other factors involved in bone formation, such as fibroblast growth factor-2 and insulin growth factor-1 have also been demonstrated in calvaria defect and tibial fracture models (Kimoto et al. 1998; Oest et al. 2007). In summary, the delivery of growth factors in micro and nanoparticles by physical entrapment allows for a more controlled delivery and more adequate approach to mimic the gradual cascade of biological events occurring during formation of vascularized bone (Ennett et al. 2006; Santo et al. 2009; Chen et al. 2009; Patel et al. 2008; Yilgor et al. 2010; Oldham et al. 2000), then simple bolus injection of growth factors in the site of regeneration.

In a different approach, Jeong et al. developed a "living" microvascular stamp engineered by encapsulating fibroblasts, which endogenously express angiogenic factors, into a three-dimensio-nally fabricated permeable poly(ethylene glycol) diacrylate/methacrylic alginate (PEGDA-MA) hydrogel via stereo lithography (SLA). The permeable hydrogel allowed for sustainable release of growth factors at pre-defined locations, after the "living" stamps were implanted onto a chick embryo chorioallantoic membrane. This approach illustrates how endogenous growth factors can also be used for therapeutic applications using simple physical entrapment methods, not only in vitro but also in vivo (Jeong et al. 2012).

5.3.1.2 Chemically Immobilized Growth Factors

Chemically immobilized growth factors have been proposed to better mimic the dynamic mechanisms of binding and selective delivery of factors that is operated in the body (Jeon et al. 2007; Lutolf and Hubbell 2005). By chemically immobilizing growth factors to the delivery vehicle, the actual factor may also be released only upon degradation of the vehicle itself, thus

providing another mechanism of controlled delivery that is not dependent upon diffusion, rather on controlled enzymatic degradation or hydrolysis (Lutolf et al. 2003).

Barbick and colleagues, in a series of papers, focused their attention in the optimization of vasculogenesis and tubular formation in PEG-based hydrogels modified with a VEGF mimetic (QK) peptide (Leslie-Barbick et al. 2009, 2011a, b). PEG-based hydrogels can undergo chemical modification to retain growth factors, adhesive ligands and degradable peptides in their matrix (Leslie-Barbick et al. 2009, 2011a, b). In a specific study, a laser scanning lithograph (LSL) technique was used to precisely control cross-linking of a polymer coupled with the VEGF mimetic growth factor at specific regions in a PEG-based hydrogel. Results showed that VEGF in the hydrogels enhanced the angiogenic processes and tubular formation in vitro, especially in the LSL pre-patterned areas after 2 days. In addition, covalent bonding and immobilization of these factors and ligands allowed the spatial distribution and local controlling on the positioning in the entire construct (Leslie-Barbick et al. 2009, 2011a, b).

Alginate hydrogels functionalized with BMPs and VEGF have also been shown to allow for sustained released of growth factors for segmental bone repair (Oest et al. 2007). Immobilizing specific matrix components, such as proteins or growth factors, or even only RGD adhesive biding sites (Alsberg et al. 2003; Comisar et al. 2007; Ratner 1996; Rowley et al. 1999), to synthetic biomaterials is arguably the most effective method to more closely mimic the natural ECM in controllably delivering molecules to improve vascularization in bone regeneration.

5.3.2 On-Chip Vascularization Studies

Although we are unaware of existing reports specifically studying bone vascularization using lab-on-a-chip methods, a number of recent publications have provided important information about vascularization of tissue-engineered constructs using on-chip approaches. A key publication on the integration of microfluidics and bone tissue engineering that requires attention, and which represented the first bone-on-a-chip model, was focused on the engineering of bone marrow, rather than bone vascularization per se (Torisawa et al. 2014). In this work, the authors first engineered new bone in vivo by implanting a bone inducing biomaterial scaffold in a subcutaneous mouse model. The engineered tissue was then retrieved surgically and cultured under continuous perfusion with culture medium in a microfluidic device. The bone marrow on-a-chip showed preserved hematopoietic stem and progenitor cell function for at least 1 week in the device, also forming an improved platform to study bone marrow toxicity under physiologically relevant culture conditions (Torisawa et al. 2014).

In another recent study of vascularization on-a-chip, Cuchiara et al. reported the co-culture of HUVECs and mesenchymal progenitors (10T1/2) cells in a hybrid matrix of collagen and fibronectin in a hybrid microfluidic device. The authors showed stable pre-vascular network formation with the 10T1/2 cells expressing a pericyte phenotype (Cuchiara et al. 2012). This co-culture system was used to create perfusable structures by integrating a microfabricated cell-laden hydrogel with a microfluidic platform (Cuchiara et al. 2012). The hydrogel containing HUVECs and 10T1/2 cells was sandwiched between two separate channels, where one channel was perfused with culture medium and the other with PBS, creating a gradient in the nutrient concentration over the thickness of the hydrogel. This enabled the cells to proliferate, degrade the matrix and remodel along the gradient to produce perfusable vascular channels.

In another attempt to replicate and study the physiologic conditions for angiogenesis and vasculogenesis on-a-chip, Nguyen et al. and Kim et al. developed very similar methods to generate perfusable 3D microvessels in vitro. Both papers utilized relatively simple microfluidic chips to generate spatially controlled gradients of angiogenic growth factors and co-cultures of endothelial cells with stromal fibroblasts, pericytes or

cancer cells. These reports not only elucidated the steps involved in microvessel formation and maturation in ECM derived 3D matrices (Kim et al. 2012, 2013, 2015), but also provided clear visualization of angiogenic sprouting originating from a larger engineered parent vessel, forming stable vascular networks that migrated towards the gradient of growth factors on chip (Nguyen et al. 2013). The advantages of on-chip models for studying angiogenesis and vasculogenesis are countless (Bischel et al. 2013; Zheng et al. 2012), and the field of vascularized bone tissue engineering can benefit tremendously from this set of techniques.

5.4 Biofabrication Approaches to Engineer Pre-vascularized Scaffolds

Although several advances have been made towards achieving vascularization in bone scaffolds, growth factor delivery and cell-based approaches generally lack control over the arrangement of the engineered vascular networks in 3D space, thus leading to inefficient distribution of oxygen, nutrients and waste products in 3D tissue constructs (Baranski et al. 2013; Chen et al. 2003). Vascular systems are inherently highly organized and hierarchically structured networks to enable efficient oxygenation of every cell in the body. Hence, coupling microenvironmental cues with microfabrication methods is an exciting alternative to engineer functional vascular networks.

An ideal fabrication platform should allow for high fidelity in architecture at microscopic scales, predictable oxygen and pressure distribution patterns, while being amenable to a wide range of biomaterials. It should also possess a fast, automated, and reproducible process. On the cellular level, the fabrication system parameters should entail high cellular viability and functionality. Multiple fabrication methodologies such as soft lithography, photopatterning and rapid-prototyping are currently being studied due to their ability to fabricate inter-connected and organized pre-vascular networks. Moreover, computational approaches to predict oxygen consumption and pressure distribution in a given scaffold are also investigated to predict the requirements of different constructs and provide guidance on the actual fabrication processes. The following sections will provide an overview of the various fabrication strategies to engineer pre-vascularized tissue constructs by taking advantage of various biofabrication methods.

5.4.1 Lithography and Microfabrication

The previously discussed techniques (i.e. growth factor delivery, cell-based approaches, etc.) have been extensively used to improve the process of vascularization for bone tissue engineering. However, these techniques usually result in the formation of randomly organized networks. For these reasons alternative methods for controlled fabrication of microvascular channels with precise and pre-defined locations have been developed.

Photolithography, for instance, is a microfabrication process derived from the semiconductor industry for generating geometrically defined micro-scale structures. Using a laser-patterned photomask for restricting UV light exposure to specific regions on pre-polymer solutions containing a photoinitiator, micropatterns can be created in a range of photocrosslinkable polymers that are compatible with cell viability (Chen et al. 2003). Soft lithography, on the other hand, comprises of various micro-molding or microcontact printing approaches, which essentially use a stamp-based technique to create the desired microscale structures (Qin et al. 2010).

Several studies have adopted these methods with natural or synthetic hydrogels to enhance ECs alignment and promote angiogenesis. For instance, West and colleagues (Leslie-Barbick et al. 2009, 2011b) used microfabrication techniques on a PEG-diacrylate (PEGDA) hydrogel to improve the vascularization process. The surface of the patterned hydrogels was modified by using cell-adhesive ligand sites, such as RGD and growth factors, in order to enhance ECs function

and morphogenesis. The patterning induced formation of cord-like structures along the prefabricated regions. Nikkhah et al. investigated organization and alignment of ECs using microfabricated methacrylate gelatin (GelMA) hydrogels. In this work, it was shown that HUVECs alignment was enhanced in patterned hydrogels (50 mm width) as compared to unpatterned substrates (Nikkhah et al. 2012). In a similar study, Raghavan et al. fabricated constructs using a micromolding method to create cell-laden collagen gels with prefabricated microchannels. In principle, a polydimethylsiloxane (PDMS) micromold with the desired channel dimensions was first manufactured and then filled with an endothelial cell-laden collagen hydrogel. A tubular organization with lumens was then observed after 24–48 h and the authors also demonstrated that the concentration of the collagen and channel width influenced the tubule diameter (Raghavan et al. 2010).

In another recent study, polydimethylsiloxane (PDMS) was micro-patterned using soft-lithography and the effect of textures on human bone marrow derived cells (hBMDCs) was analyzed under osteogenic conditions. The textured PDMS consisted of 45 μm wide curved channels with a height of 11 μm, which were separated by ridges of 5 μm. Although the expression of osteogenic phenotype was similar in both textured and non-textured substrates, the patterned PDMS triggered a denser cellular arrangement. Also, the micro-pattern induced a better alignment and high cellular aspect ratio (Mata et al. 2002). Hence, soft lithography provides a cheap screening platform for testing multiple topographic settings, the results of which could be easily applied to fabricate scaffolds with predefined patterns to guide vascular formation.

Micro-molding approaches were also utilized for directing controlled tubulogenesis. Baranski et al. utilized PDMS microchannels to self-assemble HUVECs embedded in a collagen matrix (Fig. 5.3). The cells in the microstructures rapidly reassembled within 4 h through cytoskeletal-induced contraction to generate dense tubular endothelial structures. These tubular cords were subsequently embedded in fibrin matrix and implanted in vivo. After 28 days of implantation, the vascularized fibrin bodies integrated with the host tissue and pericyte cells supported the HUVEC cords to form perfusable capillaries (Baranski et al. 2013). Mapili et al. conducted a similar study regarding the design of precise spatial patterning using a stereolithography technique (SL). PEGDMA hydrogel was dispensed and UV crosslinked using a pulsed Nd: YAG laser (10 mJ/pulse) in order to fabricate scaffolds with controlled pore size and microstrucutre (Mapili et al. 2005). Further functionalization with RGD was then provided in order to study OP-9 cells attachment on the patterned substrates. Results showed no attachment in the RGD-free structures, whereas DAPI staining revealed the opposite on the functionalized scaffolds. This technique revealed an innovative and inexpensive way to fabricate precise geometrical features and point-by-point crosslinking of the scaffold along with biological and physical factors. However, this methodology suffers of limitations such as the lack of feasibility using cells encapsulation because of slow process of fabrication.

Another recent study conducted via soft lithography (Zheng et al. 2012) used for microfabrication of type I collagen to fabricate microchannels, which were then seeded with HUVECs and cultured to achieve endothelialized lumen (Zheng et al. 2012). Results showed the spontaneous formation of tubular constructs and angiogenic sprouting when perivascular cells and SMCs were encapsulated in the matrix (Fig. 5.3). Similarly, Wray et al. (Wray et al. 2013) developed a new technology to create microchannels in a porous silk-based matrix using soft lithography. Results showed that endothelial cells reached confluence and proliferation in the highly porous areas. Moreover, co-culturing of hMVECs in the microchannels and of hMSCs embedded in the hydrogel revealed that morphogenesis and lumen formation was more active near the channel regions. These methods are certainly the strong candidates for generating more complex tissues and organs scaffolds in bone tissue engineering applications and a potent tool for future therapeutic methodologies.

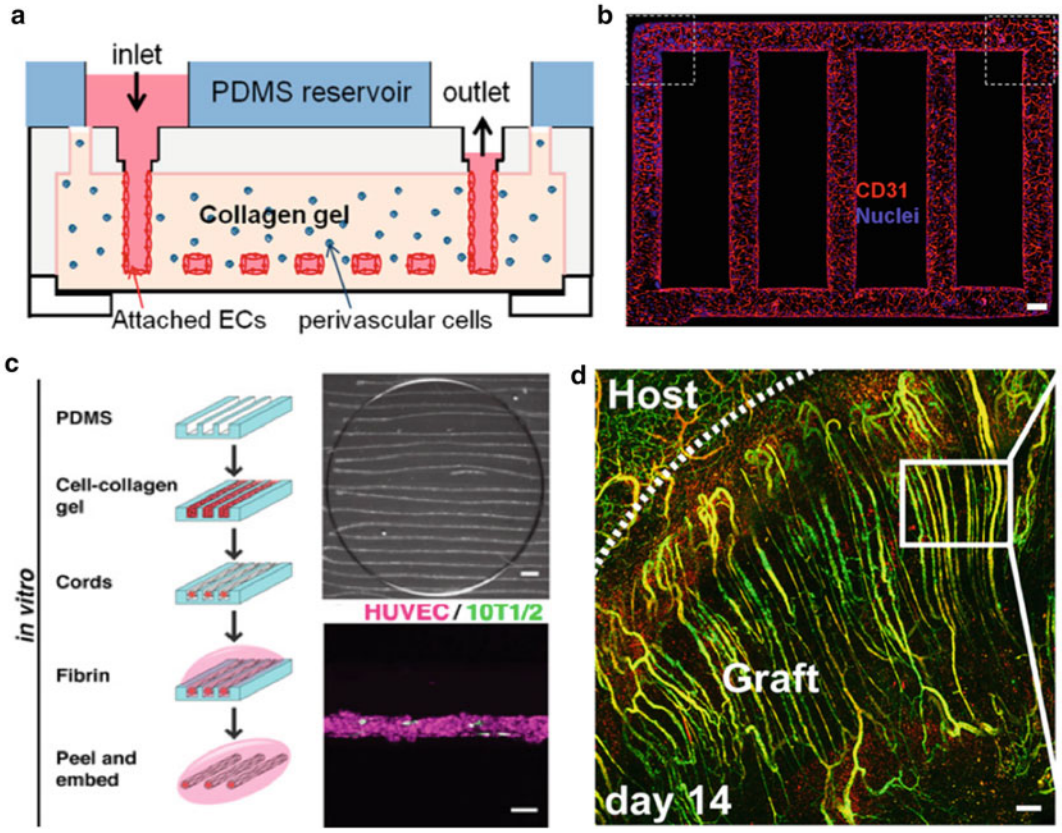

Fig. 5.3 Microfabricated pre-vascularized tissue constructs. (**a**) Collagen type I microfabricated in a microfluidic device (**b**) cultured with endothelial cells and pericytes for an in-vitro study of angiogenesis and thrombosis (Reproduced from Zheng et al. (2012) with permission. Copyright 2012. The National Academy of Science). (**c**) Micromolding of a cell-collagen gel in a fibrin matrix to engineer pre-vascularized tissues in-vitro and (**d**) in-vivo (Reproduced from Baranski et al. (2013) with permission. Copyright 2012 The National Academy of Science)

5.4.2 3D Printing

The automated deposition of biomaterials embedded with cells to fabricate three-dimensionally defined patterns giving rise to macroscopic engineered tissue construct is called 3D bioprinting (Bertassoni et al. 2014a, b; Obregon et al. 2015; Tasoglu and Demirci 2013). With high reproducibility, bioprinting offers unique and significant advantages over other traditional fabrication techniques that are restricted to planar substrates, such as lithography-based fabrication. Bioprinting generates versatile patterns by exploiting Computer Aided Design (CAD) and modeling and can be employed to fabricate a wide range of materials (Obregon et al. 2015; Derby 2012). However, rapid prototyping strategies for vascularization have, thus far, been mainly utilized to develop simple sacrificial template based approaches to engineer vascularized tissues. Still, this strategy represents one of the most effective methods for vascularizing engineered tissue constructs.

3D sacrificial template approaches basically involve printing of a primary well-defined 3D template structure through rapid prototyping, which functions as a mold for a secondary biomaterial with or without cells. Upon crosslinking of the secondary matrix, the partially embedded primary sacrificial template is then removed by physical, chemical or thermal routes, thus generating a 3D scaffold with hollow interconnected

microchannels. In one of the first studies using a sacrificial template method, Miller et al. developed a carbohydrate glass template system, where the hygroscopic behavior of the printed materials allowed for its dissolution by the application of normal cell culture medium (Miller et al. 2012). Further, to avoid the osmotic shock to the cells during the dissolution of the template, the 3D printed carbohydrate glass lattice was coated with poly(d-lactide-co-glycolide) (PDLGA). To circumvent such release of potentially toxic and cellular phenotype modifying byproducts, Bertassoni et al. printed agarose templates to engineer hollow cylindrical channels in pre-osteoblast cell-laden tissue constructs (Bertassoni et al. 2014a) (Fig. 5.4). The flexibility and poor molecular interaction of the 3D printed agarose gel with the secondary biomaterials (four types of photocrosslinkable gels were tested) contributed to the easy removal of the template simply by using a slight vacuum. Of particular interest, Bertassoni et al. demonstrated that the presence of the integrated vascular channels allowed for significantly higher viability and expression of alkaline phosphatase by the pre-osteoblast cells, thus suggesting improved differentiation towards the osteogenic lineage. When seeded with endothelial cells, the hollow channels acted as a provision for organized vasculature by allowing for sprouting of the cells lining the lumen in the surrounding matrix (Bertassoni et al. 2014a).

3D bioprinting was also used by Lee and colleagues (2014) in an attempt to build up a scaffold using collagen as matrix. A mixture of gelatin and HUVECs was dispensed on a collagen layer and then covered by a layer-by-layer deposition of collagen. The channel were then formed by liquefaction of the gelatin at 37 °C, allowing the endothelial cells to attach to the inner layer of the channel. Results revealed that the method has a great potential in tissue engineering since a well-defined vasculature was created without the necessity of printing cells in the gelatin hydrogel.

5.5 Final Remarks

For a long time, engineering vascularized tissues was arguably the main roadblock preventing tissue engineering from widespread clinical application. As we summarize in this chapter, recent developments towards engineering of vascularized tissues have changed this scenario significantly. It may be argued that engineering vascularized

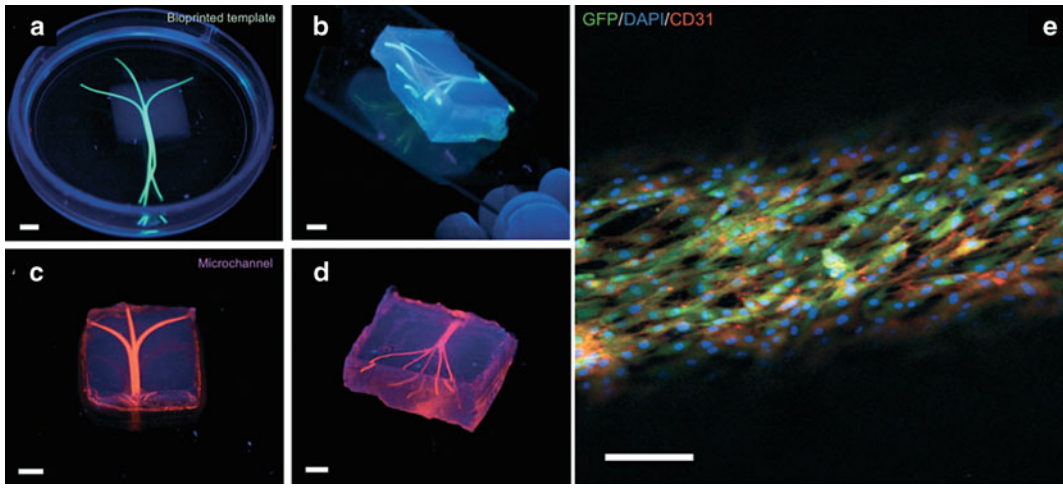

Fig. 5.4 (**a**, **b**) 3D printed microvascular network templates with various morphologies inside GelMA hydrogels and (**c**, **d**) after perfusion with a fluorescent dye. (**e**) Confocal image of endothelial cells forming a confluent monolayer inside 3D printed microchannels (Reproduced from Bertassoni et al. (2014a) with permission. Copyright 2014 The Royal Society of Chemistry)

tissues is no longer an unachievable goal. Nevertheless, understanding the methods to develop functional and clinically relevant vascularized tissues remains a great challenge in bone tissue engineering. There are a variety of methods that promote vascularization in tissue constructs. Cell-based approaches perhaps offer a more biologically relevant strategy, since they take advantage of the ability of cells to self-communicate, secrete paracrine factors and control formation of a stable vasculature in vitro. However, difficulties to control spatially organized vascular channels in macroscale and thick cell-laden constructs remains a major problem. Delivery of growth factors, via physical entrapment or chemical immobilization has also offered a myriad of answers to improve our understanding of vascular formation in engineered bone. Still, the complex cascade of multiple factors naturally occurring in the human body is yet to be artificially mimicked in vitro or in vivo. More recently, biofabrication methods, such as lithography techniques and 3D printing have provided novel and exciting avenues towards fabrication of microchannels that form artificially engineered vascular capillaries and vessels. Although these methods do not mimic the complex and dynamic steps involved in the vasculogenesis of mature and stable capillaries composed of heterotypic cells and growth factors, they allow for better controlled and on-demand fabrication of vascular networks that improve oxygen and nutrient diffusion, as well as waste removal in cell-laden tissue constructs. Perhaps the integration of these different methods, combined with novel strategies for tissue fabrication and control of cellular function in future strategies will enable true fabrication of fully pre-vascularized, biomimetic, load-bearing bone scaffolds prior to implantation.

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Morphogenic Peptides in Regeneration of Load Bearing Tissues

6

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Abstract

Morphogenic proteins due to their short half-life require high doses of growth factors in regeneration of load bearing tissues which leads to undesirable side effects. These side effects include bone overgrowth, tumor formation and immune reaction. An alternative approach to reduce undesirable side effects of proteins in regenerative medicine is to use morphogenic peptides derived from the active domains of morphogenic proteins or soluble and insoluble components of the extracellular matrix of mineralized load bearing tissues to induce differentiation of progenitor cells, mineralization, maturation and bone formation. In that regard, many peptides with osteogenic activity have been discovered. These include peptides derived from bone morphogenic proteins (BMPs), those based on interaction with integrin and heparin-binding receptors, collagen derived peptides, peptides derived from other soluble ECM proteins such as bone sialoprotein and enamel matrix proteins, and those peptides derived from vasculoinductive and neuro-inductive proteins. Although these peptides show significant osteogenic activity in vitro and increase mineralization and bone formation in animal models, they are not widely used in clinical orthopedic applications as an alternative to morphogenic proteins. This is partly due to the limited availability of data on structure and function of morphogenic peptides in physiological medium, particularly in tissue engineered scaffolds. Due to their amphiphilic nature, peptides spontaneously self-assemble and aggregate into micellar structures in physiological medium. Aggregation alters the sequence of amino acids in morphogenic peptides that interact with cell surface receptors thus affecting osteogenic

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activity of the peptide. Aggregation and micelle formation can dramatically reduce the active concentration of morphogenic peptides with many-fold increase in peptide concentration in physiological medium. Other factors that affect bioactivity are the non-specific interaction of morphogenic peptides with lipid bilayer of the cell membrane, interaction of the peptide with cell surface receptors that do not specifically induce osteogenesis leading to less-than-optimal osteogenic activity of the peptide, and less-than-optimal interaction of the peptide with osteogenic receptors on the cell surface. Covalent attachment or physical interaction with the tissue engineered matrix can also alter the bioactivity of morphogenic peptides and lead to a lower extent of osteogenesis and bone formation. This chapter reviews advances in discovery of morphogenic peptide, their structural characterization, and challenges in using morphogenic peptides in clinical applications as growth factors in tissue engineered devices for regeneration of load bearing tissues.

Keywords
BMP • Peptide conjugation • Hydrogel • Bone regeneration • Tissue engineering • Growth factor delivery • Stem cells

6.1 Introduction

Clinical needs for bone tissue regeneration are diverse and include applications arising from skeletal defects caused by fracture, trauma, infection, neurosurgical procedures, resection of tumors and spinal deformities (Di Silvestre et al. 2014; Agrawal et al. 2009; Flierl et al. 2013). There are more than 280,000 and 700,000 hip and vertebral fractures in the US annually (Brydone et al. 2010). Approximately 500,000 and 2.2 million bone grafting procedures are performed in the US and worldwide, respectively, each year (Giannoudis et al. 2005). The total number of spinal fusion procedures in the U.S. was 673,000 in 2012 (Fishman et al. 2012).

The gold standard for grafting is bone harvested from a non-load bearing site in the patient (autologous grafts). Autograft bone is osteoconductive and osteoinductive meaning that it supports osteogenic differentiation and proliferation of progenitor stromal cells migrating from the surrounding tissues to the site of injury. However, an additional surgery is required for harvesting bone from the donor site. The drawbacks include donor site morbidity, infection and pain after the operation (Goulet et al. 1997). The use of cadaveric bone (allograft) suffers from the risk of disease transmission (Fishman et al. 2012). Further, the use of devitalized allografts is limited by the risk of graft failure (60 % failure over 10 years) (Wheeler and Enneking 2005). The drawbacks of autograft and allograft bone have prompted researches to look for new strategies to accelerate bone healing and decrease the rate of graft failure.

Incorporation of osteoinductive factors in the regeneration site increases the rate of healing and reduces the risk of failure. In that regard, bone morphogenic protein-2 (rhBMP-2) and rhBMP-7 are approved by FDA for certain clinical applications (Bessa et al. 2008). rhBMP-2 protein has been used extensively in spinal fusion and sinus and alveolar ridge augmentation to accelerate bone regeneration and reduce the risk of graft failure (Mckay et al. 2007; Burkus et al. 2003). Due to its short-half-life and diffusion of protein away from the site of regeneration, doses much higher than the endogenous amount are used in clinical applications (Mckay et al. 2007; McKay and Sandhu 2002). High doses of rhBMP-2 protein in vivo cause unde-

sired side effects such as bone overgrowth, immune response, and tumor formation (Carragee et al. 2011). For example, the probability of an adverse side effect with the use of rhBMP-2 in spine fusion may be as high as 40 % (Cassar et al. 2008). An attractive approach to reduce the protein's side effects in bone regeneration is to use peptides derived from the bioactive domains of rhBMP-2 or other osteoinductive proteins (Jabbari 2013). These include peptides derived from bone morphogenic proteins (BMPs), those based on interaction with integrin and heparin-binding receptors, peptides derived from other soluble extracellular matrix (ECM) proteins such as bone sialoprotein and enamel matrix proteins, and those peptides derived from vasculoinductive and neuro-inductive proteins (Jabbari 2013). Although these peptides show significant osteogenic activity in vitro and increase mineralization and bone formation in animal models, they are not used as an alternative to morphogenic proteins in clinical orthopedic applications. This is partly due to the limited availability of data on structure and function of morphogenic peptides in physiological medium, particularly in tissue engineered scaffolds. Peptides due to their amphiphilic nature spontaneously self-assemble and aggregate into micellar structures in physiological medium (Moeinzadeh et al. 2014). Aggregation alters the sequence of amino acids in morphogenic peptides for interaction with cell surface receptors thus affecting osteogenic activity of the peptide. Aggregation and micelle formation can dramatically reduce the active concentration of morphogenic peptides with many-fold increase in peptide concentration in physiological medium. Covalent attachment or its physical interaction with the tissue engineered matrix can also alter bioactivity of morphogenic peptides and lead to lower extent of osteogenesis and bone formation. This chapter reviews the structure and property of morphogenic peptides in physiological medium and challenges in using these peptides in clinical applications as growth factors in tissue engineered devices for regeneration of load-bearing tissues.

6.2 Peptides Derived from Bone Morphogenic Proteins and Soluble Proteins of Bone Matrix

Among multiple osteoinductive peptides derived from rhBMP-2 protein, the KIPKASSVPT ELSAISTLYL peptide corresponding to residues 73–92 (hereafter referred as BMP-2 peptide) had highest alkaline phosphatase (ALP) activity and expression of osteocalcin (Saito et al. 2003). Further, the BMP-2 peptide was shown to interact with BMP receptors type IA and type II (Saito et al. 2003). The aforementioned BMP-2 peptide covalently conjugated to a succinimide functionalized alginate gel induced new bone formation after 4 weeks in a rat tibial bone defect (Saito et al. 2004, 2005). Bone formation was also induced by BMP-2 peptide grafted alginate gel when it was implanted ectopically in rat calf muscle (Saito et al. 2004). MC3T3-E1 preosteoblast cells cultured on BMP-2 peptide functionalized cobalt-chromium surfaces had twofold higher ALP activity and fourfold higher mineral deposition compared to the untreated substrate (Poh et al. 2011). Mesenchymal stem cells (MSCs) seeded in polyethylene glycol (PEG) hydrogels grafted with BMP-2 peptide and integrin-binding cell-adhesive RGD peptide showed 5 and 12-fold increase in ALP activity and calcium content after 14 and 21 days, respectively (He et al. 2008). MSCs seeded on tissue culture plates and incubated in osteogenic medium (with ascorbic acid, β- glycerophosphate, and dexamethasone) supplemented with BMP-2 peptide had higher ALP activity at day 14 and higher calcium content at day 21 compared with MSCs cultured in osteogenic medium without the peptide (Jabbari 2013). However, the ALP activity and calcium contents of MSCs were significantly higher when the osteogenic medium was supplemented with rhBMP-2 protein as opposed to BMP-2 peptide (Jabbari 2013).

Immobilization of the BMP-2 peptide on 2D substrates and incorporation in 3D alginate hydrogels increased ALP activity of clonally derived murine osteoblasts (Madl et al. 2014). The ALP

activity and mineralization of human MSCs encapsulated in PEG gels and cultured in osteogenic medium (without dexamethasone) supplemented with the BMP-2 peptide were significantly higher than those cells cultured in the osteogenic medium without peptide but lower than those cells cultured in the osteogenic medium supplemented with rhBMP-2 protein instead of peptide (Moeinzadeh et al. 2014). A BMP-7 derived peptide with sequence GQGFSYPYKAVFSTQ, corresponding to residues 100–115 of immature BMP-7, did not have a significant effect on cell viability but increased ALP activity and calcium secretion of MSCs (Kim et al. 2012). Further, the calcium secretion and expression of CD47, CD44, and CD51 osteogenic markers of the BMP-7 peptide treated MSCs were higher than those for mature BMP-7 protein treated MSCs (Kim et al. 2012). It has been shown recently that the BMP-7 peptide and cyclic RGD peptide act synergistically to regulate osteogenic differentiation of MSCs (Yin et al. 2014). A 23-mer Ac-CGGKVGKACCVPTKLSPISVLYK-NH2 peptide derived from the knuckle epitope of human BMP-9 induced osteogenic differentiation of MC3T3-E1 pre-osteoblasts and activated the Smad pathway and the expression of osteogenic markers (Bergeron et al. 2012).

A heparin binding peptide corresponding to residues 15–24 of BMP-4 (BMP-4 peptide: RKKNPNCRRH) induced osteogenesis of hMSCs through activating ERK1/2 pathway (Choi et al. 2010). Osteogenic medium supplemented with BMP-4 peptide increased ALP, osteopontin (OP) and osteocalcin (OC) expression of hMSCs but it did not significantly change the expression of collagen type I (Col I) (Choi et al. 2010). Expression of the above four markers (ALP, OP, OC, and Col I) increased with application of the same dose of the peptide (Choi et al. 2010). Further, the BMP-4 peptide did not have a significant effect on viability of the hMSCs. When the BMP-4 peptide grafted to alginate gel was implanted in the cranial defect of rabbit, the total area of new bone formation increased by fourfold compared to the gel with no peptide (Choi et al. 2010).

Osteoinductive potential of the peptides derived from soluble proteins in the bone matrix other than BMPs have also been investigated. Bone sialoprotein (BSP), a major component of the bone ECM, is involved in cell attachment, signaling, and hydroxyapatite nucleation (Ganss et al. 1999). A collagen substrate impregnated with a peptide corresponding to residues 35–62 of BSP increased ALP activity and the expression of osteogenic proteins OC, OP, and Col I of human osteoblasts (HOS) without affecting cell proliferation (Choi et al. 2013). Further, hydroxyapatite constructs coated with the BSP peptide induced bone formation in rabbit calvaria with a fourfold higher new bone area after 2 weeks compared to untreated constructs (Choi et al. 2013). A significant component of the enamel ECM is a group of proteins called amelogenins that facilitate cell adhesion, apatite nucleation and growth during enamel development (Paine and Snead 1997). Warotayanont et al. showed that the formation of mineralized matrix and osterix gene expression of mouse embryonic stem (ES) cells significantly increased by supplementing the differentiation medium with a 59-residue leucine-rich amelogenin peptide (LRAP) (Warotayanont et al. 2008). Further, the increased β-catenin expression and Wnt reporter gene activity indicated that Wnt/β-catenin signaling pathway was involved in LRAP-mediated osteogenesis of ES cells (Warotayanont et al. 2009). In addition, the increased Wnt10b expression indicated that the presence of LRAP peptide induced osteogenesis and inhibited adipogenesis of MSCs through the activation of Wnt/β-catenin signaling pathway (Wen et al. 2011). Overall, a number of peptides derived from the active domains of the BMP family or other soluble proteins in the bone matrix have demonstrated a significant osteoinductive potential without a significant cytotoxic effect and they can potentially be utilized as an alternative to osteo-inductive growth factors in orthopedic applications.

6.3 Integrin Binding Peptides

In the natural ECM, cell adhesive ligands on laminin and fibronectin bind to the integrin cell surface receptors (Cukierman et al. 2002; Gloe and

Pohl 2002) which is crucial to cell proliferation and differentiation (Aplin et al. 1998). For example, when α_2 integrins of MC3T3-E1 pre-osteoblasts were blocked with an antibody or a peptide containing the cell-binding domain of Col I, mRNA expression of OC and mineralization of pre-osteoblasts were abolished (Xiao et al. 1998). Further, a cyclic GA-CRRETAWAC-GA peptide specific to the activation of α5 integrin increased the expression of the osteogenic markers Runx2 and Col I as well as mineralization of murine C3H10T1/2 MSCs (Fromigue et al. 2012; Mould et al. 1998). The α_5 integrin-mediated osteoinduction was activated through FAK and ERK1/2-MAPKs cell signaling pathway (Fromigue et al. 2012). Subcutaneous injection of GA-CRRETAWAC-GA peptide in adult mouse cranial bone for 3 weeks increased bone thickness by twofold, the same level as that of rhBMP-2 protein (Fromigue et al. 2012). Frith et al. treated polystyrene-block-poly(ethylene oxide)-copolymer surfaces with RGD (binds to $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_{IIb}\beta_3$,$\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_V\beta_6$ (Hersel et al. 2003; Ruoslahti 1996)), IKVAV (binds to $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_6\beta_1$ (Freitas et al. 2007; Maeda et al. 1994)) YIGSR (binds to $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_6\beta_1$ (Freitas et al. 2007; Maeda et al. 1994)), and RETTAWA (binds specifically to $\alpha_5\beta_1$ (Mould et al. 1998)) peptides to investigate the effect of different integrin-binding peptides on adhesion and osteogenic differentiation of hMSCs (Frith et al. 2012). Results demonstrated that IKVAV had the highest osteoinductive potential while RGD supported the long-term viability of hMSCs (Frith et al. 2012).

A number of cell-adhesive peptides derived from laminin, fibronectin, elastin and collagen proteins have been incorporated in synthetic inert hydrogels to induce osteogenesis (Zhu 2010). The laminin and fibronectin derived Arg-Gly-Asp (RGD) adhesive peptide added to PEG hydrogels significantly increased adhesion and mineralization of the encapsulated osteoblast cells (Burdick and Anseth 2002). The viability of hMSCs was significantly higher in RGD modified PEG gels compared with the unmodified gels (Liu et al. 2010). Visser et al. showed that absorbable Col I sponges modified with collagen binding RGD peptide and loaded with rhBMP-2 protein induced ectopic bone formation with clearly defined osteocytes within the mineralized matrix after 14 days whereas those sponges without RGD peptide or rhBMP-2 protein did not show signs of osteogenesis after 21 days (Visser et al. 2014). That study indicated that synergistic action of rhBMP-2 and integrin binding ligands with cell surface receptors play a major role in bone formation.

The effect of RGD and rhBMP-2 peptides grafted to a poly(lactide-co-ethylene oxide fumarate) (PLEOF) hydrogel on DNA content (day 7), ALP activity (day 14), and mineralization (day 21) of MSCs seeded on the hydrogel surface and incubated in osteogenic medium is shown in Fig. 6.1. Groups were MSCs seeded on PLEOF gel without RGD (Ctrl), with RGD and without rhBMP-2 peptide (R), without RGD and with rhBMP-2 peptide (B) and with both RGD and rhBMP-2 peptides (B+R). DNA content of Ctrl group was lower than other three groups indicating that conjugated RGD and/or BMP-2 peptide improved cell adhesion to surface of the gel. DNA content of R group was higher than B because RGD peptide has higher cell adhesive property compared to rhBMP-2 peptide. This is consistent with several studies showing that RGD modification of hydrogels promotes adhesion of MSCs (Nicodemus and Bryant 2008; Shin et al. 2002; Yang et al. 2005). ALP activity of the MSCs increased by 2.7-folds with conjugation of RGD peptide to the gel surface. Interestingly, ALP activity of the MSCs seeded on rhBMP-2 peptide conjugated gels was lower than those conjugated with RGD. ALP activity of the MSCs seeded on PLEOF hydrogels conjugated with RGD and rhBMP-2 peptides increased by 4.8-folds compared with the untreated gels. Extent of mineralization of the MSCs seeded on PLEOF gels, as measured by calcium content, had a trend similar to ALP activity. The R and B groups had significantly higher calcium content than the Ctrl group with R group having a higher calcium content compared to B. Conjugation of RGD and rhBMP-2 peptides (B+R group) on the gel surface significantly increased calcium content of the seeded MSCs compared to B or R groups. Yang et al. reported that osteogenic differentiation of MSCs in 3D polyethylene glycol diacry-

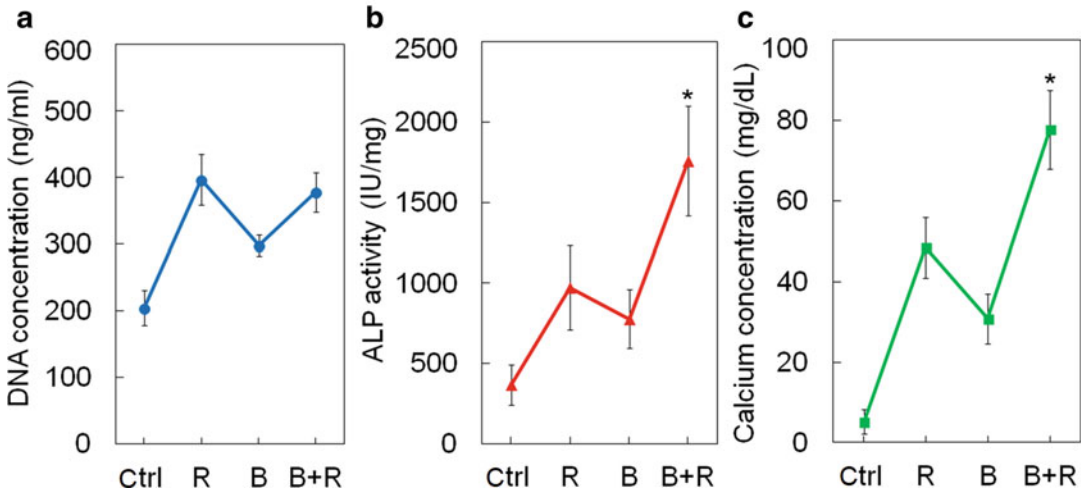

Fig. 6.1 DNA content at day 7 (**a**), ALP activity at day 14 (**b**), and calcium content at day 21 (**c**) of the MSCs seeded on peptide conjugated PLEOF hydrogel. Groups included PLEOF gel without peptide conjugation (*Ctrl*), gel conjugated with RGD (R), gel conjugated with rhBMP-2 peptide (B), and gel conjugated with RGD+rhBMP-2 peptides (B+R). *One star* indicates a statistically significant difference between the test group and all other groups for the same time point. *Error bars* correspond to means ± SD (n = 3) (Jabbari 2013)

late (PEGDA) hydrogels increased with the concentration of conjugated RGD peptide in the 0–2.5 mM range (Yang et al. 2005). Conjugation of 2.5 mM RGD peptide to PEGDA gels increased ALP activity and osteocalcin marker expressions of the seeded MSCs by 13.4- and 2.8-folds, respectively (Yang et al. 2005). Therefore, concurrent application of osteo-inductive and cell adhesive peptides synergistically enhances bone formation.

6.4 Peptides Derived from Vasculogenic and Neurogenic Proteins

In addition to soluble and insoluble proteins in the bone matrix, proteins secreted from skeletal vasculature or sensory neurons are shown to promote osteogenesis. Substance P (SP) is an 11 amino acid peptide (RPKPQQFFGLM) produced by skeletal neurons in the medullar cavity. It has been reported that SP stimulates late-stage bone formation by osteoblasts by interaction with neurokinin-1 receptor (NK1-Rs) (Goto et al. 2007). Further, inhibition of SP promoted activation of NF-κB and bone resorption by osteoclasts (Azuma et al. 2004). Wang et al. reported that SP addition to the osteogenic medium at low concentrations (10^{-12} M) increased ALP activity and osteocalcin expression of MSCs and up-regulated the expression of Runx2 protein but did not significantly affect the expression of Col I and mineralization after 21 days of cultivation (Wang et al. 2009). Conversely, SP addition to the osteogenic medium at high concentrations (10^{-8} M) significantly increased mineralization of MSCs after 21 days (Wang et al. 2009). SP treatment of bone marrow macrophages (BMMs) led to the activation of NF-κB and production of RANKL, an osteoclast differentiating factor, at concentrations that activated bone resorption (Wang et al. 2009). Further, RANKL was produced in SP treated co-cultures of MSCs and BMMs but at levels too low for induction of osteoclastogenesis (Wang et al. 2009). Therefore, SP acts synergistically on bone forming and bone resorbing cells to regulate bone turnover (Wang et al. 2009).

Endothelin-1 (ET-1) is a 21 residue amino acid peptide secreted by endothelial cells and plays a role in long-lasting vasoconstriction through tight binding to ET_A-receptors (Compeer et al. 2012; Meens et al. 2010). von Schroeder

et al. reported that ET-1 treatment of fetal rat calvaria increased cell proliferation in a dose dependent manner in the 10^{-10}–10^{-6} M concentration range (von Schroeder et al. 2003). ET-1 treatment increased cell proliferation more than treatment with dexamethasone (Dex) at the same concentration (von Schroeder et al. 2003). ALP activity and the number of bone nodules formed by the rat cells increased by 3.1-folds and 1.5-folds, respectively, with ET-1 treatment in the absence of Dex (von Schroeder et al. 2003). Further, co-treatment of fetal rat calvarial cells with ET-1 and Dex synergistically enhanced osteoinduction (von Schroeder et al. 2003).

6.5 Osteoinductivity of Peptides Versus Proteins

rhBMP-2 and rhBMP-7 are the only osteoinductive growth factors approved by FDA for specific orthopedic surgical procedures (Bessa et al. 2008). Many BMPs trigger osteogenesis in mature osteoblasts while only BMP-2, BMP-6, and BMP-9 induce osteogenic differentiation of MSCs (Senta et al. 2011). BMPs are multifunctional proteins and play important roles in the process of development including early embryonic patterning, bone morphogenesis, joint development and tissue homeostasis (Reddi 2005). Specifically, BMP-2 and BMP-7 are vital to heart morphogenesis and kidney development, respectively (Reddi 2005). BMP-2 is a negative regulator of myoblast differentiation (Aoyama et al. 2011) and a positive regulator of uterine decidualization in humans (Li et al. 2007). Over-expression of BMP-4 in the heart is associated with coronary artery disease and heart failure (Wu et al. 2014). BMP-9 increases tube formation by human umbilical vein endothelial cells (HUVECs) and human pulmonary artery endothelial cells (HPAECs) through interaction with Alk-1 receptor (Nolan-Stevaux et al. 2012; Park et al. 2012). BMP-7 inhibits telomerase maintenance and activity (Cassar et al. 2008). It also inhibits chondrocyte hypertrophy and mineralization through interaction with the periarticular region (Haaijman et al. 1999). Liver proximal tubular cells are responsive to BMP-7 (Wetzel et al. 2006). BMP-2 and BMP-7 stimulate tube formation in aortic endothelial cells (Wiley and Jin 2011). BMPs can cause adverse side effects when the exogenous administered protein diffuses way from the site of regeneration (Shields et al. 2006; Raida et al. 2005). High doses of rhBMP-2 protein in vivo cause complications such as undesired immune response, bone over-growth, and tumorigenesis (Carragee et al. 2011). The risk of undesirable side effects with the use of BMP-2 may be as high as 40 % in spinal fusion (Carragee et al. 2011). Different strategies including protein encapsulation or protein grafting to nano- and microspheres have been applied to reduce its diffusion and localize the protein to the site of regeneration (Yang et al. 2010; Mercado and Jabbari 2010; Mercado et al. 2009) but adverse side effects persist in some patients (Shields et al. 2006). Therefore, morphogenic peptides corresponding to the osteoinductive motifs of BMPs may have reduced undesired side effects compared to the parent protein when used in scaffolds for bone regeneration. Furthermore, peptides are less likely to undergo denaturation and are easier to deliver/immobilize compared to proteins (Jabbari 2013).

Several studies have reported a significantly lower bioactivity for peptides compared to the corresponding proteins (Jabbari 2013; Moeinzadeh et al. 2014; Li et al. 2011). Conformation of the peptide as a part of the protein is different from the conformation as an individual peptide in physiological or aqueous medium. Free individual peptides can aggregate in the aqueous medium due to the absence of the domains present in the protein that stabilize the peptide (Moeinzadeh et al. 2014). Li et al. compared osteoinductive potential of rhBMP-2 peptide and protein by loading the bioactive agents in nano-hydroxy apatite/collagen/poly(L-lactic acid) scaffolds and implanting the constructs in rat calvarial defect (Li et al. 2011). They reported that 3 mg BMP-2 peptide had a similar osteoinductive potential to 1 μg rhBMP-2 protein (Li et al. 2011). Therefore a 3,000-fold higher peptide concentration was required to achieve the bioactivity of the corresponding rhBMP-2 protein (Li et al. 2011).

We compared the osteoinductive effect of rhBMP-2 protein with BMP-2 peptide with MSCs seeded on tissue culture plates and incubated in osteogenic medium (with ascorbic acid, β- glycerophosphate, and dexamethasone) supplemented with protein or peptide for 21 days (Jabbari 2013). MSC cultured in osteogenic medium supplemented with rhBMP-2 protein had the highest decrease in cell number followed by that supplemented with BMP-2 peptide and the control osteogenic medium without BMP-2 protein or peptide. Furthermore, the induction of osteogenesis was faster for rhBMP-2 protein supplemented cultures than that supplemented with the BMP-2 peptide (Jabbari 2013). For example, ALP activity of rhBMP-2 protein supplemented cultures peaked at day 7 but that of BMP-2 peptide supplemented cultures peaked at day 14 (Jabbari 2013). Therefore, the BMP-2 peptide directly added to the culture medium had significantly lower osteoinductive potential compared with rhBMP-2 protein in inducing osteogenic differentiation of MSCs in 2D cultures.

We also compared the osteoinductive effect of rhBMP-2 protein and the BMP-2 peptide with human MSCs encapsulated in 3D Polyethylene glycol (PEG) hydrogels. Cell encapsulated PEG gels were incubated in osteogenic medium (with ascorbic acid and β- glycerophosphate but without dexamethasone) supplemented with rhBMP-2 protein or the BMP-2 peptide for 28 days. Figure 6.2a, b shows the effect of addition (without conjugation) of BMP-2 protein (0.01 mg/mL, group Pr in Fig. 6.2) or the BMP-2 peptide (0.01–10 mg/mL) to the hydrogel on ALP activity and calcium content of the encapsulated hMSCs over 28 days of incubation. The clinically applied concentration of rhBMP-2 protein is 1 mg/mL (Swiontkowski et al. 2006) but the protein concentration used for typical in vitro 3D experiments is in the 1–10 μg/mL range (Meinel et al. 2009; Chung et al. 2013). ALP activity of all groups had a maximum on day 14 consistent with the bimodal ALP activity in osteogenic differentiation of MSCs (Mygind et al. 2007). ALP activity of encapsulated hMSCs in the groups with the protein or peptide dissolved was significantly higher than those without the protein/peptide. ALP activity of encapsulated hMSCs in the group with 10 μg/mL rhBMP-2 protein was

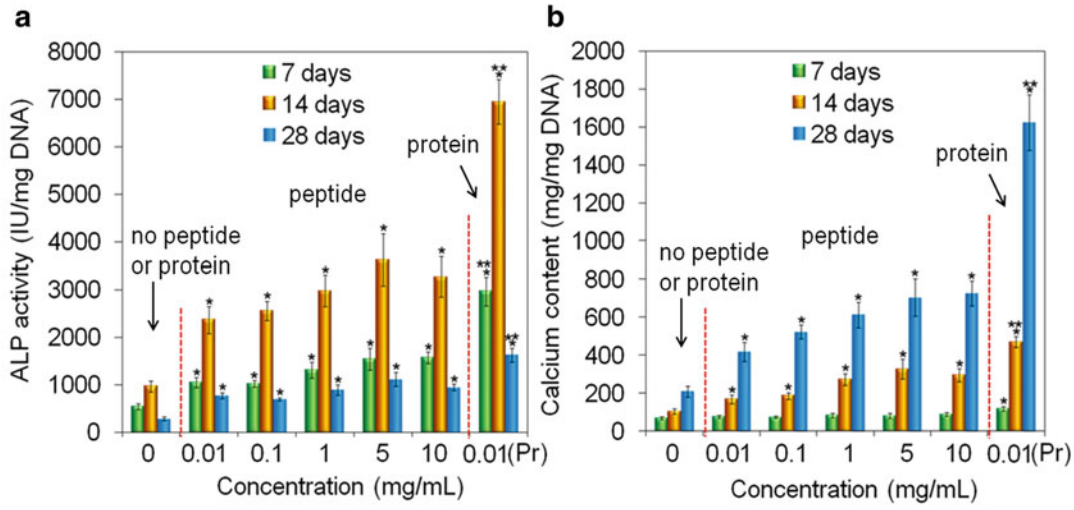

Fig. 6.2 (**a**) ALP activity and (**b**) calcium content of human MSCs encapsulated in PEGDA (15 wt.%) hydrogel with BMP-2 peptide dissolved in the matrix and medium as a function of peptide concentration in the 0–10 mg/mL concentration range or with 0.01 mg/mL BMP-2 protein (*Pr*). *Error bars* correspond to means ±1 SD for n = 3. *One star* indicates statistically significant difference ($p < 0.05$) between the test group and the group without peptide (0 mg/mL) at each time point. "*Two stars*" indicates statically significant difference between the test group and all other groups at each time point

threefold higher than the group with 10 μg/mL of BMP-2 peptide and 2.1-fold higher than the group with 10 mg/mL peptide after 14 days. Calcium content of hMSCs in all groups had an increasing trend with incubation time over 28 days. The calcium content increased from 210 ± 30 to 420 ± 50 mg/mg DNA with the addition of 10 μg/mL peptide to the gel and to 1,620 ± 140 mg/mg DNA with the addition of the same concentration of rhBMP-2 protein after 28 days. ALP activity and calcium content of the encapsulated hMSCs treated with rhBMP-2 protein were significantly higher than those cells treated with BMP-2 peptide even at 1,000-fold higher peptide concentration. Although bioactivity of osteo-inductive peptides has been shown to be lower than the corresponding proteins, higher specificity, easier delivery to the site of regeneration and lower production cost compared to the corresponding proteins may lead to the substitution of osteo-inductive proteins with peptides in clinical applications.

6.6 Dose Dependence of Osteoinductivity of Morphogenic Peptides

Results of several studies indicate a dose dependent activity for osteoinductive peptides. Kim et al. reported that ALP activity, calcium content, and expression of Runx2 and Col I of the MSCs seeded in 2D wells and cultured in osteogenic medium (with ascorbic acid, β- glycerophosphate and dexamethasone) increased monotonically with increasing concentration of the BMP-7 peptide in a 0.01−1 μg/mL concentration range (Kim et al. 2012). Yang et al. reported that ALP activity, calcium content and osteocalcin expression of MSCs encapsulated in PEG hydrogels increased with the concentration of RGD peptide grafted to the hydrogel network in the 0−2.5 mM peptide concentration range (Yang et al. 2005). We investigated the effect of concentration of the BMP-2 peptide dissolved in osteogenic medium on differentiation of hMSCs encapsulated in PEG based gels (Fig. 6.2) (Moeinzadeh et al. 2014). DNA content of the encapsulated hMSCs did not change significantly with the peptide concentration in osteogenic medium for all time points (Moeinzadeh et al. 2014). ALP activity of the peptide-treated hMSCs had a biphasic trend over 28 days with a peak at day 14 (Fig. 6.2). The effect of BMP-2 peptide concentration in the osteogenic medium on ALP activity of the encapsulated hMSCs at day 14 is shown in Fig. 6.3. ALP activity of the hMSCs did not change significantly with increasing the peptide concentration from 0 to 0.0005 mM. ALP activity of the encapsulated hMSCs increased sharply by 2.2-folds as the peptide concentration increased from 0.0005 to 0.005 mM. The rate of increase in ALP activity of hMSCs with peptide concentration decreased for concentrations above 0.005 mM.

ALP activity of the encapsulated hMSCs increased by only 1.4-folds as the peptide concentration was increased by 1,000-folds from 0.005 to 5 mM. Since the BMP-2 peptide is amphiphilic with a hydrophilic N-terminus and a hydrophobic C-terminus, it aggregated in aqueous solution to reduce the overall free energy for concentrations above the critical micelle concentration (CMC) (Moeinzadeh et al. 2014; Zana 1996). The CMC for BMP-2 peptide was 0.019 mM based on the dynamic light scattering results (dashed line in Fig. 6.3a) (Moeinzadeh et al. 2014). According to Fig. 6.3a, the rate of increase in the ALP activity with peptide concentration decreased for concentrations above CMC. In other words, peptide aggregation had a negative effect on the osteoinductivity of the peptide.

Aggregation of the BMP-2 peptides in the PEG hydrogel precursor solution was simulated via a mesoscale molecular dynamics method based on the MARTINI force field (Moeinzadeh et al. 2014; Marrink et al. 2007; Monticelli et al. 2008) that has been parameterized and applied previously to the simulation of peptides and polymer-peptide conjugates (Yang and Faller 2012). The simulated density of the total and free (non-aggregated) peptide as a function of concentration in the PEG hydrogel precursor solution is shown in Fig. 6.3b. The fraction of free peptide at concentrations above CMC decreased sharply from 0.54 to 0.06 as the peptide concen-

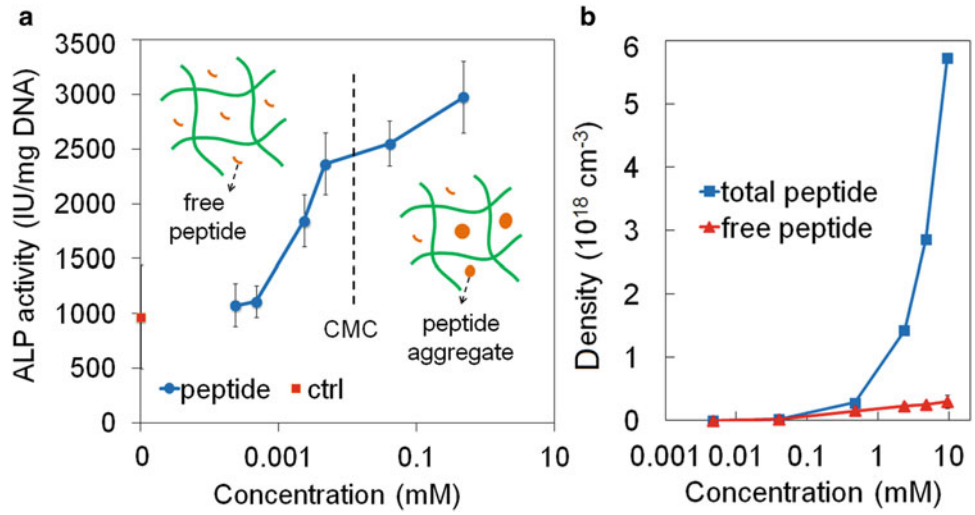

Fig. 6.3 (**a**) ALP activity of hMSCs encapsulated in PEGDA (15 wt.%) hydrogel as a function of dissolved BMP-2 peptide in the matrix and medium in the 0.00025–5 mM concentration range. The critical micelle concentration of the peptide (0.019 mM) is shown with a *dash line* in (**a**). Density of the free BMP-2 peptide and total peptide density as a function of peptide concentration in aqueous solution is shown in (**b**)

tration was increased from 0.5 to 10 mM, respectively (data not shown). However, the decrease in the fraction of free peptide was offset by an increase in the peptide number density. Therefore, the net effect of increasing the total peptide density by 20-folds was only a 1.9-folds increase in the density of free peptide (Fig. 6.3b). As the total peptide concentration was increased by ten-folds from 0.5 to 5 mM in a range well above CMC, ALP activity increased by 1.1-folds while the free peptide concentration increased by 1.6-fold (Fig. 6.3a, b). Therefore, there was a better correlation between osteogenic differentiation of the encapsulated hMSCs and the free peptide concentration.

6.7 Osteoinductive Peptide Delivery Strategies

Osteogenic proteins and peptides can be immobilized in the bone graft or they can be delivered as soluble factors within micro-/nano- carriers. Conjugation reactions have been used to immobilize osteogenic proteins/peptides in engineered scaffolds or in nanoparticles (NPs) (He et al. 2008, 2012; Cacchioli et al. 2009; Li et al. 2010). Cysteine-terminated peptides/proteins are conjugated to acrylate functionalized scaffolds through Michael's addition reaction (Moeinzadeh et al. 2014; Lutolf et al. 2001). Lysine-terminated peptides/proteins are conjugated to succinimide functionalized macromers or particles (Mercado and Jabbari 2010; Fernandez-Megia et al. 2007). Azide functionalized peptides/proteins are conjugated to propargyl functionalized macromers or surfaces through the "click reaction" (Ossipov and Hilborn 2006). In addition, peptides/proteins with free thiol functionality are conjugated to macromers or surfaces through thiol-maleimide reaction (Ananda et al. 2008). For example, rhBMP-2 protein grafted to poly(lactide fumarate) (PLAF) NPs through amine-succinimide reaction was released at a constant rate for 4 weeks and no burst release was observed when the protein was grafted to the NPs. However only 40 % of the grafted rhBMP-2 was released in the enzymatically-active conformation (Jabbari 2013). The grafted BMP-2 peptide was released at a constant rate for 30 days concurrent with matrix degradation (Jabbari 2013). Conversely, rhBMP-2 protein was released in the first 5 days of incubation with 35 % burst release by physical entrapment, without covalent attachment, in

poly(lactide-ethylene oxide-fumarate) (PLEOF) NPs. In addition 80 % of the protein was released in the enzymatically-active conformation by entrapment in the self-assembled NPs (Mercado and Jabbari 2010). Therefore, the release was slower but the fraction of bioactive growth factor released was lower when the osteoinductive rhBMP-2 was covalently attached to the NPs.

The effect of BMP-2 peptide covalently attached to a PEG hydrogel on osteogenic differentiation of hMSCs is shown in Fig. 6.4a, b. The BMP-2 peptide dissolved in the gel and in the osteogenic medium (group B in Fig. 6.4) had higher osteoinductivity indicated by higher ALP activity and higher calcium content in Fig. 6.4 than the BMP-2 peptide conjugated to the hydrogel network (group cB in Fig. 6.4). However, the presence of dissolved or conjugated BMP-2 peptide significantly increased the ALP activity and mineralization of hMSCs encapsulated in the hydrogel. The higher osteogenic differentiation of encapsulated hMSCs in the case of BMP-2 peptide dissolved in the hydrogel was consistent with the previously reported lower activity of rhBMP-2 protein covalently attached to heparin (Ruppert et al. 1996). The higher osteo-inductive potential of the dissolved BMP-2 peptide compared with that of conjugated peptide necessitates the physical or non-covalent delivery of BMP-2 peptide entrapped within micro-/nanocarriers to the site of regeneration.

6.8 Aggregation of Osteoinductive Peptides

As mentioned in previous sections, peptides due to their amphiphilic nature self-assemble and aggregate into micellar structures in physiological medium (Moeinzadeh et al. 2014). Aggregation alters the sequence of amino acids in morphogenic peptides for interaction with cell surface receptors thus affecting osteogenic activity of the peptide. Aggregation negatively contributed to osteoinductivity of the peptide. Furthermore, the osteoinductivity was correlated to the concentration of free peptides (Moeinzadeh et al. 2014). The effect of BMP-2 peptide hydrophobicity on ALP activity (day 14) and calcium content (day 28) of the hMSCs encapsulated in PEG gels are shown in Fig. 6.4a, b. Groups included gels incubated in osteogenic medium

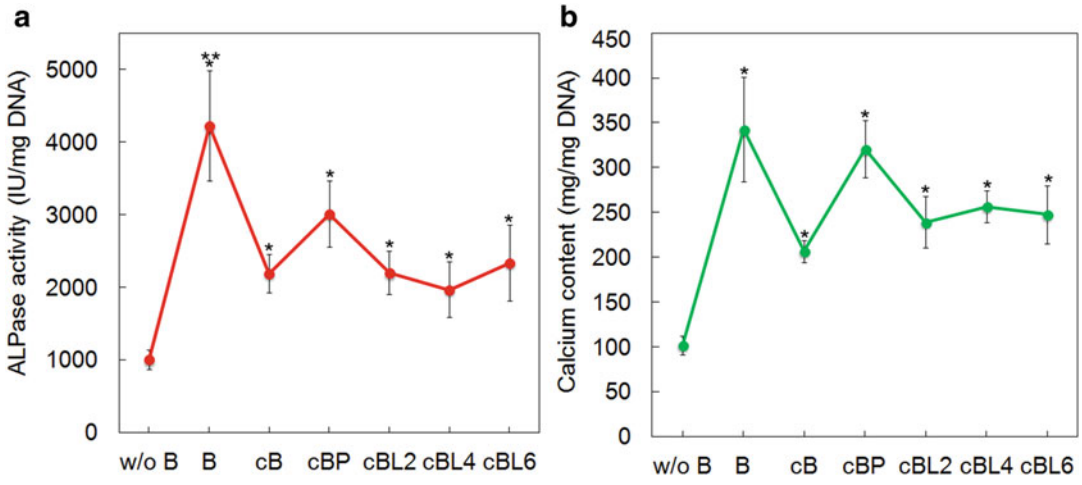

Fig. 6.4 ALP activity at day 14 (**a**), and calcium content at day 28 (**b**) of hMSCs encapsulated in PEGDA hydrogel and incubated in osteogenic medium (OM) without DEX (w/o B), OM with the BMP-2 peptide dissolved in the hydrogel (**b**), OM with the BMP-2 peptide covalently attached to the hydrogel network (cB), OM with the peptide/PEG conjugate (cBP), OM with the peptide/lactide-capped PEG conjugate with 2 (cBL2), 4 (cBL4), and 6 (cBL6) lactide units, attached to the hydrogel network. *One star* indicates a statistically significant difference ($p<0.05$) between the test and w/o B group. *Two stars* indicate a statistically significant difference between the test and all other groups

without Dex (OM) and without peptide (w/o B), OM with dissolved peptide (B), OM with peptide conjugated to the gel (cB), OM with conjugated peptide-PEG macromer (cBP) and OM with peptide/lactide-capped PEG macromer with 2 (cPL2), 4 (cPL4), and 6 (cPL6) lactides conjugated to the gel. ALP activity for cBP group (peptide-PEG conjugate attached to the gel) was 3,000 ± 450 IU/mg DNA which was higher than that for cB group (peptide attached to the gel) with 2,200 ± 260 IU/mg DNA. Further, ALP activity of the encapsulated hMSCs decreased when the peptide-conjugated PEG chain was capped with a hydrophobic lactide segment (cPL2, cPL4 and CPL6 groups).

An increase in the lactide segment length did not significantly change the peak ALP activity of the encapsulated hMSCs. Calcium content of the hMSCs encapsulated in PEG gels increased at day 28 with extension of the BMP-2 peptide with a hydrophilic PEG chain. However, extension of the peptide with a lactide-capped PEG chain (lactide segment length of two, cPL2) significantly reduced calcium content at day 28 compared to the PEG-extended peptide (cBP). Figure 6.5 shows the simulated equilibrium structure of cB, cBP and cBL6 in the PEG hydrogel precursor solution (15 wt.%). The blue and brown beads are for acrylate and lactide units, respectively, and ethylene oxide beads are shown in green. Peptides in the cB group formed irregularly shaped aggregates due to its amphiphilic nature. The hydrophobic side chains of the amino acids formed the core of the aggregates, polar and charged amino-acids positioned at the core-corona interface and ethylene oxide chains cov-

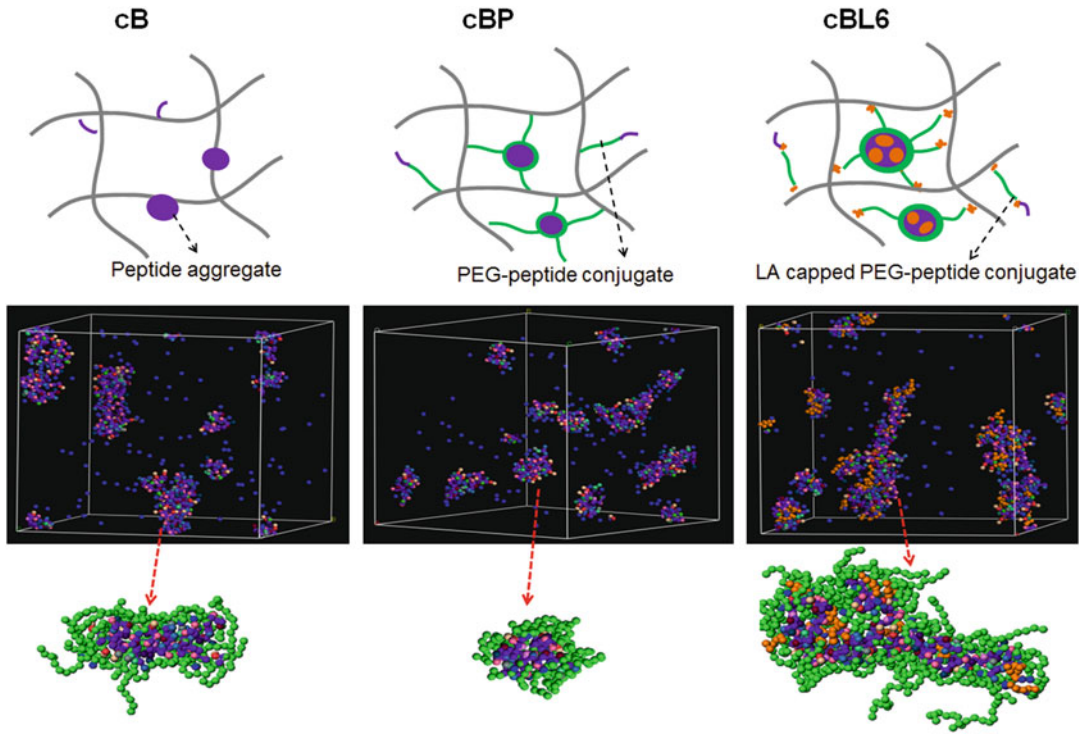

Fig. 6.5 (*top row*) Schematic representation of the peptide (shown in *purple*) covalently attached to the hydrogel network (shown in *gray*) (cB), peptide/PEG conjugate attached to the hydrogel network (cBP) and peptide/lactide-capped PEG conjugate attached to the hydrogel network (cBL6). The *purple lines* and *circles* represent the peptide chain and peptide aggregate, respectively. PEG and lactide segments are shown in *green* and *brown*, respectively. (*middle row*) The simulated BMP-2 peptide aggregates in the hydrogel precursor solution. The *blue* and *brown beads* correspond to the acrylate and lactide beads, respectively. (*bottom row*) Cross-section of one of the BMP-2 peptide aggregates in the *middle row* with *green beads* representing ethylene oxide units

ered surface of the aggregates (Moeinzadeh et al. 2014). The size and aggregation number (number of peptides per aggregate) of the aggregates decreased with conjugation of PEG to the peptide and increased with conjugation of lactide-capped PEG to the peptide. Conjugation of the peptide to the hydrophilic PEG matrix decreased aggregation and increased the free peptide density (Moeinzadeh et al. 2014). Further, capping the PEG chain with hydrophobic lactide segments increased aggregation number which can be explained by a decrease in CMC (Zana 1996; Owen et al. 2012). Therefore, osteoinductivity of the BMP-2 peptide increased with decreasing hydrophobicity as the decreased hydrophobicity increased the concentration of non-aggregated active peptide (Moeinzadeh et al. 2014). However, aggregation characteristics as well as the effect of aggregation on osteo-inductive potential of other peptides may be different from that of the BMP-2 peptide. The inhibition of peptide aggregation can potentially be utilized as a tool to modify the cell-peptide interaction and inductivity of osteogenic peptides.

6.9 Future Work

Research results demonstrate that peptides derived from the bioactive domains of bone ECM protein significantly increase osteogenic differentiation of human MSCs and that that dissolved peptide has a higher osteo-inductivity compared with the peptide attached to the matrix. However, aggregation of the peptide in aqueous physiological medium can dramatically reduce the free peptide concentration and osteoinduction. Therefore, future work should be focused on developing strategies to reduce peptide aggregation and non-specific interaction of the osteoinductive peptide with receptors on the cell membrane.

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7 Osseointegration of Plateau Root Form Implants: Unique Healing Pathway Leading to Haversian-Like Long-Term Morphology

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Abstract

Endosteal dental implants have been utilized as anchors for dental and orthopedic rehabilitations for decades with one of the highest treatment success rates in medicine. Such success is due to the phenomenon of osseointegration where after the implant surgical placement, bone healing results into an intimate contact between bone and implant surface. While osseointegration is an established phenomenon, the route which osseointegration occurs around endosteal implants is related to various implant design factors including surgical instrumentation and implant macro, micro, and nanometer scale geometry. In an implant system where void spaces (healing chambers) are present between the implant and bone

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immediately after placement, its inherent bone healing pathway results in unique opportunities to accelerate the osseointegration phenomenon at the short-term and its maintenance on the long-term through a haversian-like bone morphology and mechanical properties.

Keywords
Implants • Osseointegration • Bone healing • Tissue engineering • Bone regeneration • Plateau root form

7.1 Introduction

The phenomenon of bone presenting intimate contact to titanium implants was first reported in 1940 by Bothe et al. (1940) and later described in 1951 by Leventhal (1951). Decades later, it was characterized in more detail by a Swedish group (Branemark et al. 1969, 1977), whereas the term osseointegration was first coined only in 1981 (Albrektsson et al. 1981; Albrektsson and Johansson 2001). While its definition has constantly changed/evolved over the years, it is currently recognized as the intimate contact of material and bony tissue without the growth of fibrous tissue at the bone-implanted material interface (Dorland 2012). This phenomenon has for over five decades affected the well-being of millions of patients as the basis for dental and orthopedic rehabilitations that rely on the surgical placement of bioinert, biocompatible and osseoconductive implants followed by the adequate healing and remodeling of bone around the device (Eitner et al. 2012). During its discovery and characterization years, key factors for the successful establishment of osseointegration relied on both device (biocompatibility, osseoconductivity, sterility, and others) and patient related (local and systemic) characteristics (Albrektsson et al. 1981).

Currently, an online scientific literature search with the keyword osseointegration exceeds 10,000 references, and over 50 years the phenomenon has gone from discovery to medical device industry commodity. Such increase in fabrication scale has occurred due to the increasing world demand for cost-effective oral rehabilitation along with substantial evolution of manufacturing techniques. However, while the number of devices fabricated worldwide per year is constantly increasing, this increase is rarely founded by robust research and development strategies that result in improvements for the population in need. In contrast, when trials are performed to validate new implant designs, a decreased probability of annual failures has been associated with industry-sponsored research compared to non-industry associated trials, which not only suggests that strong bias may exist in the literature, but most importantly that a potential detour in proper decision making may be present regarding tooth preservation and governmental health care policies (Popelut et al. 2010). Such scenario has eventually led to "commodity development" in the field of implant dentistry, in the sense that the market accepts hardware versions without serious validation specific to that version (Henry 1995).

When considering the number of dental implant systems commercially available, a plethora of implantable devices with different characteristics (geometry, surface treatment, surgical instrumentation) are available. Common to all these devices is that they ultimately rely on the same phenomenon to perform their rehabilitative role in bone (Esposito et al. 2014). However, while the same intimate interaction between bone and implant is responsible for the rehabilitation system biomechanics competence, how it is achieved on the short-term may substantially vary among different implant systems and ultimately results in variations in the long-term structure/property of bone surrounding the implanted devices (Coelho and Jimbo 2014a, b). This chapter initially discusses how short- and long-term osseointegration is affected by two key

parameters that consist the implant shape and its associated surgical instrumentation dimensions especially when screw root form and plateau root form implants are concerned. Then strategies to hasten the process of osseointegration such as implant surface treatments, tissue engineering approaches, and surgical instrumentation parameters are discussed.

7.2 Early Osseointegration Pathway: Interfacial Remodeling, Intramembranous-Like Healing (Healing Chambers), and Hybrid Healing

It is a general consensus that properlycleaned and sterilized biocompatible titanium-based alloys (primarily comprised by commercially pure Ti and Ti-6Al-4V) devices will be incorporated within the bone tissue after installation (Williams 1977). While osseointegration has gone from discovery to commodity, its establishment after implant placement has endemically been thought to occur through the same pathway for any given implant system by the dental profession despite previous publications describing otherwise (Berglundh et al. 2003; Leonard et al. 2009; Coelho et al. 2010a, b, c). The difference in how surgical hardware (implant and surgical instrumentation shape) affects initial bone healing has been recently published in more detail (Coelho et al. 2010c, 2011) and the two main early healing modes are summarized below.

7.2.1 Interfacial Remodeling Healing Pathway

This early healing scenario is typically observed for tight fit screw type implants (i.e. the majority of implant systems available in the market) as these are placed in the osteotomy in intimate contact between the implant and bone throughout the device's threaded bulk. Such intimate interplay between device and osteotomy dimensions renders the system initial or primary stability where reduced biologic interplay yet exists (Leonard et al. 2009; Coelho et al. 2010c, 2011). This mechanical interlocking is variably influenced by the implant geometry and implant osteotomy site dimensions, and regulate the distribution of strain applied to the hard tissue in proximity with the implant. From a clinical standpoint an important misinterpretation occurs at this point, where some clinicians generically and equivocally assume that high insertion torque levels of any given implant design lead to proportionally high resistance to micromotion and therefore to improved osseointegration levels (Campos et al. 2014; Freitas et al. 2012). Whereas some clinicians may still quest for very high implant insertion torque, it must be acknowledged that, if the strain applied to bone in such scenario exceeds its given threshold, its stability would decrease over time due to microfractures and excessive strain that may result in pressure necrosis, since both phenomena result in bone remodeling (Campos et al. 2012; Galli et al. 2015; Jimbo et al. 2014; Coelho et al. 2013). After an interfacial cell-mediated bone resorption, subsequent bone apposition most often occurs from the pristine bone wall towards the implant surface. Such bone removal and subsequent apposition has been theoretically (Raghavendra et al. 2005) and experimentally (Gomes et al. 2013) demonstrated and is regarded as implant stability dip, where primary stability obtained through the mismatch between implant macrogeometry and surgical instrumentation dimensions is lost due to the cell-mediated interfacial remodeling to be regained through bone apposition (Coelho and Jimbo 2014a, b; Jimbo et al. 2007). This healing mode sequence concerns implant placement in sites that were surgically instrumented to dimensions that approximate the inner diameter of the implant threads (Bonfante et al. 2013) and its pathway is illustrated in detail in Fig. 7.1. In summary, a cell-mediated process whose extension is proportional to the amount of strain incurred to the bone surrounding the implant takes place prior to new bone formation. In general, such remodeling extends to several hundred micrometers away from the implant surface and this void space is filled by newly formed bone. At early

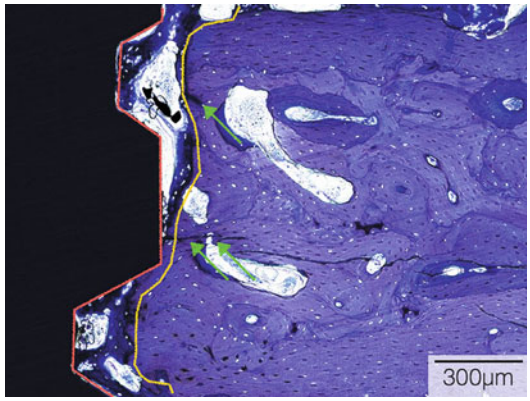

Fig. 7.1 Representative optical micrograph of a screw type implant placed in a rabbit tibia instrumented to the inner diameter of the implant thread. The *blue line* depicts the implant perimeter that was in direct contact with bone immediately after placement (the cortical plate fully occupied the region between the *blue* and *yellow lines*). The *yellow line* depicts the distance from the implant surface (*red line*) which cell mediated interfacial remodeling occurred due to osteocompression and/or bone cracking (*arrows*). The dark stained bone tissue between the *blue* and *yellow lines* is bone formed after a void space is created due to interfacial remodeling to eliminate tissue excessive strain. Toluidine Blue stain

time points, an almost continuous bone-implant interface renders the system implant primary stability. At this stage, microfractures depicting that the yield strength of bone has been exceeded due to high stress levels are visualized along with initial remodeling taking place between the implant threads due to pressure bone necrosis (Bashutski et al. 2009; Halldin et al. 2011; Verborgt et al. 2000; Chamay and Tschantz 1972). As time elapses in vivo, an extensive remodeling region is evident presenting void spaces partially filled by newly formed bone. Thus, the scenario that has been histologically observed in multiple instances confirms the theoretical and experimental basis for the initial stability rendered by mechanical interlocking between implant and bone that at some point in time after placement under stable conditions decrease due to extensive bone resorption (Fig. 7.1). Subsequently, the resorbed area will be filled by newly formed woven bone, which eventually reestablishes the contact to the implant interface (secondary stability) (Berglundh et al. 2003; Leonard et al. 2009; Coelho et al. 2010c, 2011) (Fig. 7.1).

7.2.2 Intramembranous-Like Healing Pathway (Healing Chamber Osseointegration)

The second osseointegration pathway concerns the opposite scenario of the tight fit screw type implant, where void spaces between the implant bulk and the surgically instrumented drilled site walls are formed (plateau root form implants) (Berglundh et al. 2003). These void spaces left between bone and implant bulk, often referred to as healing chambers, will be filled with blood clot immediately after placement and will not contribute to primary stability. These however, have been regarded as a key contributor to secondary stability due to its rapid ossification and morphology presenting high degrees of cellular and vascular content (Huang et al. 2015).

Berglundh et al. (2003) discussed the early healing biology and kinetics (Fig. 7.2) of bone formation in healing chambers and the effect of healing chamber size and shape (know to have a significant effect on how fast bone fills these chambers) on bone formation has also been preclinically evaluated (Coelho et al. 2011; Marin et al. 2010). Such healing chambers, filled with the blood clot, will evolve towards osteogenic tissue that subsequently ossifies through an intramembranous-like pathway (Berglundh et al. 2003). The bone forming within the healing chamber presents high cellular and vascular content at early time points, noticed by the early replacement of woven bone by lamellar bone surrounding the perimeter of primary osteons. Noteworthy is that unlike the interfacial remodeling healing pathway, healing chamber configurations do not encompass the initial cleanup process due to microfractures and excessive ostecompression (Berglundh et al. 2003; Coelho et al. 2010b, c, 2011; Marin et al. 2010; Granato et al. 2009, 2011).

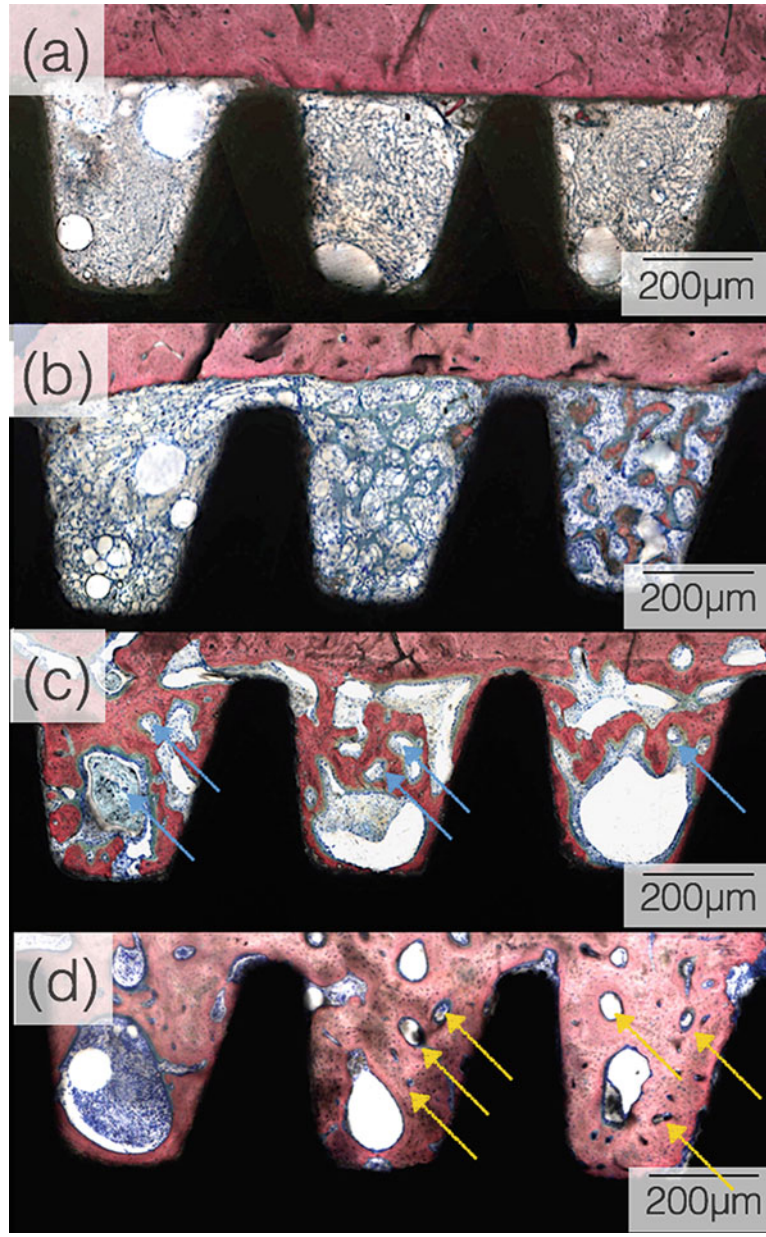

Fig. 7.2 Sequence of intramembranous-like healing osseointegration. (**a**) 1 week, (**b**) 3 weeks, (**c**) 6 weeks, and (**d**) 12 weeks in vivo in a beagle dog model. (**a**) The blood filling the space between pristine bone and device will develop towards a connective tissue network that provides a seamless pathway for cell migration within the space once filled by the blood clot. (**b**) Such healing configuration allows initial bone formation throughout the healing chamber from (**c**) all available surfaces (implant surface, instrumented bone surface) and within the chamber volume, presenting substantial deviation from the classic interfacial remodeling healing pathway observed in tight fit screw-type implant. The bone forming within the healing chamber presents high cellular and vascular content at (**a** and **b**) early time points, noticed by (**c** and **d**) the early replacement of woven bone by lamellar bone surrounding the perimeter of primary osteons (*arrows*). Stevenel's Blue Von Giesson's acid fuchsin stain

7.2.3 Current Trend: Hybrid Healing Pathway: Integrating Interfacial Remodeling and Intramembranous-Like Bone Healing Modes

Recent investigations have employed either experimental implant designs with an outer thread that provided stability while the inner thread and osteotomy dimensions allowed healing chambers (Berglundh et al. 2003; Abrahamsson et al. 2004, 2009) or alterations in osteotomy dimensions in large thread pitch implant designs (Coelho et al. 2010c; Campos et al. 2012; Vandeweghe et al. 2013). The rationale for these alterations lie upon the fact that thread designing may allow for both high degrees of primary stability along with a surgical

instrumentation outer diameter that is closer to the outer diameter of the implant allowing healing chamber formation. Since healing chambers allow rapid intramembranous-like rapid woven bone formation (Witek et al. 2013; Bonfante et al. 2011), such rapid bone growth may compensate for the implant stability loss due to compression regions where implant threads contacts bone for primary stability (Coelho et al. 2013).

7.3 Long-Term Osseointegration: Healing Pathway Effect on Osseointegration, Bone Morphology, and Bone Mechanical Property Evolution

While studies concerning early osseointegration are more easily achieved in both preclinical and clinical scenarios, tracking devices in place over the years represent a significant logistic and economic challenge (Lemons 1988a, b, 2010; Lemons et al. 2010). While a plethora of preclinical studies have shed light onto both the early and long-term osseointegration (Coelho and Jimbo 2014a, b; Coelho et al. 2009a; Elias and Meirelles 2010), clarification of the actual temporal implant-bone interaction under functional loading is a remarkable challenge as discussed in important past consensus (NIH 2000). The main difficulty arises from ethical aspects of retrieving stable implants from patients. Furthermore, sample inhomogeneity due to differences in implant design (mainly macrogeometry), surface treatment and topography, time of functional loading, loading protocols, and clinical parameters during the time of placement makes it extremely difficult to draw conclusions (Lemons 1988b; Coelho et al. 2009a). Thus, in reality, most of the existing human retrieval studies are based on failed implants or on limited number of stable implants that were extracted for different reasons such as inadequate position within the arch (Iezzi et al. 2012, 2013a, b, c, 2014; Mangano et al. 2013; Degidi et al. 2009a, b, c; Shibli et al. 2007; Grassi et al. 2007), which eventually limit the amount of variables required to provide an informed design rationale for device engineering and associated surgical and prosthetic guideline developments.

While limited in the number of reports and sample size, histologic human retrieval studies report that over the first year under functional loading, bone around implants undergoes remodeling and the initial woven bone in proximity of the implant surface is replaced by bone presenting higher degrees of organization in a lamellar configuration (Degidi et al. 2009a, b, c; Grassi et al. 2007). The same body of literature also supports that as time further elapses with implants in function, higher degrees of organization are achieved, which is reflected by multiple remodeling sites within the lamellar bone (Iezzi et al. 2012, 2013a, b, 2014).

The difficulty in obtaining human retrieved samples is such that studies comprising samples retrieved from the same implant system placed under the same surgical guidelines and latency period prior to prosthetic loading are limited in number (four only) (Baldassarri et al. 2012; Coelho et al. 2009b, 2010d; Gil 2015).

7.3.1 Long-Term Morphology of Implants That Undergo Interfacial Remodeling

Screw root form implants are typically placed in drilled sites that are narrower than the implant diameter (Coelho and Jimbo 2014a, b). According to preclinical studies, this mechanical interlocking due to differences in screw root form implant and drilled site diameter results in a substantial bone volume under compression immediately after placement that is subsequently remodeled prior to further bone apposition (Berglundh et al. 2007; Davies 2003). Following osseointegration of a screw root form implant, it is a general consensus that further bone remodeling occurs under functional loading resulting in higher degrees of bone organization (Coelho and Jimbo 2014a, b). Remodeling seems to be more evident at the coronal aspect of implants, where most of the occlusal forces are concentrated. Morphologically, however, bone surrounding these implants has been often described as compact mature lamellar bone with

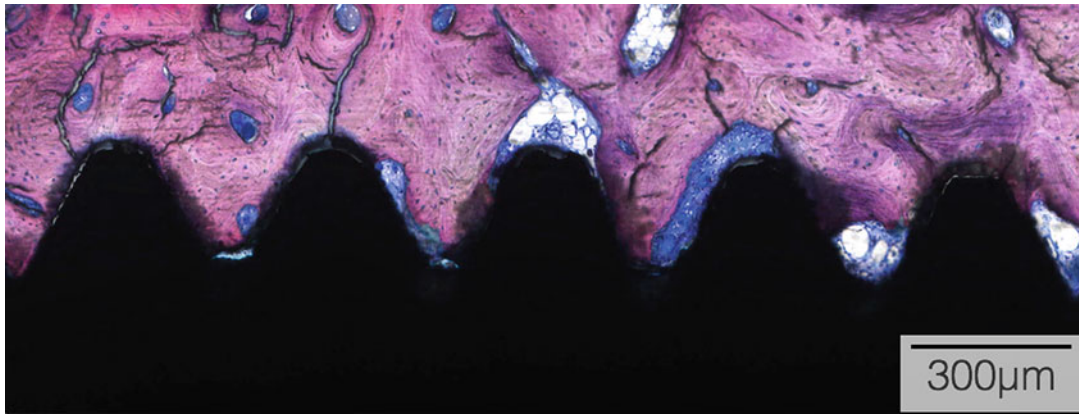

Fig. 7.3 A human retrieved sample showing direct agreement with other reports for screw type implants placed in undersized drilled sites. The bone surrounding these implants present a compact mature lamellar bone with few and small marrow spaces. Stevenel's Blue Von Giesson's acid fuchsin stain

few and small marrow spaces (Iezzi et al. 2012, 2013b, 2014; Mangano et al. 2013) (Fig. 7.3). To date, no study regarding screw root form implant has presented sufficiently large sample size to determine the time course alteration of osseointegration or to make a clear assessment of bone mechanical property evolution as a function of implantation time.

7.3.2 Long-Term Morphology, Bone Mechanical Property, and Temporal Osseointegration of Implants That Undergo Intramembranous-Like Healing

Four sizable implant retrieval studies are currently available, concerning plateau root form implants (Baldassarri et al. 2012; Coelho et al. 2009b, 2010d; Gil 2015). The first two studies reported the basic morphologic and morphometric evaluation of textured titanium surface and hydroxyapatite coated plateau root form implants as a function of implantation time (Coelho et al. 2009b, 2010d). While these studies provided valuable data regarding the unique bone morphology presenting cortical-like characteristics irrespective of implant and patient-dependent characteristics, the sample size was not sufficient for an appropriate assessment of osseointegration measurable parameters as a function of functional loading time. The results of these two studies showed that regardless of functional loading time and any other implant/clinical parameter, similar bone morphologic scenarios were observed between specimens. Intimate contact between bone and implant surface was observed at all regions of the implant perimeter. Varying degrees of bone filling were observed between plateaus for all samples. No signs of epithelial migration or connective tissue was observed. In general, bone morphology around implants that were loaded up to 1 year presented a mixture of woven and lamelar bone embedding primary osteons as presented in Fig. 7.4a. For all other implants, which were retrieved after approximately one and half years of implant placement, a remarkably similar structure was observed as presented in Fig. 7.4b, c. All optical micrographs showed that the predominant bone morphology observed between plateaus presented a lamellar configuration in varied directions depicting multiple remodeling cycles in a cortical-like fashion throughout the implant life under loading.

The third study considered various implant surfaces for 30 implants that were under functional loading for as long as over 20 years (Baldassarri et al. 2012). While the sample size was also not sufficiently powerful to determine

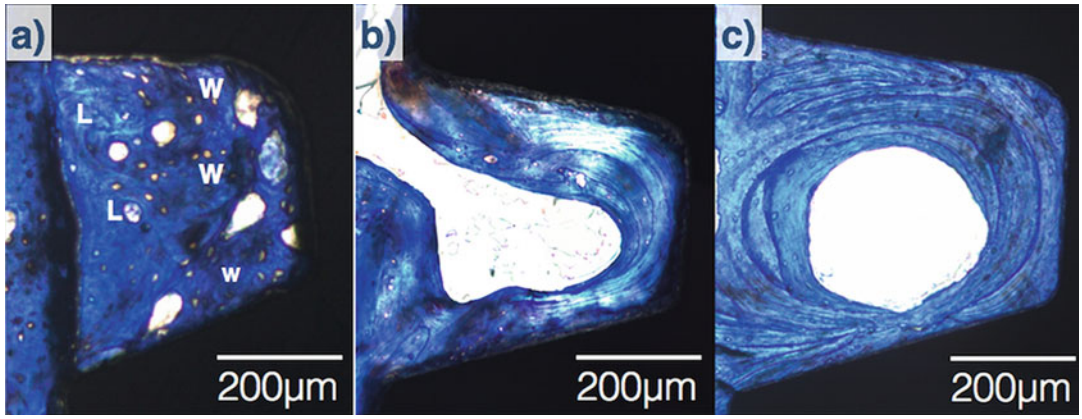

Fig. 7.4 Optical micrographs representative of (**a**) implants that were loaded up to 1 year in vivo demonstrated a mixed bone morphology with regions of woven (w) and lamellar bone. Implants that remained loaded for longer periods of time such as in (**b**) 8 years and (**c**) 17 years primarily presented a cortical-like lamellar structure. Toluidine Blue stain

the status of osseointegration over time, nanoindentation measurements of bone around implants was able to depict that bone mechanical properties (hardness and elastic modulus) did increase over time, strongly suggesting that osseointegration is a dynamic phenomena and that bone is always adapting to functional loading over time to increase the overall system biomechanics. In summary, this mechanical study determined that cortical-like bone properties are achieved and maintained after 5 years of implantation time irrespective of patient-specific and implant design variables (Fig. 7.5).

These three studies, each presenting between 24 and 30 implants, resulted in limited power when one attempts to correlate the effect of clinical and dental implant design variables in osseointegration and bone measurable parameters (Baldassarri et al. 2012; Coelho et al. 2009b, 2010d). Among these three studies, only one has presented sufficient statistical power to determine the positive effect of time under functional loading on bone mechanical property evolution (Baldassarri et al. 2012). Thus, while insightful, these studies lack appropriate sample size for appropriate statistical inferences regarding the effect of patient-dependent and implant parameter variables on osseointegration. The most valuable feature of all these studies is that the surgical and prosthetic protocols were comparable and thus one study may be directly compared to the other and over time the samples can be collapsed for more statistically comprehensive analyses.

Thus, the fourth and most comprehensive study retrieved over a period of approximately 15 years, 210 human samples that were under functional loading from 0.3 to 24 years due to retreatment reasons (Gil 2015). All implants were retrieved for prosthetic reasons (primary treatment planning alteration due to tooth loss that required different treatment modalities; no clinically failing implants were considered). A retrospective patient chart review revealed that all retrieved implants were restored with a single crown and subjected to functional loading approximately 6 months after surgical placement. Data regarding implant design parameters such as surface, length, and diameter and patient related characteristics as jaw placement (maxilla vs. mandible), region (anterior vs. posterior), and functional loading time were collected. The final analysis considered 93 implants—72 hydroxyapatite (HA, Integra-CP, Bicon LLC, MA), 8 titanium plasma sprayed (TPS, Bicon LLC) and 13 uncoated (Integra-Ti, Bicon LLC). On average, these implants were retrieved after about 6 years (SD = 5.2); 21.5% were retrieved within a year, and the longest retrieval interval was almost 18 years. Analysis of variance (ANOVA) was used to evaluate relationships between outcome measures of bone-to-implant contact (BIC), bone area fraction occupancy (BAFO), and ranked

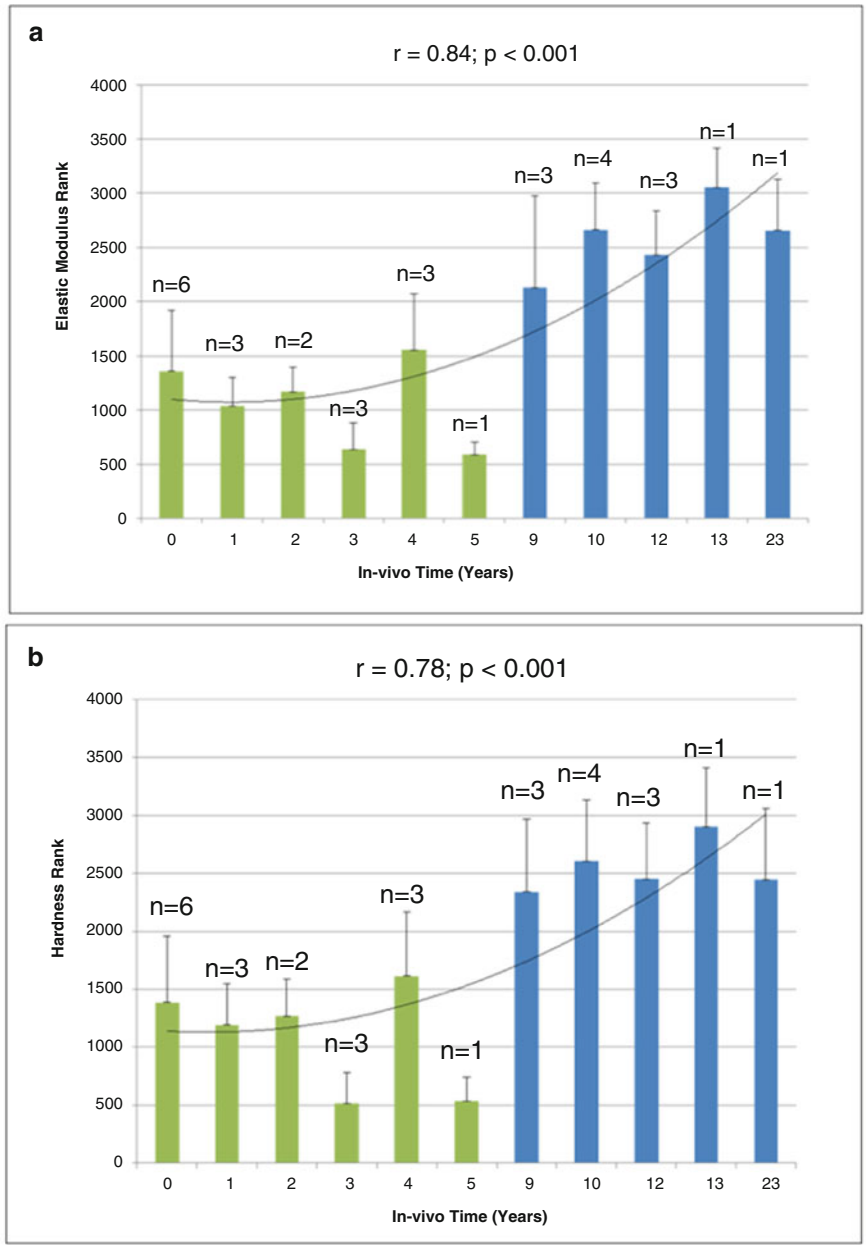

Fig. 7.5 (**a**) Elastic Modulus (GPa) vs. implantation time and (**b**) hardness (GPa) vs. implantation time. Each bar shows mean and standard deviation for specimens with similar time in function. Note the significant increase in bone mechanical property around the implants after 5 years of implantation time

functional loading time and independent variables of surface material, implant diameter, implant length, arch, and anterior/posterior location. Patient age and gender were excluded from the analysis as these presented non-significant influence in any dependent variable. Linear regression analyses (IBM, Armonk, USA) were used to evaluate the relationship between time of functional loading, BIC, and BAFO.

The results of this study (Gil 2015) is presented in Table 7.1 that shows mean levels of BIC, BAFO, and functional loading time as a function of implant characteristics and clinical independent variables. Mean functional loading

Table 7.1 Mean (SD) BIC, BAFO, and time in vivo (ranked) as a function of surface material, implant diameter, implant length, region and arch Different supercript letters indicate statistically homogeneous groups

Measure	N	BIC %	BAFO %	Loading time (ranked)
Surface				
HA	72	65.4 (13.6)	63.2 (17.2)	40.5 (25.5)[a]
TPS	08	72.0 (11.0)	56.3 (14.1)	70.5 (9.1)[b]
Uncoated	13	72.6 (9.3)	71.0 (11.5)	68.3 (24.3)[b]
Diameter (mm)				
4.0	38	69.6 (12.2)	67.4 (15.9)	60.3 (26.9)[a]
4.5	21	61.4 (14.4)	60.3 (18.6)	28.7 (19.4)[b]
5.0	34	67.5 (13.0)	61.6 (15.8)	42.7 (24.3)[c]
Length (mm)				
6.0	24	64.2(13.0)	62.1 (15.6)	26.8 (15.7)[a]
8.0	39	65.1 (13.8)	62.9 (18.7)	38.3 (24.3)[b]
11.0	30	71.6 (11.9)	65.9 (14.7)	72.8 (15.4)[c]
Region				
Anterior	28	63.8 (12.5)	63.2 (16.3)	40.3 (23.5)
Posterior	65	68.4 (13.2)	63.9 (16.7)	49.9 (28.0)
Arch				
Maxilla	40	68.5 (12.7)	65.8 (15.9)	53.0 (28.6)
Mandible	53	65.9 (13.4)	62.1 (16.9)	42.5 (25.0)

time was significantly lower in HA than other implants; significantly increased with implant length; was highest in 4 mm diameter implants, lowest in 4.5 mm implants and intermediate in 5 mm implants (significantly different between 3 diameters); and did not vary with either anterior/posterior region or arch. No implant and patient related characteristics were statistically related to either BIC or BAFO. Figure 7.6 shows that both BIC ($R=0.38$, $p<0.001$) and BAFO ($R=0.25$, $p<0.02$) increased as a function of (ranked) functional loading time.

The overall BIC and BAFO results presented by the 93 implants evaluated in the described study (Gil 2015) were in direct agreement with previous studies concerning plateau root form implants that were under functional loading from 0.3 to over 20 years (Baldassarri et al. 2012; Coelho et al. 2009b, 2010d). The substantially larger sample size evaluated also allowed a more powerful analysis regarding how BIC and BAFO were possibly affected by different implants design and patient-related clinical parameters.

While preclinical studies concerning the effect of plateau root form implant surface on early osseointegration have shown that calcium-phosphate coated implants presented higher degrees of osseointegration over time (Coelho et al. 2010b; Granato et al. 2009, 2011; Suzuki et al. 2009, 2010), no difference in BIC and BAFO were detected between the three different surfaces evaluated in the described human retrieval study involving the same implant design (Gil 2015). In addition, concerning specific implant design parameters evaluated, no individual or combination of parameters (implant diameter, length, and surface) resulted in higher BIC or BAFO, suggesting that after osseointegration is successfully achieved, implant diameter, length, and surface have minimum to no effect on its temporal fate. Specific to implant diameter and length, despite being design features that are well known to affect the overall bone-implant environment biomechanics, the results obtained in that human retrieval study (Gil 2015) suggest that the bone configuration and mechanical properties were sufficient to maintain clinically sufficient levels of osseointegration. Of special interest were the clinical independent variables evaluated: maxilla or mandible, and whether the

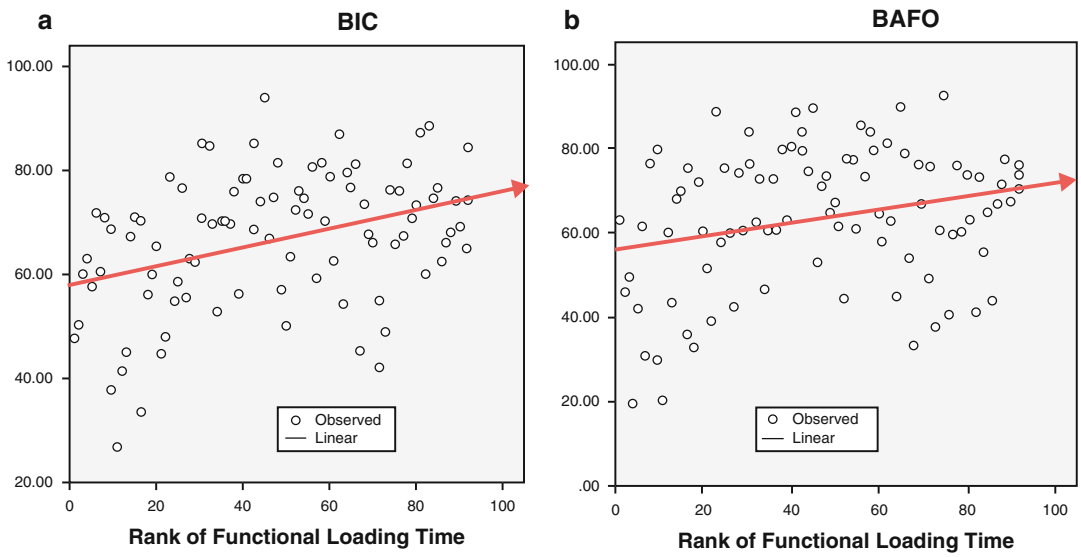

Fig. 7.6 Human retrieved implants (**a**) BIC and (**b**) BAFO as a function of rank of functional loading time

implants were placed in the anterior or posterior regions of the arch. Surprisingly, statistical analyses showed that none of these parameters affected either BIC or BAFO. While it is known that denser bone is usually available in the mandible relative to the maxilla, and anteriorly relative to posterior, the long-term bone-implant interfacial interactions are not affected by the bone quality in this specific implant geometry (plateau root form implants) (Gil 2015).

Finally, when BIC and BAFO were collapsed over all implant design and clinical independent variables, both BIC and BAFO regression models showed an increase as a function of (ranked) functional loading time (Gil 2015). These data suggest that, over time and functional loading, osseointegration is a cumulative process in plateau root form implants (Fig. 7.6). Statistical analysis also showed that increased integration as a function of functional loading time is not specifically related to any implant design parameter and clinical variables such as arch location and anterior/posterior position. Thus, the longer a plateau root form implant stays under functional loading, osseointegration is expected to increase (Fig. 7.6).

7.4 Hastening the Osseointegration Process

While promising developments have been made over the last five decades regarding implant hardware designing and how it dictates early bone healing and long-term bone morphology around implants, it is widely recognized that other design features do in fact hasten bone healing around implants and can further increase the performance of implant hardware (Coelho and Jimbo 2014a, b; Coelho et al. 2009a; Palmquist et al. 2010). For instance, lower length scale design parameters have been designed in an attempt to change the degree of intimacy between host biofluids and implant surface while also changing cell phenotype to hasten biological response (Hamilton and Brunette 2007; Leclercq et al. 2013; Yang et al. 2013; Zambuzzi et al. 2011). However, their early effects are directly related to their strategic hierarchical placement as a function of implant hardware design since, as previously presented, healing mode and kinetics drastically shift as a function of the macrometer

scale variables. It is thus intuitive that implant hardware should be strategically designed to maximize interaction between the host biofluids and the implant surface.

For instance, the reduced length scale design features intended to improve the establishment and maintenance of continuous pathway for bone forming cell migration towards the implant surface will not be as effective in hastening bone healing in regions where cell-mediated interfacial remodeling initially occurs after placement due to initial hardware design interaction with bone (Gomes et al. 2013; Bonfante et al. 2013). Thus, implant hardware designs that allow healing chamber formation are more suited to deliver adequate conditions for improved micrometer and the nanometer length scale design features performance in hastening early osseointegration (Coelho and Jimbo 2014a, b).

A plethora of preclinical studies concerning surface modifications and tissue engineering strategies concerning plateau root form implants have been conducted to date (Leonard et al. 2009; Coelho et al. 2010b, c, 2011; Marin et al. 2010; Granato et al. 2009, 2011; Suzuki et al. 2009, 2010). Common to all these studies is the fact that modifications of implant design at the micrometer and nanometer scale with or without biomolecular tagging have been performed in an attempt to improve the early bone response to plateau root form implants.

The initial studies comprised direct biomechanical and histometric comparison between an alumina-blasted/acid-etched surface (IntegraTi™) that has been available for the Bicon implant system for several decades (used as control), and a variety of candidate surfaces that primarily comprised thin coatings of calcium-phosphate based bioactive ceramics (Suzuki et al. 2009, 2010). For this series of studies, a cylindrical prototype implant macrogeometry was utilized and promising results were histometrically detected for the bioactive ceramic thin-coated implants relative to the uncoated IntegraTi™. The significantly higher osseoactivity around thin-coated implants was approximately 35 % higher than controls. When the plasma-sprayed calcium-phosphate coating (PSCaP, IntegraCP™) was compared to IntegraTi™ and thin bioactive ceramic coated implant groups, a significant increase in biomechanical fixation and bone mineral apposition rate was detected for the PSCaP coated implants, indicating that relatively thicker coating hastened bone healing to a higher degree compared to the other surfaces, warranting investigations utilizing plateau root form implant prototypes. Then, a second series of studies also compared the IntegraTi™ and thin bioactive ceramic coatings on plateau root form implant prototypes (Granato et al. 2009, 2011). As expected, due to the favorable environment provided by the surface treated healing chambers, measurable osseointegration indicators depicted a more sizable effect in both biomechanical and histometric parameters for the thin-coated implant relative to IntegraTi™ when differences were compared to the previous studies considering cylindrical implant prototypes that mostly healed through the interfacial remodeling pathway (Coelho and Lemons 2009; Coelho et al. 2009c, d).

A third series of studies comparing IntegraTi™, thin-coated bioactive ceramics, and IntegraCP™ was conducted on plateau root form implant prototypes. This series of preclinical studies in different animal species unequivocally demonstrated the superiority of the IntegraCP™ surface treatment when improvements in early bone healing around pleateau root form is concerned (Coelho et al. 2010b; Bonfante et al. 2012; Quaranta et al. 2010).

Subsequently, attempts to hasten early bone healing around plateau root form implants comprised strategies to improve the early interaction between the blood clot and implant surface in an attempt to provide a seamless pathway for ostegenic cells to migrate through the osteogenic connective tissue present in the healing chambers towards the implant surface. Such approach utilized an argon-based atmospheric pressure plasma (APP) source applied to the implant immediately prior to implantation (Giro et al. 2013; Coelho et al. 2012). When such strategy was applied in a beagle dog model, osseointegration levels increase over 300 % for the APP-treated IntegraTi™ surface relative to the untreated IntegraTi™ surface (Coelho et al.

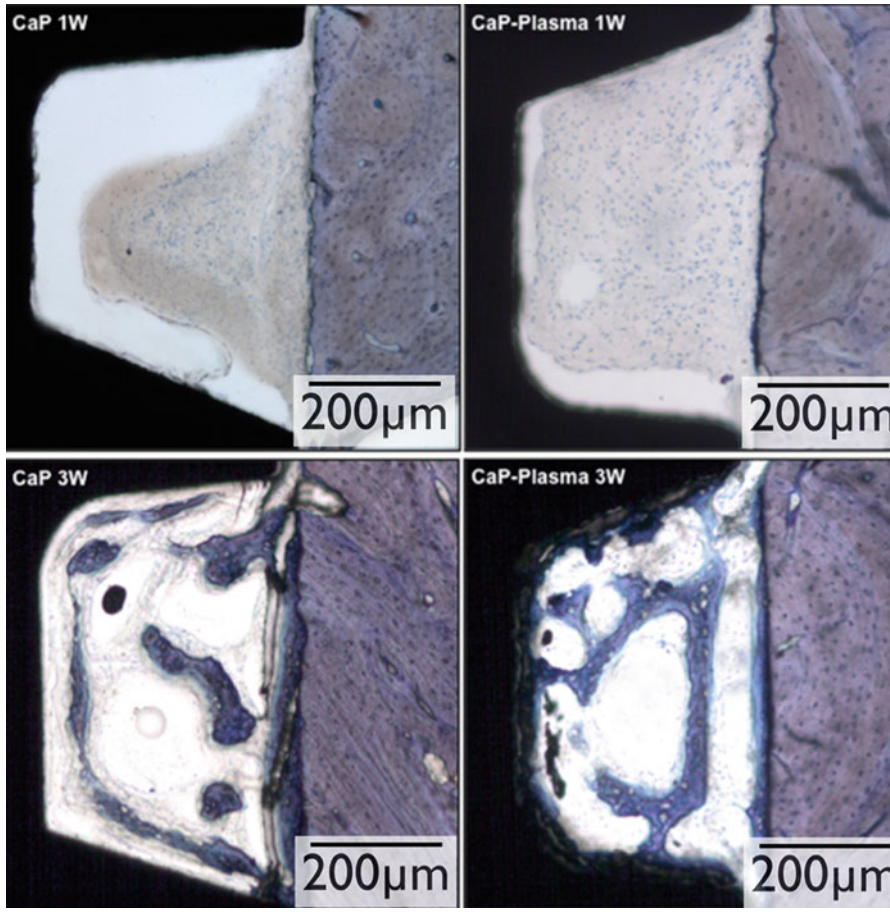

Fig. 7.7 Representative overview of the histological micrographs of PSCaP coated plateau root form implant groups at 1 and 3 weeks in vivo. At 1 week, the histologic sections of the CaP-plasma group showed initial signs of bone formation adjacent to the implant surface and the presence of layers of early connective tissue (stroma) in intimate contact with the implant surface. In contrast, the CaP group presented the stroma collapsed to the center of the plateau. At 3 weeks, bone formation was observed throughout the healing chambers of both groups. Note the gap between tissue and implant surface was pronounced for the 1 week samples. Toluidine Blue stain

2012). When the approach was applied to IntegraCP™ surfaces, an increase in 80 % osseointegration was obtained for the APP-treated integraCP™ compared to untreated integraCP™ surface (Giro et al. 2013). The story telling histologic sections presented in Fig. 7.7 showed that the APP treatment of both IntegraTi™ and IntegraCP™ surfaces resulted in a more intimate interaction between the ostegenic tissue derived from the blood clot relative to untreated surfaces. Such panorama resulted in a seamless pathway for ostegenic cells to reach the implant surface compared to untreated surfaces.

To date, a tissue engineering approach has also been utilized to accelerate early bone healing around plateau root form implants. This strategy involved dip coating both IntegraTi™ and IntegraCP™ implants in rhBMP-2 prior to implantation in the hip of sheep (a bone that presents very low density) (Yoo et al. 2014). The results (Fig. 7.8) demonstrated that increases over 100 % and 50 % osseintegration was observed for the rhBMP-2 IntegraTi™ and IntegraCP™ groups, respectively, relative to their controls. Worth noting is that a difference of approximately 600 % was observed between the

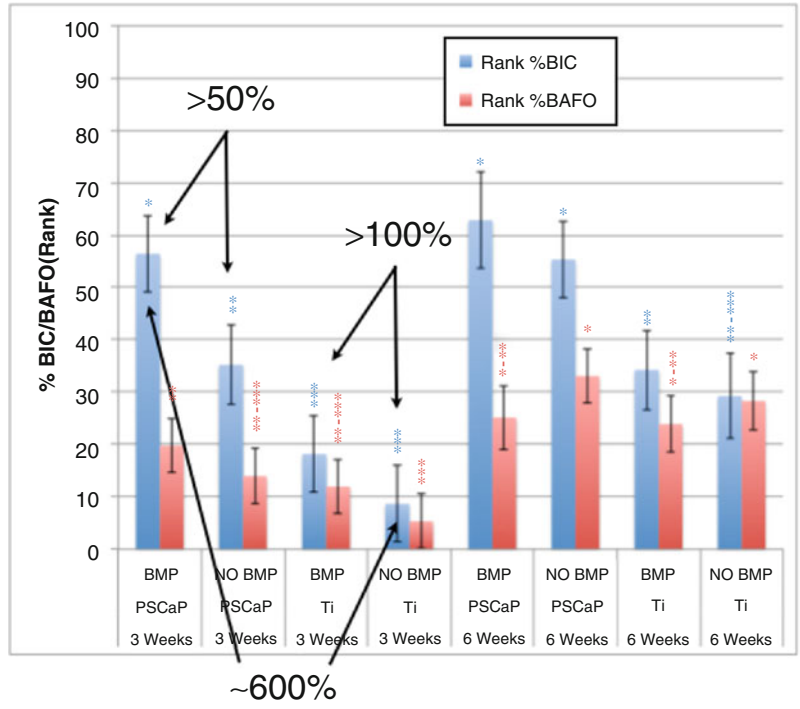

Fig. 7.8 Statistical results summary (mean ±95 % CI) concerning ranked %BIC and %BAFO considering presence of rhBMP-2 (BMP), implant surface type, and time *in vivo*. Note that the number of asterisks depicts statistically homogeneous groups

rhBMP-2 IntegraCP™ group and regular IntegraTi™ were compared, demonstrating the efficacy of the combination of a PSCaP coating loaded with rhBMP-2 in increasing osseointegration levels of plateau root form implants (Yoo et al. 2014).

7.5 Final Remarks

Over the last 50 years, the phenomenon of osseointegration has not only revolutionized but also led to significant developments in dentistry and orthopedics, resulting in life-changing clinical outcomes that have not only provided or improved esthetics and function, but also promoted quality of life to millions of individuals worldwide. Research has attempted to maximize, accelerate, and perfect the physiologic events associated with the process of osseointegration. Significant improvements in short- and long-term osseointegration have been achieved over the years, with the most substantial, consistent, and reproducible results being associated with more careful and gentle instrumentation protocols, enhanced implant surface treatments, and variations in implant macrodesign. While properly cleaned and sterilized biocompatible titanium-based alloys are capable of achieving osseointegration, implant surface treatments and coating methods such as alumina-blasted/acid-etched surface, plasma-prayed calcium-phosphate, argon-based atmospheric pressure plasma, and rhBMP-2 dipping have shown to substantially accelerate and enhance osseointegration, allowing for faster patient rehabilitation. While interfacial remodeling healing pathway still is accepted by many as the process likely to result in superior clinical outcomes, intramembranous-like healing or hybrid pathways are likely to change this misconception since such implant macrodesigns leading to the latter pathways have been shown to facilitate the enhancement of micrometer/nanometer and tissue engineering to hasten early bone healing around implants while permitting continuous osseointegration increase over time. Considering

that long-term stability of osseointegration currently is one of the major concerns in implant dentistry, this field of research is likely to gain even more attention in the future.

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Dentin Matrix Proteins in Bone Tissue Engineering

8

Sriram Ravindran and Anne George

Abstract

Dentin and bone are mineralized tissue matrices comprised of collagen fibrils and reinforced with oriented crystalline hydroxyapatite. Although both tissues perform different functionalities, they are assembled and orchestrated by mesenchymal cells that synthesize both collagenous and noncollagenous proteins albeit in different proportions. The dentin matrix proteins (DMPs) have been studied in great detail in recent years due to its inherent calcium binding properties in the extracellular matrix resulting in tissue calcification. Recent studies have shown that these proteins can serve both as intracellular signaling proteins leading to induction of stem cell differentiation and also function as nucleating proteins in the extracellular matrix. These properties make the DMPs attractive candidates for bone and dentin tissue regeneration. This chapter will provide an overview of the DMPs, their functionality and their proven and possible applications with respect to bone tissue engineering.

Keywords

Dentin matrix proteins • DMP1 • DPP • DSP • FAM20C • DMP4 • Tissue engineering • Bone regeneration

8.1 Introduction

Bone and dentin are mineralized hard tissues. The primary inorganic component of these two tissues is crystalline hydroxyapatite and the primary organic component is type I collagen. Type I collagen forms a dynamic and instructive template for the deposition of calcium phosphate polymorphs and their subsequent transformation into crystalline hydroxyapatite crystals. However, the process of hydroxyapatite nucleation and collagen mineralization is highly complex and is controlled by proteins that are categorized as noncollagenous proteins (NCPs) (Young et al. 1992). Although they only constitute a fraction of the bone and dentin volume, without the NCPs, it

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would be impossible to achieve bone formation and remodeling. Dentin matrix proteins (DMPs), osteocalcin, osteopontin and bone sialoprotein (BSP) are some examples of NCPs found in bone and dentin.

Bone tissue engineering, which consists of repair and regeneration, is an area that has generated a lot of interest. The fast pace of development of materials for bone tissue engineering suggests that the potential to be clinically viable would be sooner than other fields of tissue engineering. Products such as demineralized bone matrices (DBMs) and other allograft bone materials are already in clinical use (Campana et al. 2014; Wang et al. 2007). On the other hand, the incidence of bone disorders and the repair of deformed bones is an ever- increasing problem owing to many factors such as, lifestyles, global wars and increase in devastating accidents worldwide. Current clinical practices cannot address many of the regenerative aspects of bone injuries satisfactorily and tissue engineering has been seen as the logical alternative (Amini et al. 2012).

As proposed more than two decades ago by Langer and Vacanti, tissue engineering of any tissue requires three components, namely: Cells, scaffolds and growth factors (Langer and Vacanti 1993). These components are often referred to as the tissue engineering triad. Several types of scaffolds have been used for bone tissue engineering including artificial polymers, natural polymers and synthetic self assembling peptides (George and Ravindran 2010). The focus of this chapter will be on the use of DMPs as signaling molecules and hydroxyapatite nucleating agents for bone tissue engineering.

Dentin matrix proteins (DMPs) are a group of NCPs found in the extracellular matrix of dentin and bone albeit in different quantities. Currently, this group consists of four different proteins, namely: Dentin matrix protein 1 (DMP1), dentin phosphophoryn (DPP) or dentin matrix protein 2 (DMP2), dentin sialoprotein (DSP) and DMP 4 (also known as Fam20C) (Hao et al. 2007, 2009). Over the years, these DMPs have been shown to play multiple roles that control important functions ranging from attachment, proliferation and differentiation of stem cells and preostoblasts to matrix mineralization. The multifunctional properties of these DMPs make them as attractive agents to be incorporated within an appropriate scaffold to enable stem cell based bone tissue engineering. Although primarily isolated as calcium binding and hydroxyapatite nucleating proteins, the DMPs can be used successfully as signaling molecules to direct stem cell recruitment, attachment and differentiation.

The overall goal of this chapter is to highlight the important functions of these DMPs and provide an overview of their utilization in bone tissue engineering.

8.2 Expression and Localization of DMPs in Bone

During bone repair several of the development processes are recapitulated. Therefore, it is important to identify the localization pattern of the dentin matrix proteins during bone development, as it is indicative of its function. In general, the dentin matrix proteins are expressed both intracellularly and extracellularly during development. Based on their localization patterns each of these molecules perform different functions. Such knowledge would be useful when designing strategies for bone tissue engineering.

8.2.1 DMP1

DMP1 was the first of the DMPs to be discovered and was initially thought to be specific to the dentin matrix (George et al. 1993, 1994). However, several studies confirmed that DMP1 is expressed in many tissues at a basal level and in high quantities by tissues undergoing biomineralization such as bone and dentin (Hao et al. 2004; Huang et al. 2008). Figure 8.1 shows the expression of DMP1 in 3-month-old mouse femur section. The figure is a 3D reconstruction of z stack confocal images. The red staining represents DMP1. Note the high expression levels in the bone matrix and also in the osteoblasts. The DMP1 protein in the extracellular matrix (ECM) is seen as two

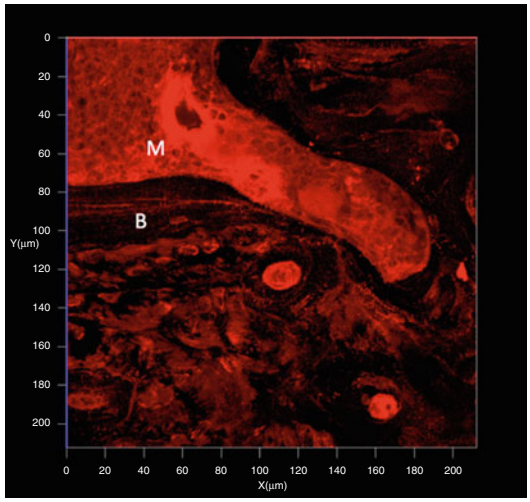

Fig. 8.1 Expression of DMP1 in the femur of 3-month-old mouse: 3D rendering of z-stack confocal micrographs showing immunofluorescence staining of DMP1 (*red stained areas*). *M* denotes the bone marrow and *B* represents bone matrix

processed fragments: the 37 KDa N-terminal fragment and the 57 Kda C-terminal fragment (Qin et al. 2003). The N-terminal fragment of DMP1 is glycosylated at the Ser74 residue and is important for DMP1 to carry out its functions in vivo (Qin et al. 2006). However, this glycosylated form of DMP1 fragment is a negative regulator of hydroxyapatite nucleation (Gericke et al. 2010). Processing of DMP1 into the two fragments is critical for its function in vivo.

Overexpression of DMP1 that cannot be processed in DMP1 null mice failed to rescue the defective mineralization of dentin, bone and cementum observed in the knockout mice, showing the need for proteolytic processing of DMP1 for it to be functional *in vivo* (Sun et al. 2011).

Of the two fragments of DMP1, the 57 KDa C-terminal fragment is the most biologically active. Studies with knockout mouse models revealed that this fragment alone could restore the functionality of full-length DMP1 (Lu et al. 2011). The two fragments of DMP1 also show varied distribution in intracellular compartments as well as in the extracellular space (Maciejewska et al. 2009). The C-terminal fragment of DMP1 is primarily localized to the mineralizing areas of bone and dentin in the ECM. Inside the cell, the C-terminal fragment accumulates in the nucleus of the mesenchymal cells. On the other hand, the N-terminal fragment of DMP1 is localized to the predentin, a non-mineralizing tissue and in the articular cartilage in the ECM. Intracellularly, it was predominantly localized to the cytosol and plasma membrane. This varied distribution suggests that both fragments of DMP1 may play different biological roles in the intra and extracellular environments.

8.2.2 DPP or DMP2

DPP is expressed by odontoblasts and is considered as a marker for terminally differentiated odontoblasts. Recent report indicates that DPP is expressed using an internal ribosome entry site (IRES) present in the DSPP gene (Zhang et al. 2014). However, several other studies have shown that DSPP is a substrate for proteases such as bone morphogenetic protein 1 a tolloid-related protein to be proteolytically processed into dentin sialoprotein (DSP), DPP and dentin glycoprotein DGP (Ritchie et al. 2012). In reality, it could be a combination of both processes. Interestingly, the proteolytic cleavage process does not account for the increased presence of DPP and the absence of DSPP precursor protein in the matrix. On the other hand, the IRES theory cannot account for the presence of the small DGP protein. Therefore, it is reasonable to propose that a combination of processing methodologies might account for the abundant presence of DPP in the matrix.

During development and maturation of the odontoblasts, the expression level of DPP increased and was maintained at high levels in the dentin matrix (Hao et al. 2004, 2009). Although initially thought to be uniquely specific to the dentin matrix, DPP has also been identified in the bone matrix (Qin et al. 2002). The red immunofluorescence staining in Fig. 8.2 shows the expression of DPP in the mineralizing dentin (marked as D in the figure). Furthermore, DPP has also been identified in other non-mineralizing tissues albeit in very small amounts (Prasad et al. 2011).

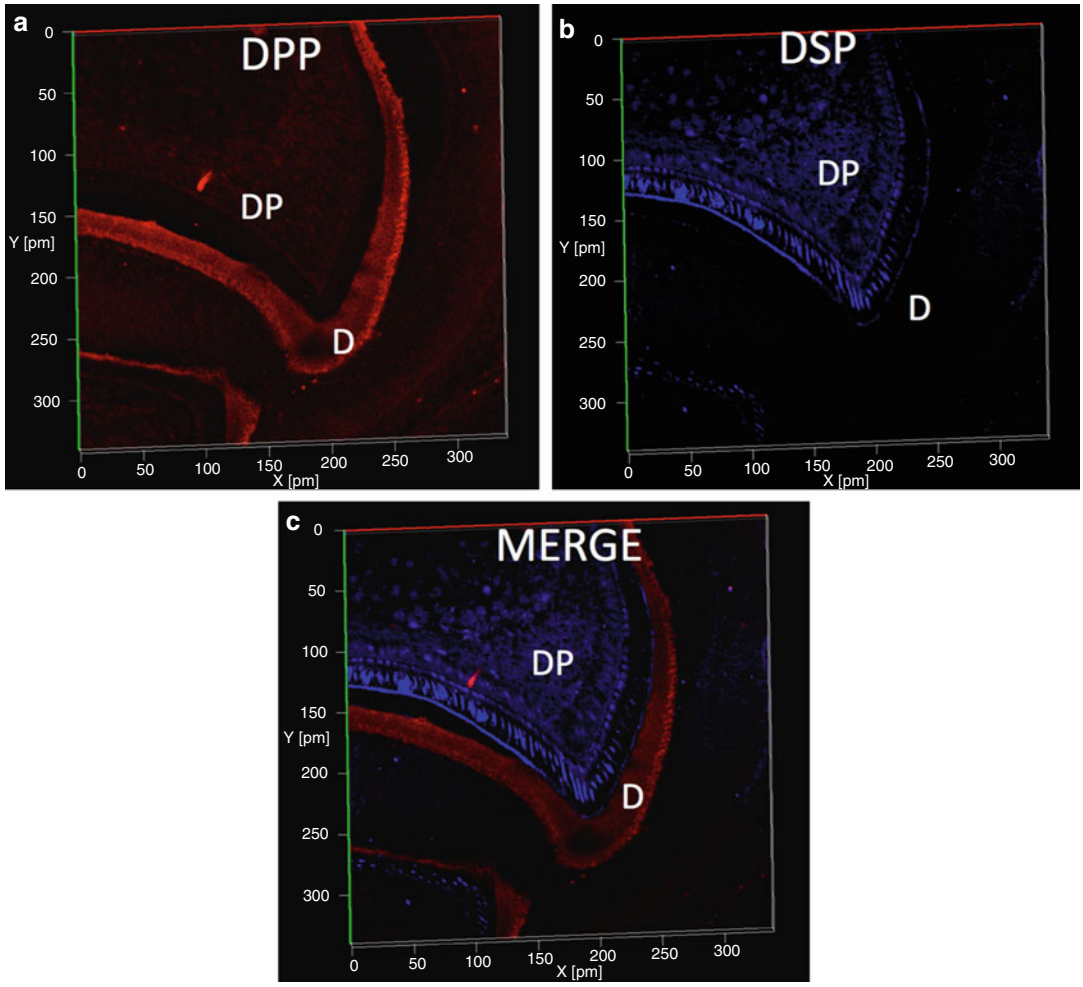

Fig. 8.2 Expression of DPP and DSP in the unerupted molar of 7 day old mice: Images are 3D rendering of z-stack confocal images of sections stained with the DPP (*red*) and DSP (*blue*) antibodies. Note the difference in the expression pattern of the two proteins. *DP* represents the dental pulp and *D* represents dentin. Note the predominant expression of DPP in the mineralizing dentin. (**a**) Expression of DPP; (**b**) Expression of DSP; (**c**) Merged image of (a) and (b)

8.2.3 DSP

DSP is the second abundant noncollagenous protein next to DPP and along with DPP is considered as a phenotypic marker of dentin and secretory odontoblasts. DSP and DPP are proteins encoded by the gene DSPP. Although encoded by the same gene, the amounts of protein present at any given time vary significantly. DPP accounts for about 50 % of the total NCPs present in dentin whereas DSP constitutes only 5 % (Qin et al. 2001; Yamakoshi et al. 2006). As they are expressed via the same gene, the expression patterns of DSP and DPP coincide although the protein half-life of DSP may be shorter due to its susceptibility to proteolytic cleavage. Thus the difference in the amount of DPP and DSP protein levels could be a direct demonstration of the functional difference between these two proteins. Our experiments also indicate a differential expression of DSP compared to DPP. In Fig. 8.2, the blue immunohistochemical staining shows predominant DSP expression in the odontoblast cells and to some extent in the dental pulp. However, it is not observed in the dentin matrix.

8.2.4 DMP4/FAM20C

DMP4 is the newest member of the DMP family of proteins It was discovered as a novel protein via a subtractive hybridization technique (Hao et al. 2007). It is a 579 amino acid long secretory protein that contains a known calcium-binding domain. In situ hybridization techniques showed that the mRNA for DMP4 is highly expressed in odontoblasts and osteoblasts.

Localization studies by in situ hybridization and immunohistochemistry identified the spatial and temporal expression patterns of this protein during development of osteogenesis and odontogenesis (Wang et al. 2010). These studies revealed that during osteogenesis, DMP4/Fam20C is expressed in the chondrocytes, osteoblasts and osteocytes. The intracellular localization is predominantly cytoplasmic. In the ECM, the protein was localized in the long bone as well as in the alveolar bone matrices. During odontogenesis, the mRNA expression was noted in odontoblasts, ameloblasts, cementoblasts and periodontal ligament fibroblasts. The protein expression was however, noted only in odontoblasts, ameloblasts and cementoblasts (cytoplasmic localization). In the ECM, the protein has been identified in dentin, enamel, cementum and bone. Figure 8.3 shows immunohistochemical localization of Fam20C in the femur of 30-day-old mice. The white arrows in the image point to ECM localization. Figure 8.3b is a zoomed in image of the boxed area in Fig. 8.3a. Overall, the expression pattern of this protein suggests a strong role for this protein in biomineralization.

Recently, it was shown that DMP4 is a kinase that phosphorylates the family of secreted calcium binding proteins that consists of the matrix proteins osteopontin, DMP1 and DSPP (Tagliabracci et al. 2012, 2013). This further suggests that phosphorylation events, catalyzed by secreted kinases may be a critical component of regulatory mechanisms in the ECM.

From the localization patterns of the dentin matrix proteins, it is clear that the organic matrix synthesized by either osteoblasts or odontoblasts is not homogenous in composition and that in a mineralizing tissue there are well-defined zones with different properties. The dentin matrix proteins are a heterogeneous mixture and they operate within a well-defined and controlled program to facilitate cell adhesion and differentiation and in the extracellular matrix to nucleate, direct crystal placement, stabilize the mineral phase and regulate the crystal size.

8.3 Functions of DMPs

The localization pattern of the DMPs suggests that these proteins are multifunctional. In this section, we will elucidate the multifunctional

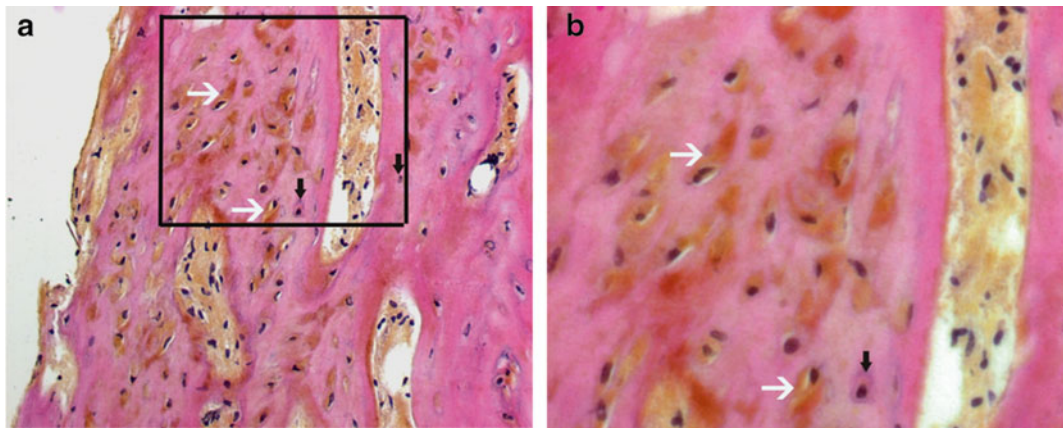

Fig. 8.3 Expression of DMP4/Fam20C in the femur of 1-month-old mouse. *Arrows* in (**a**) point to positive staining in the ECM. (**b**) Is an enlarged image of the *boxed area* in ((**a**))

properties of the DMPs that justify them as potential candidates for use in bone tissue engineering applications.

8.3.1 DMP1

The first multifunctional protein identified from the dentin matrix is DMP1. DMP1 belongs to a family of non-collagenous proteins categorized as small integrin- binding ligand, N-linked glycoproteins (SIBLING) (Ravindran and George 2014). Loss of DMP1 results in defective biomineralization of bone and dentin, particularly the phenotype exhibits hypophosphatemic rickets and osteomalacia (Feng et al. 2006). Recent studies have shown that DMP1 regulates the hormone FGF23 (Feng et al. 2006) and plays critical role in osteoblast differentiation and biomineralization. Both of these functions will be highlighted below.

8.3.1.1 Biomineralization Function of DMP1

DMP1 is a highly phosphorylated protein with a strong affinity for calcium. As a result, its primary function in the ECM is to facilitate nucleation of hydroxyapatite crystals. In vitro experiments indicate that DMP1 can self assemble into a β-sheet template and facilitate the nucleation of hydroxyapatite (He et al. 2003; Bhatia et al. 2012). Additionally, DMP1 can bind specifically to the N-telopeptide region of type I collagen. Nucleation of hydroxyapatite was exclusively found in regions where DMP1 bound to type I collagen (He and George 2004). Thus, DMP1 functions as a nucleating protein in the extracellular matrix of bone and dentin. Further, overexpression of DMP1 accelerated biomineralization and significantly altered the biomechanical properties of cortical bone (Bhatia et al. 2012). The active HAP nucleating domain was found to reside at the C-terminal end of the molecule and overexpression of this fragment alone, could rescue the defective mineralization phenotype observed in DMP1 null mice (Lu et al. 2011).

8.3.1.2 DMP1 and Stem Cell Differentiation

DMP1 contains an RGD tripeptide. RGD domain serves as ligands for integrin receptors on the cell membrane and can regulate cell adhesion and cytoskeletal dynamics. Published studies show that the RGD domain in DMP1 promotes selective cell attachment (Kulkarni et al. 2000). Furthermore, the RGD domain in DMP1 binds to α5β1 integrin on the cell surface and activates the extracellular signal-regulated kinase (ERK) 1/2 pathway to stimulate osteoblastic differentiation of mesenchymal stem cells including periodontal ligament stem cells (PDLSCs) (Chandrasekaran et al. 2013; Eapen et al. 2011). This DMP1 mediated signaling cascade can occur via DMP1 tethered to the ECM via its collagen binding domains facilitating cell attachment and differentiation.

DMP1 in the ECM can undergo receptor-mediated endocytosis via its receptor GRP-78 (Ravindran et al. 2008). Endocytosis of DMP1 triggers calcium mediated regulation leading to the activation of the mitogen activated protein kinase (MAPK) signaling cascade resulting in osteogenic differentiation of mesenchymal stem cells (Eapen et al. 2010). GRP-78 is also a multifunctional protein. GRP-78 binds type I collagen and DMP1 in the ECMand in conjunction with DMP1 can initiate the nucleation of calcium phosphate to facilitate matrix mineralization (Ravindran et al. 2011).

DMP1 is a substrate for the matrix metalloprotease 2 (MMP2) enzyme. The proteolytic fragments of DMP1 generated via MMP2 cleavage also have the potential to trigger mesenchymal stem cell differentiation (Chaussain et al. 2009). This scenario can be envisaged as one wherein bone remodeling is occurring resulting in release of several NCPs that are acted upon by proteases. These proteolytic fragments can then prove to be useful in recruiting and directing differentiation of stem cells enabling new bone formation.

Apart from its obvious roles in stem cell differentiation and in biomineralization, DMP1 has an intriguing presence in the nucleus of mesenchymal cells (Siyam et al. 2012; Narayanan et al. 2003). Studies show that nuclear DMP1 can bind

to DSPP promoter and initiate transcription of DSPP gene (Narayanan et al. 2006), which in turn can stimulate DPP secretion resulting in accelerated mineralization of the organic extracellular matrix.

Collectively, DMP1 is a multi-faceted protein that plays a critical role in native bone and dentin physiology. Using DMP1 for bone tissue engineering may promote accelerated bone formation and differentiation of mesenchymal stem cells to osteoblast precursors and fully functional osteoblasts facilitating biomineralization.

8.3.2 DPP

Like DMP1, DPP also belongs to the SIBLING family of proteins and contains an RGD sequence for integrin binding and this domain is conserved among most species. DPP is the most acidic protein ever discovered with an isoelectric point of 1. DPP is highly phosphorylated and contains multiple aspartic acid-serine-serine repeats (DSS repeats) contributing to its acidic nature (George et al. 1996). DPP is also a multi-functional protein with both intra and extra cellular functions.

8.3.2.1 DPP Mediated Hydroxyapatite Nucleation

DPP is the most abundant noncollagenous protein in the dentin matrix and it is also present in bone albeit in relatively smaller quantity. Studies have shown that the abundant levels of phosphoserine and aspartic acid in DPP might be responsible for calcium binding in an ordered manner. The calcium phosphate clusters initially formed by immobilized phosphate esters have short range order based on the ionic and coordination binding of the successive layers. These clusters grow until they coalesce with adjacent clusters into apatite nuclei (George and Hao 2005; Veis 1993; Prasad et al. 2010). Presence of DPP in the alveolar bone at later stages of development suggests that this ECM protein might be involved in regulating crystal size at later stages of osteogenesis (Hao et al. 2004). However, phosphorylation of DPP is essential for its function as a mediator of biomineralization (He et al. 2005).

8.3.2.2 Signaling Roles of DPP

Recently several studies have identified many signaling functions for DPP resulting in mesenchymal stem cell differentiation. In the ECM, DPP can mediate cell adhesion and integrin signaling via the RGD peptide (Eapen et al. 2012a, b). Integrin signaling in turn plays a significant role in stem cell differentiation and cytoskeletal rearrangement. Additionally, DPP can also trigger calcium and calmodulin mediated signaling cascades in undifferentiated mesenchymal cells guiding their differentiation process (Eapen et al. 2013). All of these signaling roles of DPP will prove to be crucial if it has to be utilized effectively as a component of the tissue engineering triad.

Recently, the acidic domain of DPP consisting of multiple DSS repeats was identified as an endocytic peptide capable of cellular endocytosis via a pathway that is not receptor mediated (Ravindran et al. 2013). The acidic domain by itself does not possess any signaling function and hence it can be used as a carrier molecule for targeted protein delivery.

Collectively, all these studies point to yet another multi-functional DMP that is an enticing target both as a signaling molecule as well as a facilitator of targeted protein delivery.

8.3.3 DSP

As discussed previously, the 5′ region of DSPP encodes DSP. Although DSP is expressed at the same time as DPP, its quantity in mineralized tissues constitutes only a fraction of the amount of DPP. On the other hand, functional studies have identified that DSP serves as a negative regulator of biomineralization and thereby bringing balance and control to the action of DPP (Gibson et al. 2013). Published reports also confirm that DSP is involved in the formation and maintenance of tooth structures such as the peridontium (Baba et al. 2004).

Although DSP is not as functionally diverse as DMP1 and DPP, it is still an important contributor to biomineralization by regulating mineralization. It is therefore, important to consider the use

of this protein in tissue engineering applications from this perspective to achieve a biomimetic setup.

8.3.4 DMP4/Fam20C

8.3.4.1 Calcium-Binding Property of DMP4/Fam20c

A characteristic functional domain identified in the DMP4 sequence is the Greek-key calcium-binding motif located at the C-terminal end of the molecule. Conformational analysis indicates that the entire molecule adopts a flexible random structure, but in the presence of calcium, the Greek key domain could bind calcium ions resulting in ordered structures.

A role for DMP4 in the extracellular matrix was confirmed by mineral nodule formation assay after its overexpression. Silencing DMP4 inhibited mineralization of the ECM organic matrix. Phenotype of the DMP4 null mice exhibited hypophosphatemic rickets confirming a significant role that this molecule plays in the formation of physiological bone (Wang et al. 2012). Published studies also indicate that Fam20C is a new regulator of FGF23 along with DMP1 and plays a significant role in biomineralization. However, when DMP1 is overexpressed in the Fam20C knockout mice, the phenotype is not rescued indicating that DMP1 and Fam20C do not perform redundant functions *in vivo* (Wang et al. 2014). Mutations in Fam20C are associated with Raine syndrome, an autosomal recessive skeletal dysplasia characterized by osteosclerosis and ectopic calcifications often leading to neonatal death (Simpson et al. 2007). This further attests to the importance and uniqueness of Fam20C function during biomineralization.

8.3.4.2 Fam20C/DMP4 and Osteoblast Differentiation

Gain of function studies were performed to elucidate the function of DMP4 in pre- osteoblasts and undifferentiated mesenchymal stem cells. Results from these studies found an increase in osteogenic gene expression levels. Similarly, silencing DMP4 showed down-regulation in the mineralization cascade (Hao et al. 2007). A recent report suggests that Fam20C is also a secreted kinase that phosphorylates extracellular proteins that regulate biomineralization (Tagliabracci et al. 2012, 2013).

Apart from its role in dentin mineralization, Fam20C is also involved in the process of enamel formation and amelogenesis (Wang et al. 2013). This fact shows that its role in biomineralization is ubiquitous and is not limited to type I collagen-based biomineralization. It is to be noted that enamel formation and biomineralization is a completely different phenomenon when compared to bone and dentin. The primary difference being the complete absence of type I collagen in enamel. However, in dentin and bone, type I collagen is the primary organic component. Further studies are required to understand the complexities of this novel biomineralization regulator.

Overall, the dentin matrix proteins are unique and play a regulatory role in calcified tissue formation. The calcium binding domains in each of these proteins are different and play a combinatorial and important role in the assembly of hard tissues. Besides the extracellular function they initiate intracellular events that aid in cell differentiation and synthesis of a functional bone matrix.

8.4 Tissue Engineering Strategies Using DMPs

Tissue engineering bone requires recreating the complexities that nature has devised for physiological bone formation. Several studies have shown that the bone repair processes resemble normal development of the skeleton during embryogenesis. Therefore, bone repair and regeneration should be greatly influenced by the intimate interactions between the cells and the matrix. There are several signaling molecules used for inductive bone regenerative engineering. Among them recombinant bone morphogenetic proteins have been used clinically. However, several detrimental clinical outcomes raise concerns. In the previous sections, we introduced and

briefly summarized the various functionalities of the members of the DMP family. In this section, we will look at how these proteins can be exploited in bone repair and regeneration.

8.4.1 DMP1

Due to its multi-functional nature, DMP1 is a very attractive protein to be used as native protein in its entirety or its processed forms in tissue engineering applications. To this end, DMP1 has been used as a signaling molecule in biomimetic collagen constructs to regenerate soft tissues such as the dental pulp tissue (Alsanea et al. 2011; Prescott et al. 2008). With respect to bone regeneration, biomineralization motifs from DMP1 have been used to generate engineered peptides with the ability to directly transform amorphous calcium phosphate (ACP) to crystalline hydroxyapatite (HAP) (Tsuji et al. 2008). Nucleation of crystalline HAP with a calcium to phosphorus ratio of 1.6 similar to that of native bone is absolutely critical for bone tissue engineering applications to generate high quality engineered tissues capable of withstanding the mechanical loading that bones are normally subjected to. In this regard, the ability of DMP1 derived peptides to induce transformation of ACP to crystalline HAP is a handy tool in our quest for a signaling molecule to be incorporated within the biomimetic scaffold.

Along similar lines, genetically engineered non toxic phage bio-nanofibers containing the nucleating motifs from DMP1 have been generated to serve as building blocks of self assembling fibers that can be used to recreate the complex structure of bone (Xu et al. 2011). In addition, the presence of DMP1 motifs in these constructs facilitated the nucleation of crystalline HAP.

More recently, synthetic peptides that contain the type I collagen binding domain and the nucleating domains of DMP1 were generated for repair of carious dentin (unpublished study). The function of the collagen-binding domain was to tether the peptides to the N-telopeptide region of type I collagen and the nucleating domain would facilitate calcium binding and subsequent transformation to crystalline hydroxyapatite. These peptides are non-toxic, easy to synthesize and can be incorporated into any collagen based scaffold designed for bone tissue engineering.

In a recent study, using biomimetic strategy, we have developed a self-assembling leucine zipper chimeric protein that has the ability to self-assemble into a biomimetic scaffold for tissue engineering applications (Huang et al. 2014). The primary feature of this molecule is its ability to be customized for several tissue-engineering applications. One of the variants that we have developed for bone tissue regeneration is the leucine zipper chimeric protein that contains the DMP1 nucleating motifs. In vivo and in vitro studies show that these scaffolds can support cell differentiation and facilitate hydroxyapatite nucleation (Padovano et al. 2015). Figure 8.4 shows the ability of the self-assembling, DMP1 calcium-binding motif containing leucine zipper hydrogel to nucleate crystalline hydroxyapatite. When subjected to in vitro nucleation, these the nucleated particles contained hydroxyapatite morphology (Fig. 8.4a). Element analysis (arrows in Fig. 8.4b) revealed the presence of calcium and phosphorus and finally, small angle diffraction experiments using TEM confirmed the crystalline nature of the nucleated calcium phosphate (Fig. 8.4c).

One underrated function of DMP1 that has not been significantly explored in bone tissue engineering is its signaling function in osteogenesis. Osteogenesis is a dynamic process initiated by mesenchymal stem cells in coordination with signaling molecules. As DMP1 can stimulate MSCs to differentiate into functional osteoblasts, therefore incorporating the osteogenic signaling portion of DMP1 might be of importance to induce osteogenic differentiation of adult stem cells. These signaling properties of DMP1 are recent discoveries that will in time find its way into tissue engineering applications much like its nucleating properties. Depending on the end use, various combination of the functional domains of DMP1 can be developed into powerful tools that can be incorporated in scaffolds to elicit responses for mineralized tissue regeneration.

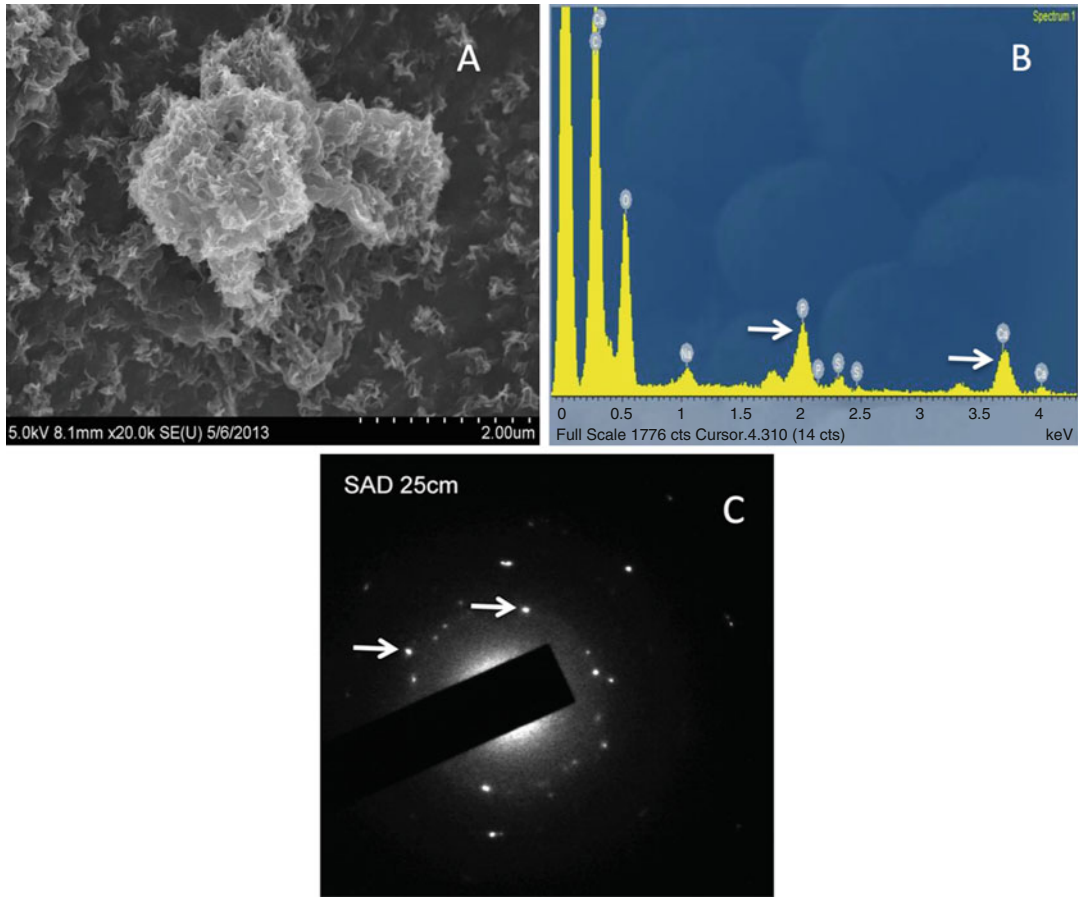

Fig. 8.4 Nucleation of crystalline hydroxyapatite by 3D self-assembling leucine zipper scaffolds containing the nucleating domains of DMP1. (**a**) is an SEM image of the nucleated crystals. (**b**) shows energy dispersive X-ray element analysis of the crystals showing the presence of calcium and phosphorus ions (*white arrows*). (**c**) is a small angle diffraction image from a TEM showing the presence of crystalline calcium phosphate (*arrows* pointing to *dots* represent crystals)

8.4.2 DPP

Similar to DMP1, the primary function of DPP is biomineralization. Recent studies have shown that type I collagen scaffolds containing DPP was able to generate hydroxyapatite containing mineralized nodules similar to those found in bone when incubated with simulated body fluid solution at 37 °C (Zurick et al. 2013). The other NCPs used in this study were osteopontin (OPN) and bone sialoprotein (BSP). It is to be noted that although the other NCPs generated calcium phosphate deposits, none of them resembled mineral nodules present in bone. From this study it is clear that DPP would be a prime candidate to be incorporated in bone tissue engineering scaffolds to facilitate nucleation and growth of HAP.

Our recent report shows the ability of the DSS polypeptide domain from DPP to penetrate the plasma membrane of several cell types including mesenchymal, epithelial and endothelial cell types (Ravindran et al. 2013). This DSS domain is endocytosed in a non-classical manner that is not receptor mediated. Additionally, the mechanism of endocytosis is also not via clathrin or caveolar endocytic pathways. These properties provide the DSS polypeptide with the ideal characteristics of a carrier protein. Results show the ability of this polypeptide to transport the osteoblast transcription factor Runx2 to the nucleus of

mesenchymal cells (arrows in Fig. 8.5a). Further, the transcriptional activity of Runx2 remains intact upon cell entry and nuclear translocation (Fig. 8.5b). These results show the utility of this acidic polypeptide to function as a carrier molecule for targeted protein delivery to induce osteogenic differentiation of mesenchymal stem cells.

The minimum DSS repeats required to achieve endocytosis was found to be 6. Therefore, the carrier peptide is easy to synthesize, and when conjugated with a target protein forms a potent tool for direct intracellular transport of transcription factors to elicit required cell response. Another key feature of this study is that the DSS polypeptide by itself does not trigger cellular differentiation. Therefore, it can serve as an effective transport molecule not only for Runx2, but several other factors as well. In this regard, the tissue engineering implications for this polypeptide are far reaching. It is possible to envisage the use of multiple fusion proteins consisting of early and late transcription factors tagged to the DSS polypeptide. These fusion proteins can be delivered in a controlled manner at different stages to effectively control stem cell fate and tissue regeneration. Such approaches will be at the forefront of bone tissue engineering applications and further studies are required to identify and standardize the different parameters that might influence the success of this approach.

8.4.3 DSP

DSP is one of the least studied DMPs and its primary function seems to be inhibition of mineralization and influencing the role of DPP. DSP has so far not been used in any tissue engineering applications. However, one possible application of DSP could be its use along with DPP to control the rate and quality of biomineralization. Several NCPs existing in the bone and dentin matrix function as negative regulators of biomineralization. Their primary function is to work in tandem with the positive regulators to ensure appropriate and high quality mineral deposition. The function of these inhibitors is just as important as other positive regulators and cannot be ignored when designing biomimetic scaffolds for bone tissue engineering.

We therefore envisage DSP working in tandem with DPP in the same ratio as they exist

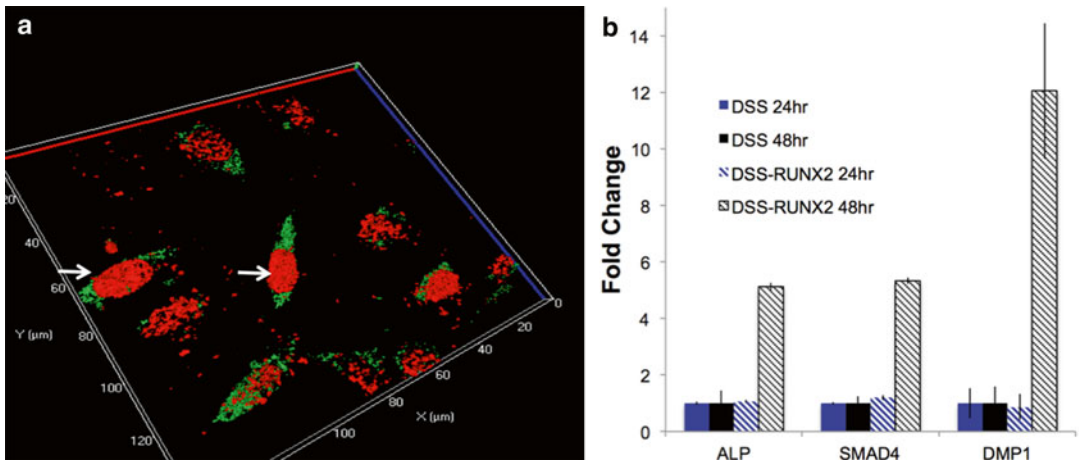

Fig. 8.5 Protein delivery using the DSS polypeptide: (**a**) represents 3D reconstruction of z-stack confocal images showing endocytosis of quantum dot tagged DSS polypeptide expressed as a fusion protein with the osteoblast transcription factor Runx2 (*red*). Note the nuclear localization of the transcription factor (*arrows*). The *green* staining represents EEA1 (early endosome *antigen* 1) staining, a marker for endosomes. Note that the endocytosed protein is not routed to the endosomes for degradation. (**b**) represents the qRT PCR data showing expression levels of alkaline phosphatase (*ALP*), SMAD 4 and DMP1 that are down stream targets for Runx2. Increase in gene expression at 48 h post endocytosis indicates that the endocytosed Runx2 retains its transcriptional activity

in vivo. The appropriate physiological concentrations of these molecules might be necessary to control the rate and quality of biomineralization bringing balance and protecting against hyper mineralization events. However, additional research needs to be performed to validate this hypothesis before DSP can be used as a regulatory molecule in bone tissue engineering.

8.4.4 DMP4/Fam20C

Like the other DMPs such as DMP1 and DPP, Fam20C is a positive regulator of biomineralization. The recent finding that Fam20c is a Golgi kinase opens up avenues for the use of this protein in tissue engineering applications. Protein phosphorylation is one of the most important cellular control mechanisms. Several of the proteins that function to regulate biomineralization are phosphoproteins. Phosphorylation of these proteins makes the proteins highly polyanionic and are thought to play a role in regulating biomineral morphology. The current theory is that the negatively charged proteins could selectively interact with the crystallographic faces that would alter the growth kinetics at the growing step edges in specific directions thereby altering the crystal shape. Therefore, kinases that could phosphorylate matrix proteins could have potential applications in tissue engineering bone. It is therefore possible to envision the use of this protein for such a purpose.

8.5 Conclusion

Formation of the vertebrate biomineral requires the structural arrangement of the HAP crystals. At the nanostructural level the HAP crystals are crystallographically oriented nearly parallel to the long axis of the collagen fibrils. This structural arrangement leads to the formation of intertwined organic-inorganic composite that imparts bone with high strength and rigidity. The noncollagenous proteins of which the dentin matrix proteins form a subset of proteins play an important role in the biomineralization process. These proteins can help in the orientation and reinforcement of the nanocrystals by the formation of a transient amorphous precursor phase leading to their transformation into a crystalline phase.

The use of the dentin matrix proteins in bone tissue engineering applications can help recreate the architecture of the mineralized collagen fibril by regulating the kinetics of the amorphous to crystalline transition. This is an important step toward reproducing the hierarchical organization of natural bone. Nature uses multiple proteins to achieve controlled mineralization. Understanding the physiological function of the dentin matrix proteins would help in tissue engineering bone by promoting crystallographic alignment of apatite crystals as well as facilitating spatial control of mineralization in specific locations on the organic collagenous matrix. Our understanding of the biomineralization process can help in providing cues for bone tissue repair and regeneration.

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Multiphasic, Multistructured and Hierarchical Strategies for Cartilage Regeneration

9

Clara R. Correia, Rui L. Reis, and João F. Mano

Abstract

Cartilage tissue is a complex nonlinear, viscoelastic, anisotropic, and multiphasic material with a very low coefficient of friction, which allows to withstand millions of cycles of joint loading over decades of wear. Upon damage, cartilage tissue has a low self-reparative capacity due to the lack of neural connections, vascularization, and a latent pool of stem/chondroprogenitor cells. Therefore, the healing of articular cartilage defects remains a significant clinical challenge, affecting millions of people worldwide. A plethora of biomaterials have been proposed to fabricate devices for cartilage regeneration, assuming a wide range of forms and structures, such as sponges, hydrogels, capsules, fibers, and microparticles. In common, the fabricated devices were designed taking in consideration that to fully achieve the regeneration of functional cartilage it is mandatory a well-orchestrated interplay of biomechanical properties, unique hierarchical structures, extracellular matrix (ECM), and bioactive factors. In fact, the main challenge in cartilage tissue engineering is to design an engineered device able to mimic the highly organized zonal architecture of articular cartilage, specifically its spatiomechanical properties and ECM composition, while inducing chondrogenesis, either by the proliferation of chondrocytes or by stimulating the chondrogenic differentiation

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of stem/chondro-progenitor cells. In this chapter we present the recent advances in the development of innovative and complex biomaterials that fulfill the required structural key elements for cartilage regeneration. In particular, multiphasic, multiscale, multilayered, and hierarchical strategies composed by single or multiple biomaterials combined in a well-defined structure will be addressed. Those strategies include biomimetic scaffolds mimicking the structure of articular cartilage or engineered scaffolds as models of research to fully understand the biological mechanisms that influence the regeneration of cartilage tissue.

Keywords
Cartilage regeneration • Hierarchical scaffolds • Multiphasic scaffolds • Chodrogenesis • Stem cells • Tissue engineering

9.1 Introduction

Articular (hyaline) cartilage is the thin connective tissue covering the bearing surface of long bones in diarthrodial (synovial) joints, which represent important organs of the musculoskeletal system. An healthy articular cartilage tissue ensures physiological mobility by providing a lubricated and smooth surface, as well as by dissipating energy during the articulation of the joints, which encounter forces of several times the body weight (Mow et al. 1992). The unique composition and highly organized structure of this inhomogeneous, anisotropic, multiphasic, and viscoelastic tissue are the key elements for its complex mechanical properties (Malda et al. 2012). Articular cartilage damage can occur either by degenerative injury or by trauma, which can be acute or repetitive micro trauma from years of use. However, this complex and hierarchical tissue has a very limited capacity of self-repair or regenerate through endogenous healing due to its relatively hypocellular structure, with approximately 100×10^6 chondrocytes/cm^3 on average (Chiang and Jiang 2009), as well as to the lack of neural connections, vascularization, and a latent niche of stem cells/chondroprogenitors (Ge et al. 2012). In current clinical practice, different cartilage repair techniques are being used to address tissue damage. While conservative treatment aims to alleviate pain and slow down or prevent the progress of osteoarthritis (OA), surgical interventions aim to stimulate the natural repair process to rebuild the damaged tissue (Chiang and Jiang 2009; Gomoll and Minas 2014). However, the outcomes of these clinical treatments remain unpredictable and numerous surgical cases have not yet proven to be successful for long-term applications (Chiang and Jiang 2009). Lesions left unrepaired or which have undergone improper repair, subsequently form tissues of inferior mechanical strength, leading to the loss of a suitable mechanical function and progressive OA degeneration, resulting in significant pain and disability.

Cartilage tissue engineering (TE) has emerged as an alternative treatment method of this uniquely challenging tissue. The main challenge to produce functional devices that successfully regenerate cartilage is to mimic its highly organized zonal architecture, specifically its spatio-mechanical features and extracellular matrix (ECM) composition. Cartilage TE has employed a variety of techniques to produce scaffolds, frequently composed by multiple components, which are assembled into a final biomimetic three-dimensional (3D) shape. The biomaterials employed include a wide range of synthetic and/or natural materials, which act as a carrier of and delivery vehicle for, alone or co-cultured, chondrocytes and stem/chondro-progenitor cells towards cartilage formation (Ge et al. 2012).

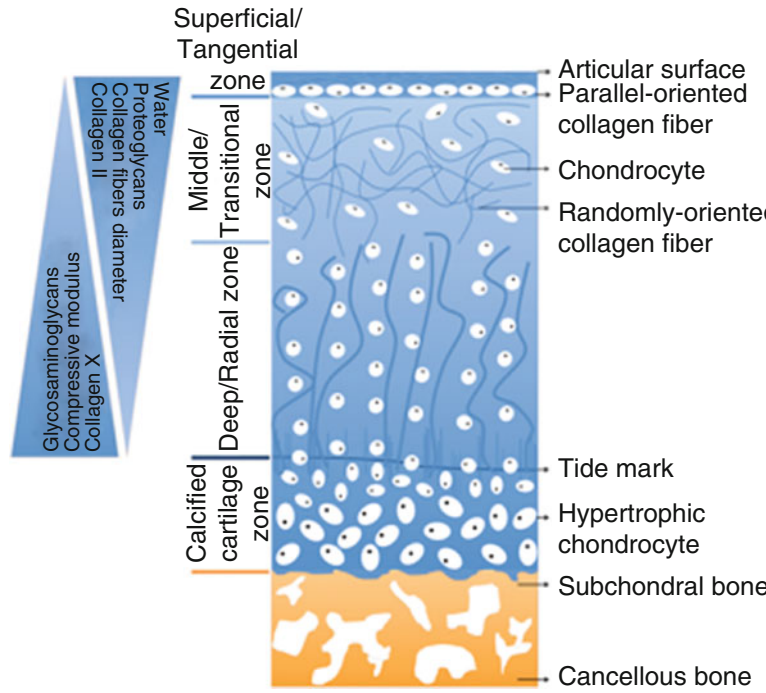
In this chapter, recent research progress on multistructured strategies to regenerate cartilage is addressed. The reported multistructured strategies

are divided into four main sections, namely multiphasic, multiscale, multilayered, and hierarchical strategies. Recent progress in this field is summarized and future directions for the development of functional biomaterials are proposed.

9.2 The Hierarchical Composition of Articular Cartilage

In the past decade, significant advances have been made in cartilage TE strategies. Most of these efforts have been focused in mimicking its hierarchical and highly organized zonal architecture. In fact, it is well accepted by the scientific community that functional cartilage regeneration can only be achieved through a well-orchestrated interplay of biomechanical properties, unique hierarchical structures, ECM composition, and bioactive factors to stimulate the proliferation of chondrocytes within the engineered biomaterials (Ge et al. 2012). Therefore, to propose successful biomimetic strategies to regenerate cartilage it is required to fully understand, anatomically and functionally, this complex tissue from its macro to nanostructure.

Articular cartilage is a composite and organic solid matrix saturated with water and mobile ions. The stiff and elastic crosslinked collagen fibers present within the ECM of cartilage, predominately type II, provide resistance to shear and tensile forces. Additionally, cartilage is composed of glycoproteins, specifically the proteoglycan aggrecan, that with its highly sulfated glycosaminoglycan (GAG) chain attached to collagen fibers captures large amounts of ions and water molecules via negative charges (Gomoll and Minas 2014). Overall, water constitutes 70–80 % of the wet weight of healthy cartilage, with collagen and to a lesser extent glycoproteins making up the remaining weight. Furthermore, the solid matrix of articular cartilage has a highly hierarchical structural arrangement consisting of four spatially distinct zones, namely (i) the superficial/tangential zone, (ii) the middle/transitional zone, (iii) the deep/radial zone, and (iv) the calcified zone (Poole et al. 2001). Each individual zone is characterized by specific ECM composition and organization, and thus distinct mechanical properties. In the superficial/tangential zone, collagen fibers are oriented parallel to the articular surface and impart high tensile strength to withstand the tensile stress encountered under joint loads, as well as a low coefficient of friction to assure a smooth articulation. This zone has high levels of collagen II and low levels of GAGs. Nevertheless, the relative small amount of proteoglycan molecules in the ECM region contribute to the compressive mechanical properties by producing high osmotic pressure within the tissue (Kleine et al. 2009). In the middle/transitional zone, the collagen fibers are randomly oriented and aggrecan reaches its maximal concentration. Here, the collagen II content decreases, while GAG concentration increases. The deep/radial zone contains the highest concentration of GAGs and the lowest concentration of collagen II fibers. In this zone the collagen fibers are oriented perpendicular and attached to the calcified cartilage zone. Finally, the calcified cartilage zone has high levels of collagen X and integrates the cartilage to the subchondral bone (Bhosale and Richarson 2008). The mechanical properties of articular cartilage are sensitive to this depth-varying ECM composition. Although the total collagen content per wet weight is unchanged with depth (Malda et al. 2012), hydroxylysine and hydroxylysyl crosslinking increase. This feature combined with the presence of other minor collagen isoforms, such as type IX and XI, play a critical role in regulating collagen fibers diameter, as well as inter-fibers crosslinking, and interactions with cartilage proteoglycans, thereby contributing to the complex mechanical features of the tissue (Bank et al. 1998). Therefore, from the articular surface to the middle/transitional zone, as the concentration of these proteoglycans aggregates increases, the swelling pressure and water content also increase, leading to the ultimately increase of the compressive modulus (Williamson et al. 2003). Besides the depth-varying mechanical features of cartilage, the superficial/tangential, middle/transitional, and deep/radial zones have distinct cell morphology and metabolic activity, with distinct



Scheme 9.1 Illustration of articular cartilage tissue. From the superficial/tangential to the deep/radial zones of articular cartilage the increasing trend of water, proteoglycans, and collagen II contents, and collagen fibers diameter are represented. On the opposite direction, the increasing trend of glycosaminoglycans and collagen X content, and compressive modulus values are also illustrated

gene and microRNA expression profiles (Grogan et al. 2013). Chondrocytes in the superficial/tangential zone are flattened and horizontally clustered at a relatively high density, in the middle/transitional they are more rounded and randomly oriented in the ECM, and in the deep/radial zone chondrocytes are larger and organized in vertical columns. Scheme 9.1 summarizes the described spatially-varying native articular cartilage anatomy. From the superficial/tangential to the deep/radial zones of articular cartilage, the decreasing trend of water, proteoglycans and collagen II content, as well as fibers diameter are represented. On the opposite direction, the decreasing trend of GAGs and collagen X content, as well as compressive modulus values are also illustrated.

9.3 Research Progress on Cartilage Regeneration Strategies

Among the different employed biomaterials to produce scaffolds for cartilage TE, naturally occurring polymers, such as collagen and polysaccharides, have been widely used since they resemble the natural ECM composition of cartilage, rich in GAGs. Examples of natural biomaterials frequently used in cartilage TE strategies are collagen I and II, alginate, agarose, chondroitin sulfate, chitosan, hyaluronic acid, fibrin, among others, as summarized in the literature (Chung and Burdick 2008). Although natural biomaterials are recognized and interact with cells via surface receptors, consequently regulating or directing their biological behavior and phenotype, they also stimulate an immune system response of the body upon implantation. Therefore, antigenicity and disease transfer are often a concern when scaffolds composed by natural polymers are implanted. Additionally, natural biomaterials are subject to variable enzymatic host degradation and thus have a fast degradation rate. To address those issues, they are often combined with synthetic polymers, which are more controllable and predictable, since their chemical and physical properties can be modified to alter mechanical and degradation characteristics. A wide range of synthetic polymers have been used for different biomedical applications as summarized in the literature (Tian et al. 2011). While natural biomaterials provide the biological cues to promote cell adhesion and proliferation, and mimic the natural ECM

composition of cartilage, synthetic polymers allow mimicking the adjacent complex mechanical cues. Different combinations of natural biomaterials and synthetic polymers to produce scaffolds for cartilage tissue engineering are reported in the literature. For example, chitosan was grafted to poly(ε-caprolactone) (CH-PCL) to produce grafting copolymers with a tailored mechanical and degradation properties (Wan et al. 2010). Recently, CH-PCL grafting copolymers were blended with collagen II to fabricate stratified and multilayered porous scaffolds for cartilage TE (Zhu et al. 2014). In fact, the production of an engineered cartilage tissue has encountered specific difficulties because articular cartilage has a stratified structure, with an ECM composition and mechanical properties varying in an apparently anisotropic fashion. The classical TE approach of creating homogeneous tissue replacements for articular cartilage has failed to achieve widespread clinical effectiveness because the bulk properties of the homogeneous tissue substitutes do not mimic native tissue function. Considering that the properties of cartilage are dictated by its spatially-varying structural organization, current TE approaches are focused in reproducing the native architecture and function of cartilage. Therefore, the manufacture of multistructured and hierarchical scaffolds with satisfactory structures and properties that mimic those found in the native tissue is now a hot topic in cartilage TE and regenerative medicine.

In this section we discuss different strategies reported in the literature to produce multistructured and hierarchical 3D structures for cartilage regeneration. Depending on the concept adopted, those strategies are divided in four sections, namely (i) multiphasic, (ii) multiscale, (iii) multilayered, and (iv) hierarchical strategies. Mainly, these strategies aim to create 3D constructs with variable molecular compositions and mechanical properties, mimicking the heterogeneous ECM composition and consequently zone-variable mechanical properties of native cartilage. Additionally, strategies to produce multistructured or hierarchical scaffolds as research models to fully understand the biological cues that dictate the successful regeneration of cartilage were also included.

9.3.1 Multiphasic Strategies

Hydrogels are widely used in cartilage TE due to their inherent biocompatibility, high water content, and resemble to the natural cartilage ECM. However, hydrogels have limited mechanical strength, which is a main issue for cartilage regeneration. The recreation of the complex mechanical properties of native healthy cartilage is of great importance, especially since upon implantation the construct must provide ability to withstand physiologic joint loading. Alternative strategies have been explored to increase the strength of hydrogel constructs, e.g. through the creation of interpenetrating networks (IPN), incorporation of solid particles, fibers and/or tubes. This lead to the rise of new strategies for cartilage regeneration comprising the combination of biomaterials in different states, namely soft and rigid biomaterials, assembled in a single 3D structure, here termed as **multiphasic strategies**.

An example of a multiphasic strategy in cartilage TE is the composite 3D woven PCL scaffolds with interpenetrating network (IPN) hydrogels proposed by Liao et al. (2013). A 3D scaffold that combined two material states, namely IPN hydrogels and rigid fibers, was developed. Commonly used hydrogels for cartilage regeneration, such as alginate, agarose, gelatin (Awad et al. 2004), hyaluronic acid or poly(ethylene glycol) (PEG) (Chung et al. 2009), generally consist of polymers of single networks and exhibit relatively low stiffness and wear properties. To address the harsh biomechanical requirements of the joint, IPN hydrogels in different combinations of alginate, fibrin, and polyacrylamide (PAAm) were combined with 3D woven PCL scaffolds. The proposed IPN hydrogels showed a significant improvement in the Young's aggregate and dynamic moduli, as well as low coefficients of friction similar to native cartilage. Additionally, the combination with 3D PCL woven scaffolds ensured compressive properties similar to those found in the native tissue, thus indicating the successful resistance to joint loading after implantation procedures. In the same concept, other examples of multiphasic cartilage TE strategies with improved mechanical

properties are the co-electrospinning of soft and rigid fibers composed by gelatin and poly(L-lactic acid) (PLLA) (Torricelli et al. 2014), respectively, or gelatin methacrylamide (gelMA) hydrogels reinforced with 3D printed scaffolds composed by a thermoplastic polymer blend between poly(hydroxymethylglycolide-co-ε-caprolactone) (pHMGCL) and PCL. pHMGCL/PCL was also functionalized with methacrylate groups (pMHMGCL). The 3D composite scaffold with and without methacrylate groups (Ma+ and MA−, respectively) were tested in a PLLA model of the femoral condyle of the human knee for a focal articular cartilage defect (Boere et al. 2014).

Multiphasic cartilage TE strategies can also comprise controlled drug release strategies that take advantage of the combination of different material states in the same 3D construct. Spiller et al. (2012) developed a complex multiphasic strategy to regenerate cartilage taking in consideration the importance of cartilage formation and integration, the sustained release of cartilage growth factors, and the required mechanical properties of the engineered device to successfully regenerate cartilage. For that, they produced different building blocks, namely a poly(glycolic acid) (PGA) fiber mesh scaffold, a poly(vinyl alcohol) (PVA) hydrogel, and poly(lactic-co-glycolic acid) (PLGA) microparticles loaded with insulin-like growth factor-1(IGF-1). The PLGA microparticles loaded with IGF-1 were then encapsulated in the PVA hydrogel, and the whole 3D construct was finally wrapped with PGA fibers. *In vivo* results showed that the outer layer composed by PGA fibers improved cartilage formation and integration, while the inner core composed by PVA/PLGA hydrogel provided mechanical support while releasing IGF-1 in a sustained fashion.

Figure 9.1 summarizes the referred examples found in literature comprising multiphasic strategies to regenerate cartilage.

9.3.2 Multiscale Strategies

Collagen, proteoglycans, noncollagenous proteins, and tissue fluid compose the ECM of articular cartilage. In particular, collagen II, VI, IX, and XI, are found in articular cartilage, although 90–95 % of total collagen is composed of type II. Collagen types II, IX and XI form fibrils and create an interweaved fiber mesh in the ECM environment, where fibril diameter increases with depth (Temenoff and Mikos 2000). Aiming to mimic this multiscale organization of articular cartilage, different strategies have been proposed, here termed as **multiscale strategies**.

Fibrous scaffolds are widely used in TE due to their characteristic high porosity and interconnected pores that facilitate cellular infiltration. Previous reports have indicated that the size scale of fiber scaffolds influence tissue development. Specifically, nanoscale features are desirable because resemble better the collagen fibers of the native ECM (Li et al. 2005). However, scaffolds only composed by nanofibers have a limited cellular infiltration due to a closed pore network mesh (Pham et al. 2006). Therefore, the logical progression was the development of scaffolds combining fibers of variable sizes. Levorson et al. (2014) developed electrospun scaffolds with fibers of two diameter scales interspersed evenly throughout an entire 3D construct. For that, nano and micro fibers of PCL, or nano and micro fibers of fibrin and PCL, respectively, were produced. Results showed that the presence of both nanofibers, either synthetic (PCL) or natural (fibrin), within a PCL microfiber mesh improved the proliferation of cells in serum-free conditions, as well as aided the deposition of GAGs compared with scaffolds composed solely of microfibers. In a similar multiscale cartilage strategy, Moroni et al. (2008) combined rapid prototyping fabrication technique of 3D fiber deposition (3DF) with electrospinning (ESP) to fabricate integrated macro and micro polymeric scaffolds. Scaffolds consisted of integrated 3DF periodical macrofiber and random ESP microfiber networks (3DFESP) were produced with block-co-polymers of poly(ethylene oxide terephthalate) (PEOT) and poly(butylene terephthalate). While 3DF macro scaffolds provided structural integrity and enhanced mechanical properties, the ESP micro network allowed cell entrapment and resembled the nano features of native cartilage ECM. Results showed that

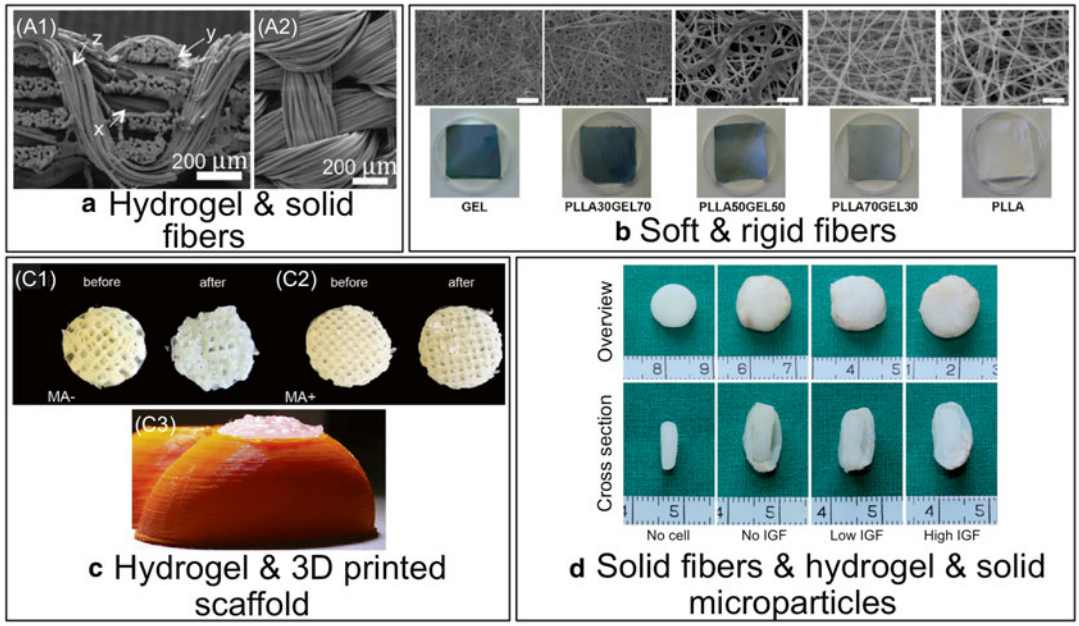


Fig. 9.1 Examples of multiphasic strategies to regenerate articular cartilage. (**a**) 3D woven PCL/IPN composite composed by (*A1*) stacked layers, namely seven layers of *x* and *y* fiber bundles of PCL, which were interlocked with a third set of PCL fibers (*z* -direction) that were passed vertically through the layers following a continuous and repeated path as showed in (*A2*) (Adapted from Liao et al. 2013). (**b**) Scanning electron microscopy images and photographs of crosslinked mats composed by co-electrospun fibers of gelatin (GEL) and PLLA. Scaffolds containing different amounts of PLLA and gelatin (nominal PLLA/gelatin weight ratio: 0/100, 30/70, 50/50, 70/30, 100/0) were produced. *Scale bar* is 10 µm (Adapted from Torricelli et al. 2014). (**c**) Gelatin methacrylamide hydrogels reinforced with a thermoplastic polymer blend of poly(hydroxymethylglycolide-co-ε-caprolactone)/PCL functionalized with methacrylate groups (MA+) or without (MA−). Scaffolds were photographed (*C1*) before and (*C2*) after applying increasing axial compressive forces. (*C3*) The 3D composite scaffolds were tested in a model (*side view*) of the femoral condyle of the human knee composed by PLLA (*orange*) (Adapted from Boere et al. 2014). (**d**) PGA fiber mesh scaffold wrapping a PVA hydrogel encapsulating PLGA microparticles loaded with IGF-1. Different formulations of composite scaffolds were tested namely a control without cells (No cell), with unloaded microparticles (No IGF), with a low concentration of IGF-1 (Low IGF), and with a high concentration of IGF (High IGF) (Adapted from Spiller et al. 2012)

3DFESP scaffolds had a superior cell entrapment, GAG/DNA ratio, and expression of sulfated GAGs by safranin-O staining compared to 3DF scaffolds. Other groups besides varying the fiber diameter also tested the orientation of the fibers. McCullen et al. (2012) reported the fabrication of a trilaminar scaffold by sequential electrospinning and varying fiber size and orientation in a continuous construct, to create scaffolds that mimicked the structural organization and mechanical properties of the collagen fiber network of cartilage. By preparing PCL solutions with different concentrations and by varying the linear velocity of the collector, fibers with variable diameters (1 and 5 µm) and aligned or randomly oriented were produced. Three different zones aiming to mimic the superficial, middle, and deep natural zones of cartilage composed the developed trilaminar construct. Results demonstrated that the trilaminar scaffold displayed superior mechanical properties compared to homogeneous scaffolds, and supported *in vitro* cartilage formation.

Besides exploring the fibers diameter from a macro to a nanoscale, other groups included carbon nanotubes to regenerate cartilage. Carbon nanotubes mimic the dimensions of the constituent components of tissues, in which cells interact with nanofibrous proteins. This feature makes carbon nanotubes excellent candidates when

employed as implants (Tran et al. 2009). In particular, carbon nanotubes can be used as a secondary phase for high load bearing such as cartilage applications, due to their superior mechanical properties. Additionally, the unique chemical properties of carbon nanotubes allows them to be functionalized with different chemical groups to, for example, improve cell adhesion and proliferation. Holmes et al. (2013) combined wet and electrospinning to fabricate poly(L-lactic acid) (PLLA) fiber scaffolds with a controlled fiber dimension and combined with multi-walled carbon nanotubes (MWCNTs). The MWCNTs used were treated or untreated with hydrogen (H_2). *In vitro* studies with mesenchymal stem cells (MSCs) showed that PLLA scaffolds composed of fibers with smaller diameters had improved cell adhesion. More importantly, the incorporation of MWCNTs in the PLLA fiber scaffolds had a drastic increase in mechanical strength and a compressive modulus similar to native cartilage. Ultimately, the chondrogenic differentiation of MSCs was enhanced by combining H_2MWCNTs-PLLA fiber scaffolds with poly(L-lysine) (PLL) coating, resulting in the highest GAGs content compared to the control scaffolds, namely PLLA, MWCNTs-PLLA, and H_2MWCNTs-PLLA.

Another type of a multiscale strategy to regenerate cartilage is to create in the same structure a gradient pore size as reported by Zhang et al. (2013). A collagen porous scaffold by using ice particulates as the porogen material with four ranges, namely 150–250, 250–355, 355–425, and 425–500 μm, within the same construct was developed. The objective of the study was not to mimic the multistructured organization of cartilage, but to create a research model to study in the same construct, and thus under the same culture experiment conditions, the effect of pore size on cartilaginous matrix production and cartilage regeneration.

Multiscale strategies also include the encapsulation of smaller structures within macrostructures, which is a frequently employed cartilage TE strategy, particularly in drug delivery systems. The most common approach is the encapsulation of hydrogel microparticles within a macro hydrogel structure. Bian et al. (2011) reported a multiscale delivery system able to support human MSCs chondrogenesis and neocartilage formation. The system was composed of alginate microparticles coated with transforming growth factor-β3 (TGF-β3) nanofilm and encapsulated within a hyaluronic acid (HA) hydrogel. The sustained release of TGF-β3 from microparticles resulted in an enhanced expression of chondrogenic markers and higher levels of cartilage specific matrix deposition by human MSCs in HA hydrogels compared to uncoated microspheres (negative control) and, more importantly, with HA hydrogels without microparticles and, thus, with TGF-β3 directly encapsulated within the matrix core of the hydrogel. Additionally, the *in vivo* results showed that the sustained release of TGF-β3 provided by the multiscale strategy was essential to neocartilage formation compared to the burst release of TGF-β3 directly loaded in the core of HA hydrogels. In the same concept, Nanda et al. (2014) reported a strategy of a porous scaffold encapsulating microparticles for cartilage regeneration. A freeze-dried collagen porous scaffold with a controlled porous structure and incorporating insulin loaded PLGA microparticles was developed. The proposed hybrid scaffold demonstrated a high mechanical strength and a sustained release of insulin for 4 weeks, ensuring the *in vitro* survival and proliferation of chondrocytes.

Figure 9.2 summarizes the referred examples found in literature comprising multiscale strategies to regenerate cartilage.

Fig. 9.2 (continued) cells (MSCs) and alginate microparticles with a TGF-β3 nanofilm. (*F1*) Schematic representation of the photoencapsulation (UV light) to produce loaded MeHA hydrogels. (*F2*) Fabricated HA hydrogel disk and fluorescent and bright field microscopic images of MSCs (membrane labeled with *red dye*) and alginate microspheres (containing FITC-labeled protein) encapsulated in HA gels (Adapted from Bian et al. 2011). (**g**) Scanning electron microscopy images of (*G1*) freeze-dried collagen porous scaffolds (control) and (*G2*) scaffolds incorporating insulin loaded PLGA microparticles. (*G3*) and (*G4*) are high magnification images of (*G1*) and (*G2*), respectively. The *yellow arrows* at (*G4*) represent the integrated PLGA microparticles in the porous collagen matrix (Adapted from Nanda et al. 2014)

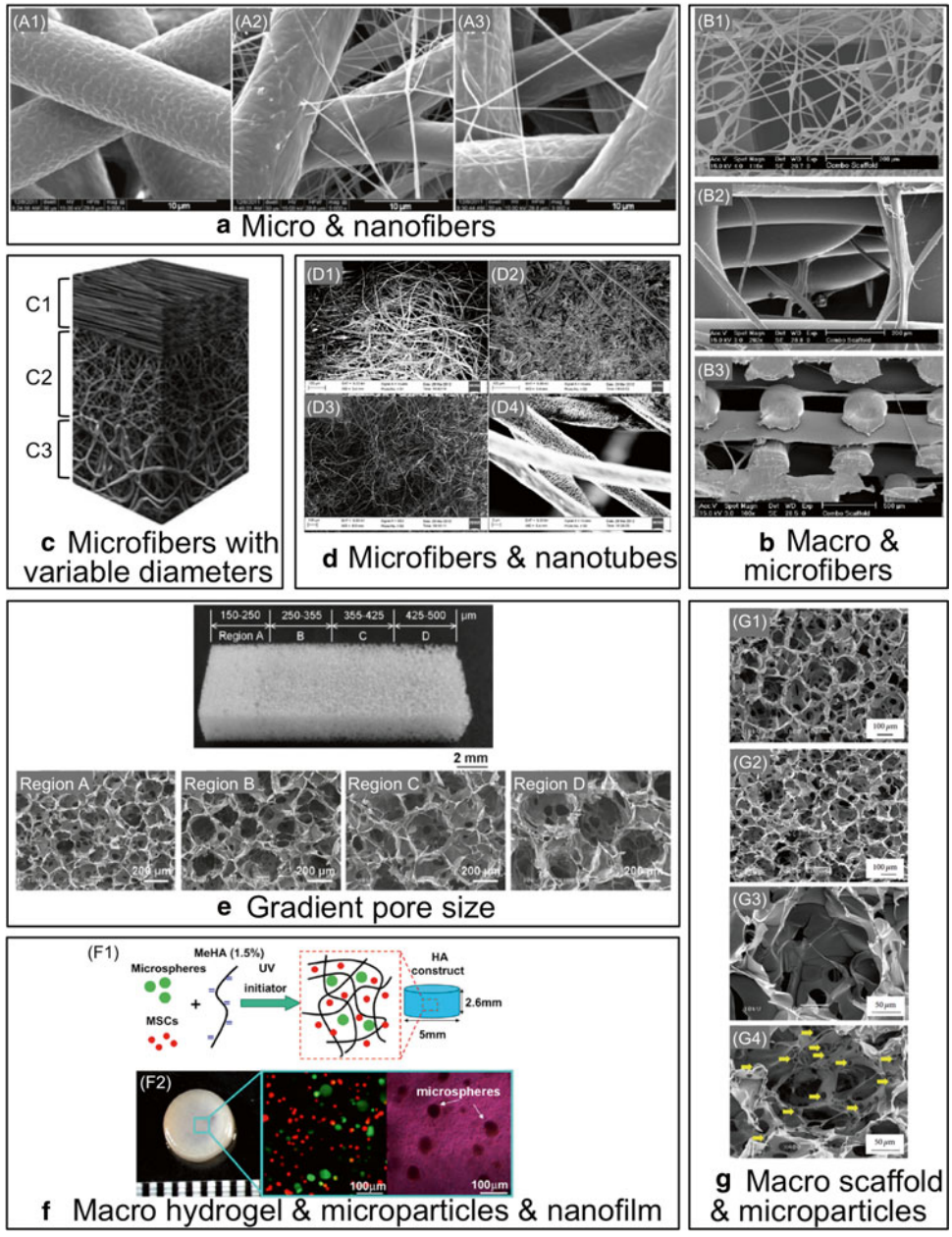


Fig. 9.2 Examples of multiscale strategies to regenerate articular cartilage. (**a**) Electrospun scaffolds with fibers of two diameter scales interspersed evenly throughout an entire 3D construct. (*A1*) PCL microfibers, (*A2*) PCL micro and nanofibers, and (*A3*) PCL microfibers and fibrin nanofibers (Adapted from Levorson et al. 2014). (**b**) Macro scaffolds of integrated 3D fiber (*3DF*) deposited by rapid prototyping technique and electrospun (*ESP*) microfibers with block-co-polymers of poly(ethylene oxide terephthalate) (*PEOT*) and poly(butylene terephthalate). ESP network spun for (*B1*) 2 min or (*B2*) 30 s every two 3DF deposited layers. *Scale bar* is 200 µm. (*B3*) Cross section of (*B2*). *Scale bar* is 500 µm (Adapted from Moroni et al. 2008). (**c**) Trilaminar scaffold produced by sequential electrospinning. The 3D construct was composed by three different zones by varying the diameter and organization of PCL microfibers: (*C1*) superficial zone with aligned 1 µm fibers, (*C2*) middle zone with random 1 µm fibers, and (*C3*) deep zone with random 5 µm fibers (Adapted from McCullen et al. 2012). (**d**) Scanning electron microscopy images of varying PLLA electrospun fibrous scaffolds with (*D1*) 0.5 % multi-walled carbon nanotubes (MWCNTs), treated with (*D1*) 0.5 % or (*D2*) 1 % of hydrogen. (*D4*) High magnification picture of (*D1*) (Adapted from Holmes et al. 2013). (**e**) Collagen porous scaffolds with gradient pore sizes by using ice particulates as a porogen material. The ice particulates had diameters of 150–250, 250–355, 355–425 and 425–500 µm, originating four different regions through the 3D scaffold, namely region A, B, C, and D, respectively (Adapted from Zhang et al. 2013). (**f**) Methacrylated hyaluronic acid (MeHA) hydrogels encapsulating mesenchymal stem

9.3.3 Multilayered Strategies

Inspired by the depth-dependent multi-zone morphology of articular cartilage,with varied mechanical properties and function, scaffolds composed by organized layers were developed. The most common approach is to vary the content of a biomaterial and create a multilayered 3D scaffold, either in porous scaffolds or hydrogels. Zhu et al. (2014) proposed a porous scaffold composed by collagen II blended to CH-PCL copolymers. By varying the content of collagen II and chitosan in opposite trends, a four-layered porous scaffold similar to the ECM of articular cartilage in terms of composition, porous architecture, water content, and compressive mechanical properties, was developed. Nguyen et al. (2011) developed a three-layer poly(ethylene glycol) (PEG)-based hydrogel with chondroitin sulfate (CS) and matrix metalloproteinase-sensitive peptides (MMP-pep) in the top layer, CS in the middle layer, and HA in the bottom layer. Mouse MSCs were encapsulated within the multilayered hydrogels and their biological outcome was assessed. Results demonstrated that the developed hydrogels not only induced the MSCs to differentiate into the chondrogenic lineage but also that the phenotype and matrix production profile could be tailored to specific zones of articular cartilage by altering the material composition alone. Most importantly, the authors showed that the spatial organization of specific biomaterials affected collagen II expression and created a composite tissue structure with spatially-varying collagen II levels, increasing from the superficial layer to the deep layer. Creating layers by varying the shape of the biomaterial used is also an example of a multilayered strategy. Steele et al. (2014) developed a PCL two-zone approach with electrospun fiber articulating surface deposited onto a particulated-leached foam. The final two-layered 3D scaffold was composed by an aligned fiber zone to mimic the morphology of the superficial zone of articular cartilage, laminated to a bulk porous particulate-template scaffold to allow cellular infiltration and extensive ECM deposition. Results showed that the incorporation of aligned fibers significantly enhanced the tensile mechanics of the laminated porous scaffold. Additionally, the fibers also reduced the surface roughness of the porous scaffolds, mimicking the smooth articular surface of native cartilage. On the other hand, the porous scaffolds contributed to the infiltration of seeded chondrocytes, resulting in high rates of proliferation and GAG production. Ng et al. (2009) proposed a layered agarose hydrogel by varying the population of encapsulate chondrocytes. Chondrocytes from the superficial (SZC) and middle (MZC) zones were isolated and encapsulated to form bilayered constructs with 2 or 3 % agarose. Two different layered formulations were tested, namely 2 %SZC/2 %MZC and 3 %SZC/2 %MZC. For SZC, GAG and collagen production increased with increased agarose concentration and when layered with MZC. For MZC, GAG production and cell proliferation increased when layered with SZC.

Another type of multilayered strategies is the creation of gradients by the incorporation of biomolecules found in the ECM, such as proteins and GAGs, which are known to organize cytokines, chemokines, and growth factors, thus guiding cell migration, growth, and differentiation in various biological processes. However, controlling the spatial distribution of these biomolecules to mimic native tissue remains very challenging. Chow et al. (2014) proposed the incorporation of a peptide-polymer conjugate system to functionalize the surface of scaffolds with selected peptides that specifically and dynamically bind GAGs to guide their spatial arrangement. Combining this functionalization approach with electrospinning, a single and dual opposing gradients of peptide concentrations that directed the spatial organization of GAGs through the thickness of the scaffold was developed. Two binding peptides, namely HA-binding and chondroitin sulfate (CS)-binding peptides-PCL conjugate, were incorporated by co-electrospinning into PCL fibers, producing HAbind-PCL and CSbind-PCL fibers, respectively. Sequential electrospinning of opposing concentrations of HAbind-PCL and CSbind-PCL created peptide gradients, that specifically organized contrasting gradients of HA and CS through the scaffold

thickness. Interestingly, the gradient in peptide concentrations organized GAGs into a depth gradient through the scaffold thickness. Gradients of biomolecules can also be simply achieved by the encapsulation of varying concentrations within a hydrogel. Smith Callahan et al. (2013) developed a poly(ethylene glycol dimethacrylate) (PEGDM) hydrogel system with a gradient of arginine-glycine-aspartic acid peptide (RGD) concentrations to correlate primary human osteoarthritic chondrocyte proliferation, phenotype maintenance, and ECM production. In higher RGD concentration regions, the cell number and the expression of chondrogenic phenotype markers decreased, while, on the other hand, in lower RGD concentration regions both were maintained. Additionally, ECM content was higher in lower RGD concentration regions. The design of gradients through 3D structures was also explored by Thorpe et al. (2013), by modulating the oxygen tension and mechanical environment thorough the depth of MSCs seeded agarose hydrogels. The 3D constructs were radially confined of half their thickness and subjected to dynamic compression (DC). Results showed that confinement led to low oxygen levels in the bottom of the construct, while the application of DC increased strain at the top. These spatial changes correlated with GAGs accumulation in the bottom of the construct and led to increased collagen accumulation in the top, as well as a suppression of hypertrophy and calcification throughout the construct. Therefore, by modulating gradients of the environment through the depth of agarose hydrogels, it was possible to suppress MSCs chondral progression and to engineer tissues with zonal gradients mimicking certain aspects of native articular cartilage.

Figure 9.3 summarizes the referred examples found in literature comprising multilayered strategies to regenerate cartilage.

9.3.4 Hierarchical Strategies

Hierarchical strategies to regenerate cartilage comprise organized bottom-up approaches, in which smaller components are assembled into greater 3D constructs in an attempt to mimic the native hierarchical organization of cartilage, from its macro- to nano-stratified cues. For this propose, different assembling techniques have been explored, such as layer-by-layer (LbL) and peptide self-assembly, inverted crystal colloidal (ICC), cell sheets, and magnetic force-based approaches.

The most common approach in hierarchical strategies for cartilage TE is to assembly microparticles and create microparticles-based 3D scaffolds. For example, Silva et al. (2013) developed nanostructured 3D constructs combining LbL technology and template leaching as scaffolds for cartilage regeneration. Paraffin spheres were previously modified with poly(ethyleneimine) (PEI) and used as templates to produce the LbL membrane composed by chitosan and chondroitin sulphate as polyelectrolytes. Once the LbL membrane was built, the paraffin was leached out and samples were freeze-dried. The obtained 3D constructs had high porosity and water uptake, and a viscoelastic nature. Cultures of bovine chondrocytes (BCH) and MSCs were performed up to 21 days. Both cells adhered and proliferated on the surface of the samples. Additionally, during the time of the culture tested, BCH maintained their chondrogenic phenotype and MSC were able to differentiate into the chondrogenic lineage upon chondrogenic medium stimulation. Singh et al. (2010) explored another assembly approach to produce microparticles-based scaffolds by using subcritical carbon dioxide (CO_2) as a sintering agent to assemble poly(D,L-lactide-co-glycolide) (PLG) microparticles. The CO_2 sintering technique for manufacturing microparticles-based scaffolds is suitable for producing cell-containing, shape-specific matrices, such as scaffolds, under relatively mild conditions via a single-step sintering of microparticles in the presence of cells, with high cell viability. In vitro assays using chondrocytes and human umbilical cord mesenchymal stromal cells (HUCMSCs) demonstrated the viability of the method and, more importantly, that the macro constructs were suitable for cartilage tissue engineering applications.

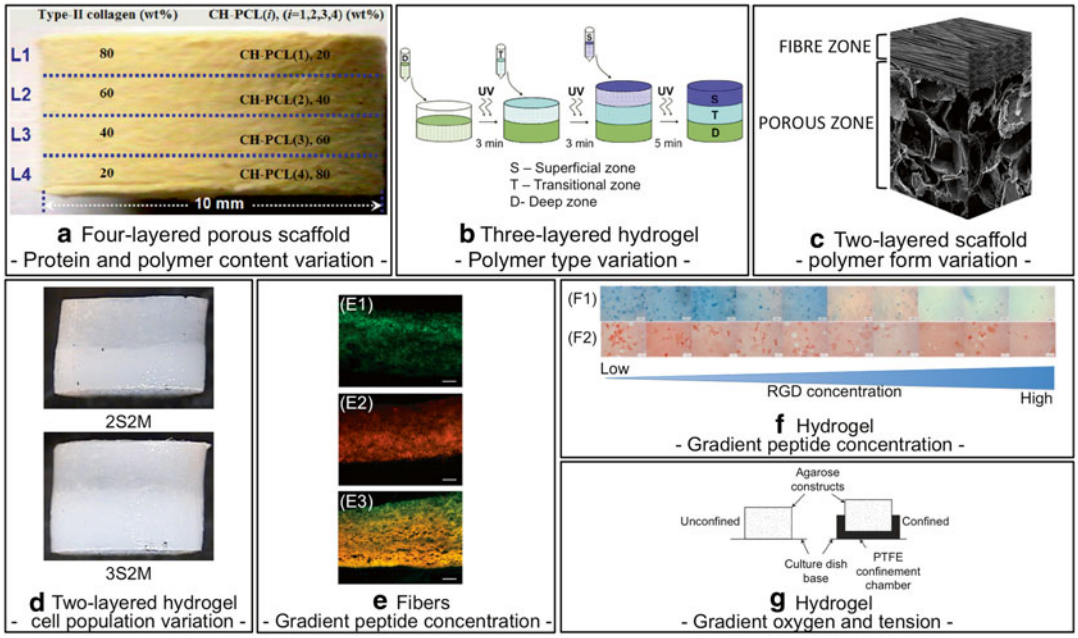


Fig. 9.3 Examples of multilayered strategies to regenerate articular cartilage. (**a**) Image of a cylindrical collagen II/chitosan-PCL scaffold with the schematic representation of its four layers (L1, L2, L3, and L4), which were achieved by varying the content of collagen II and chitosan in opposite trends. The *dotted lines* indicate the interface zones of the layers (Adapted from Zhu et al. 2014). (**b**) Multilayered hydrogel construct fabrication with three distinctive layers, each corresponding to the superficial (*S*), transitional (*T*), and deep (*D*) zones of articular cartilage (Adapted from Nguyen et al. 2011). (**c**) Diagram illustrating the two-layered PCL scaffold composed by an electrospun PCL fiber zone laminated to a bulk porous particulate-template PCL scaffold (porous zone) (Adapted from Steele et al. 2014). (**d**) Chondrocytes from the superficial (SZC) and middle (MZC) zones were isolated and encapsulated to form two-layered hydrogels with 2 or 3 % agarose. Two different layered formulations were tested, namely 2 %SZC/2 %MZC (2S2M) and 3 %SZC/2 %MZC (3S2M) (Adapted from Ng et al. 2009). (**e**) Fluorescence microscopy of cross sections of gradient scaffolds formed by sequential electrospinning concentrations of peptide-PCL conjugates of (*E1*) HAbind-PCL labeled with fluor-HA (*green*), (*E2*) CSbind-PCL labeled with rhod-CS (*red*), and (*E3*) opposing concentrations of HAbind-PCL and CSbind-PCL labeled with fluor-HA and rhod-CS (Adapted from Chow et al. 2014). (**f**) Poly(ethylene glycol dimethacrylate) (*PEGDM*) hydrogel system with a gradient of arginine-glycine-aspartic acid peptide (*RGD*) concentrations. (*F1*) and (*F2*) are histological sections of alcian blue and sirius red stainings, respectively. Images of the extracellular matrix production by human chondrocytes after 3 weeks of culture where taken every 5 mm down the length of the gradient. *Scale bar* is 200 μm (Adapted from Smith Callahan et al. 2013). (**g**) Experimental design of agarose hydrogel constructs press-fitted into custom made PTFE wells. The 3D constructs were radially confined of half their thickness and subjected to dynamic compression, creating a gradient of oxygen tension and mechanical environment thorough the depth of the hydrogels (Adapted from Thorpe et al. 2013)

Other methodology used to assemble microparticles in hierarchical cartilage TE applications is the production of ICC scaffolds. Organized colloids with hexagonal structure are often described as photonic crystals, photonic band gap materials, and artificial opals since close-packed particles display unique optical properties via the diffraction of visible light. By organizing arrays of microparticles as an ordered reverse replica, a 3D scaffold with inverted lattice pore topography was produced, named ICC scaffolds. Kuo and Tsai (2011) proposed the development of heparinized ICC scaffolds to generate uniform tissue-engineered cartilage. Highly viscous heparin, chitin, and CH gels were infiltrated repeatedly into the voids among self-assembled polystyrene (PS) microparticles. *In vitro* assays with chondrocytes showed that cells proliferated, secreted GAGs, and produced collagen. Importantly, the infused cells could migrate and

proliferate in and near the core of the ICC scaffolds. This uniform cell growth was mainly because the regular pore geometry of ICC scaffolds was able to reduce the resistance to cell migration and nutrient transport. Moreover, immunocytochemistry for collagen II also showed a uniform distribution of cartilaginous components.

In a different approach from microparticles-based scaffolds, Liu et al. (2011) explored the assembly of nanofibers to produce hollow microparticles without using any prefabricated template. Poly(amidoamine) (PAMAM) dendrimers were used as initiators to synthesize star-shaped PLLA (SS-PLLA), which self-assembled into nanofibrous hollow microparticles. The engineered microparticles were evaluated as injectable cell carriers for cartilage regeneration. *In vitro* results showed that cells adhered to the surface of the nanofibrous microparticles and migrated to the inside of the hollow structure, while remaining rounded in morphology, which is characteristic of the chondrocyte phenotype. Additionally, high production of GAGs and high levels of aggrecan and collagen II expression were detected. The capacity of the nanofibrous hollow microparticles as an injectable scaffold to fill cartilage defects was also tested *in vitro* using a mold with the shape of a rat femoral condyle. After 4 week of chondrocytes culture, a piece of cartilage tissue, filling the entire mold could be obtained. The engineered tissue was composed by abundant amounts of GAG and collagen II, confirmed by safranin-O and immunohistochemistry, respectively.

In a different assembly approach from engineered materials assembly, cell based therapies, such as cell sheet technology or magnetic-based approaches, are also included in hierarchical strategies for cartilage repair. Cell sheet technology using temperature-responsive culture dishes was first reported by Okano et al. (1993), and nowadays is used widely in different regenerative medicine fields. Cell sheets as hierarchical systems comprise the assembly of multiple layers of cell sheets, which then originates a 3D engineered tissue from micro to macroscales. Kushida et al. (2000) reported the production of 3D cell sheets. The engineered tissue structures had intact ECM and adhesion factors, allowing the production of a 3D construct without the use of scaffolds. Particularly for cartilage TE, Mitani et al. (2009) produced cell sheets composed by chondrocytes, with a consistent cartilaginous phenotype and adhesive properties. Sato et al. (2014) reported different studies that successfully regenerate cartilage *in vitro* by cell sheet technology. Additionally, in this study they solved some drawbacks reported by other authors, and confirmed the safety and efficacy of cell sheet technology for cartilage defects repair. The authors stated that a clinical study with layered 3D chondrocyte sheets is currently ongoing. Likewise cell sheet technology, a promising alternative for shaping multicellular organization is the use of magnetic forces. This approach requires individual cell magnetization through the internalization of magnetic nanomaterials. Fayol et al. (2013) reported the formation of stem cells aggregates at the millimetric scale guided by home-designed miniaturized magnetic devices, and the production of 3D cellular patterns of defined sizes and shapes. The proposed magnetic confinement was an alternative approach to the standard centrifugation for the production of cell aggregates. The *in vitro* differentiation of MSCs into the chondrogenic lineage and nanoparticles with a maghemite core-citrate coated were chosen as the model system. With this approach the potential use of stem cells magnetic confinement for building large cartilage tissue substitutes of defined geometry and high tissue integration potential was demonstrated.

Figure 9.4 summarizes the referred examples found in literature comprising multilayered strategies to regenerate cartilage.

All the different referred studies found in literature regarding multiphasic, multiscale, multilayered, and hierarchical strategies to regenerate cartilage tissue are summarized in Table 9.1. In each example the shape and composition of the components used, the respective assembling technique to produce 3D constructs, as well as, the cell phenotype and isolation source used to performed *in vitro* and/or in vivo tests were described.

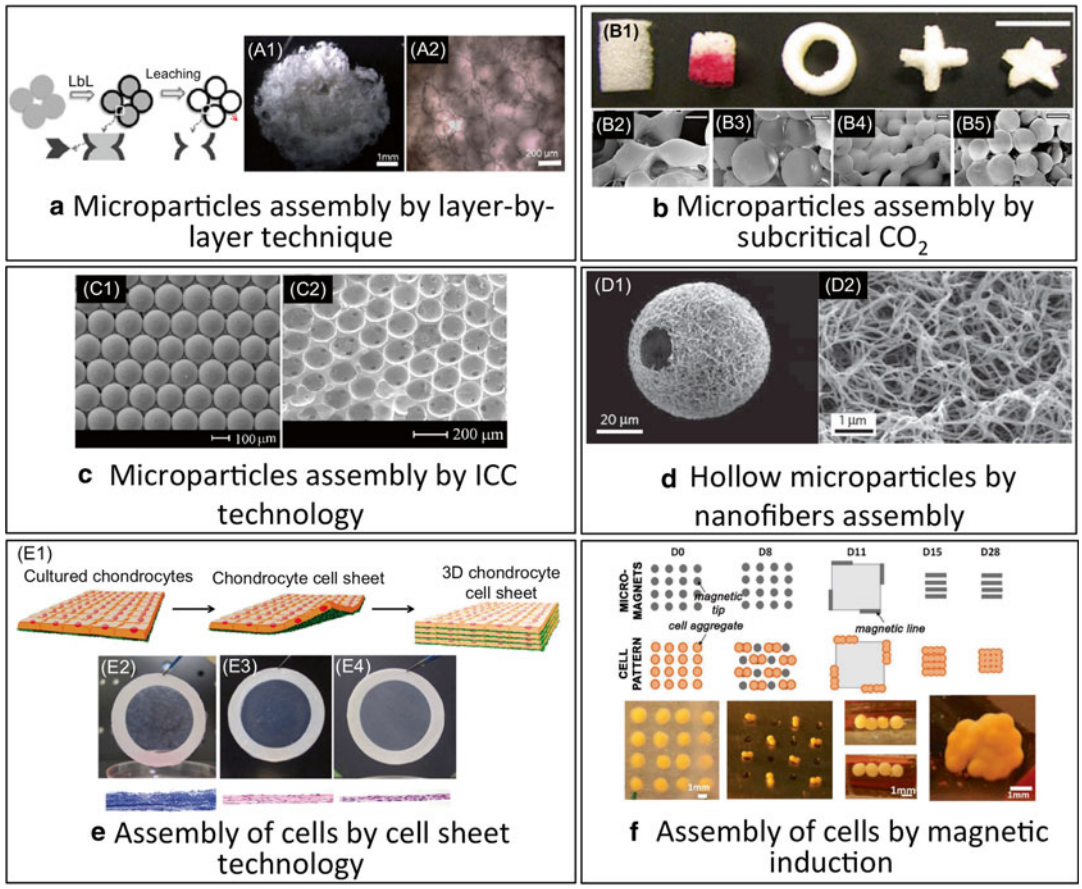


Fig. 9.4 Examples of hierarchical strategies to regenerate articular cartilage. (**a**) Scheme of the production steps of nanostructured 3D constructs. (*A1*) Picture and (*A2*) optical microscopy of the obtained microparticles-based scaffolds fabricated combining LbL and leaching of free-packet paraffin spheres (Adapted from Silva et al. 2013). (**b**) Microparticles-based scaffolds by using subcritical carbon dioxide (CO_2) as a sintering agent to assemble poly(D,L-lactide-co-glycolide) (PLG) microparticles. (*B1*) Various shape-specific scaffolds produced by using rubber molds of different shapes filled with PLG microspheres (140 µm diameter) using CO_2 at subcritical conditions (15 bar for 1 h at 25 °C followed by depressurization at 0.14–0.21 bar.s^{-1}). From *left* to *right*: cylinder, bilayered cylinder, tube, plus sign, and star shapes. *Scale bar* is 1 mm. (*B2*) Characteristic scanning electron microscopy (SEM) images of PLG scaffolds at CO_2 at subcritical conditions. Sizes of the microspheres used were (*B2* and *B3*) 240 µm, (*B4*) 175 µm, and (*B5*) 140 µm (Adapted from Singh et al. 2010). (**c**) Microparticles assembling organized in colloids with hexagonal structure by inverted colloidal crystals (ICC). SEM images of (*C1*) inverted colloidal crystals and (*C2*) heparinized ICC scaffolds in pure ethylene glycol (Adapted from Kuo and Tsai 2011). (**d**) Assembly of nanofibers to produce hollow microparticles without using any prefabricated template. SEM images (*D1*) of a nanofibrous hollow microparticle, showing the nanofibrous architecture and a hole of approximately 20 µm on the microparticle shell, and (*D2*) showing the nanofibers, which have an average diameter of about 160 nm (Adapted from Liu et al. 2011). (**e**) Cell sheets technology for articular cartilage regeneration. (*E1*) Schematic representation of the production of temperature-responsive three-dimensional (3D) chondrocyte sheets. 3D cell sheets composed by chondrocytes isolated from different sources and used at different passages, namely (*E2*) from rabbits at passage 0, (*E3*) from mini-pigs at passage 1, and (*E4*) from humans at passage 1. Each cell sheet presented has its corresponding histological image showing the respective ECM deposition (bellow). Cell sheets with the same number of layers, namely three, but with chondrocytes isolated from different sources have different amounts of ECM deposition (Adapted from Sato et al. 2014). (**f**) Sequential magnetic fusion for the formation of a cartilage sheet. A network of magnetic tips was used to form 16 spheroid aggregates of 2.5×10^5 cells (day 0, D0), which were placed in contact on day 8 (D8), leading to the formation of eight doublets upon spheroid fusion. At day 11 (D11), four quadruplets were formed upon doublet fusion. Ultimately, at days 15 and 28 (D15 and D28), the four quadruplets were fused to form the final 3D structure (Adapted from Fayol et al. 2013)

Table 9.1 Examples of multiphasic, multiscale, multilayered, and hierarchical strategies for cartilage regeneration

	Components shape/composition	3D assembling technique	Cell phenotype/source	Reference
Multiphasic	Fiber/PCL	Vacuum infuse	–	Liao et al. (2013)
	Hydrogel/alginate-PAAm			
	Fiber/gelatin	Co-electrospinning	Chondrocytes/human knee joint	Torricelli et al. (2014)
	Fiber/PLLA			
	Hydrogel/gelatin methacrylamide	Photopolymerization	Chondrocytes/human talus bone	Boere et al. (2014)
	Porous scaffold/pMHMGCL-PCL			
	Hydrogel/PVA	Wrapping	Chondrocytes/piglet knee joint	Spiller et al. (2012)
	Microparticle/PLGA-IGF-1			
	Fiber/PGA			
Multiscale	Micro and nano fibers/PCL, PCL and fibrin	Electrospinning	MSCs/human umbilical cord blood	Levorson et al. (2014)
	Gradient porous scaffold/collagen I	Freeze-drying	Chondrocytes/bovine	Zhang et al. (2013)
	Fiber/PCL	Sequential electrospinning	Chondrocytes/calf lower leg joint	McCullen et al. (2012)
	Macro scaffolds and microfibers/ PEOT-PBT bock co-polymers	Bioplotter and electrospinning combination	Chondrocytes/bovine knee joint	Moroni et al. (2008)
	Microfibers/PLLA	Wet electrospinning	MSCs/human iliac crest bone marrow	Holmes et al. (2013)
	Nanotubes/hydrogen-treated MWCNTs			
	Hydrogel/methacrylated HA	Photopolymerization	MSCs/human bone marrow	Bian et al. (2011)
	Microparticle/alginate-TGF-β3 Nanolayer/ PAH/PSS			
	Porous scaffold/collagen I	Freeze-drying	Chondrocytes/calf knee joint	Nanda et al. (2014)
	Microparticle/PLGA-insulin			

(continued)

Table 9.1 (continued)

	Components shape/composition	3D assembling technique	Cell phenotype/source	Reference
Multilayered	Porous scaffold/PCL	Electrospinning with surface deposited onto a particulate-leached foam	Chondrocytes/calf lower leg joint	Steele et al. (2014)
	Fiber/PCL			
	Porous scaffold/chitosan-PCL-collagen II	Temperature gradients, collimated photothermal heating, and freeze-drying combinatorial technique	Chondrocytes/rabbit knee joint	Zhu et al. (2014)
	Hydrogel/PEG-CS-MMP-pep, PEG-CS, and PEG-HA	Photopolymerization	D1 line/mouse bone marrow	Nguyen et al. (2011)
	Hydrogel/PEGDM-RGD	Photopolymerization	Chondrocytes/human tibial plateau and femoral condyle	Callahan et al. (2013)
	Fiber/PCL-HA and PCL-CS bind peptides PCL conjugated	Sequential electrospinning	–	Chow et al. (2014)
	Hydrogel/agarose	PTFE confinement chamber	MSCs/porcine femora bone marrow	Thorpe et al. (2013)
	Hydrogel/agarose	Ionic crosslinking	Chondrocyte/bovine calf knee	Ng et al. (2009)
Hierarchical	Microparticle/paraffin-PEI	Layer-by-layer assembly	Chondrocytes/calf legs patellofemoral groove	Silva et al. (2013)
	Nanolayer/chitosan and CS		MSCs/human hip bone marrow	
	Fiber/PLLA-PAMAM-OH	Self-assembly	Chondrocytes/rabbit	Liu et al. (2011)
	Microparticle/polystyrene	Inverted colloidal crystal	Chondrocytes/calf knee joint	Kuo et al. (2011)
	Hydrogel/chitin-chitosan-heparin			
	Sheet/cells	Cell-sheet technology	Chondrocytes/human knee joint	Sato et al. (2014)
	Nanoparticle/maghemite-core citrate coated	Magnetic aggregation	MSCs/human bone marrow	Fayol et al. (2013)
	Microparticle/PLG	Subcritical CO_2	Chondrocytes/hog ankle and mandibular condyle	Singh et al. (2010)
			MSCs/human umbilical cord	

To each example, the shape and composition of the components of the strategy, as well as the technique to assemble the respective components into the final three-dimensional (3D) structure, are listed. The cell phenotype and isolation source used to performed *in vitro* and/or *in vivo* tests are described

Abbreviations: *CO_2* carbon dioxide, *CS* chondroitin sulfate, *HA* hyaluronic acid, *IGF-1* insulin-like growth factor-1, *MMP-pep* metalloproteinase-sensitive peptides, *MSCs* mesenchymal stem cells, *MWCNTs* multi-walled carbon nanotubes, *PAAm* polyacrylamide, *PAH* poly(allylamine hydrochloride), *PAMAM-OH* poly(amidoamine) dendrimers with hydroxyl groups on the surface, *PBT* poly(butylene terephthalate), *PCL* poly(ε-caprolactone), *PEG* poly(ethylene glycol), *PEGDM* polyethylene glycol dimethacrylate, *PEI* poly(ethyleneimine), *PEOT* poly(ethylene oxide terephthalate), *PGA* Poly(glycolic acid), *PLG* poly(D,L-lactide-co-glycolide), *PLGA* poly(lactic-co-glycolic acid), *PLLA* poly(L-lactic acid), *pMHMGCL* methacrylate poly(hydroxymethylglycolide-co- ε-caprolactone), *PSS* poly(sodium 4-styrenesulfonate), *PTFE* poly(tetrafluoroethylene), *PVA* poly(vinyl alcohol), *RGD* arginine–glycine–aspartic acid peptide, *TGF-β3* transforming growth factor-β3

9.4 Summary and Future Directions

Articular cartilage is a highly organized and hierarchical tissue that provides to the bearing surface low friction and wear-resistance. The characteristic anisotropic mechanical properties of cartilage tissue are a result of depth-dependent differences in the composition of its ECM. Once damaged, cartilage tissue has a low self-repair potential. In current clinical practice, different cartilage repair techniques are being used to address tissue damage. However, their outcome remains unpredictable and long-term success is questionable. Cartilage tissue engineering has emerged as an alternative method to regenerate cartilage defects. At present, the success of cartilage TE requires a detailed understanding of the contribution of biological factors, architectural and mechanical properties, and biochemical/material composition in the regeneration of cartilage. Cartilage TE with the research and development of innovative biomaterials and research models, contributed to a more extensive understanding of cartilage biology. Particularly, either the development of biomimetic scaffolds and/or research models has contributed to the regulation and/or stimulation of different biological processes of cells, facilitating ECM deposition, alignment and functionality. Future strategies to fabricate biomaterials for cartilage TE can now have their development base on the wide range of scientific data available. Additionally, the detailed analysis of the engineered tissue using well-known techniques, such as histology, immunocytochemistry, and mechanical assays, can now be complemented with new and sophisticated characterization techniques, including nanotechnology and novel biofabrication methods, thus providing informative insights about the structure-function relationship of cartilage from its macro to nanostructure.

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Anterior Cruciate Ligament: Structure, Injuries and Regenerative Treatments

10

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Abstract

Anterior cruciate ligament (ACL) is one of the most vulnerable ligaments of the knee. ACL impairment results in episodic instability, chondral and meniscal injury and early osteoarthritis. The poor self-healing capacity of ACL makes surgical treatment inevitable. Current ACL reconstructions include a substitution of torn ACL via biological grafts such as autograft, allograft. This review provides an insight of ACL structure, orientation and properties followed by comparing the performance of various constructs that have been used for ACL replacement. New approaches, undertaken to induce ACL regeneration and fabricate biomimetic scaffolds, are also discussed.

Keywords

Anterior cruciate ligament • Autograft • Allograft • Ligament prostheses • Tissue engineering

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Abbreviations

45S5 BG	45S5 bioglass®
ACL	Anterior cruciate ligament
ACLR	Anterior cruciate ligament reconstruction
ACP	Amorphous calcium phosphate
BFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BPTB	Bone-patellar tendon-bone
EGF	Epidermal growth factor
GAG	Glycosaminoglycan
HA	Hyaluronan
HAp	Hydroxyapatite
HT	Hamstring tendon
HYAFF-11®	Benzylic ester of hyaluronic acid
IGF	Insulin-like growth factor
OA	Osteoarthritis
PAAm	Polyacrylamide
PCL	Poly(ε-caprolactone)
PCLDLLA	Poly(ε-caprolactone-co-D,L-lactide)
PDGF	Platelet-derived growth factor
PDLLA	Poly(D,L-lactide acid)
PDMS	Poly(dimethyl siloxane)
PDTDDD	Poly (desaminotyrosyl-tyrosine dodecyl dodecanedioate)
PDTEC	Poly (desaminotyrosyl-tyrosine ethyl ester carbonate)
PE	Polyester
PEEK	Poly(ether ether ketone)
PEGDA	Poly(ethylene glycol)-diacrylate
PEU	Poly(ester-urethane)
PEUUR	Poly(ester urethane-urea)
PG	Phosphate glass
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLCL	Poly(L-lactide-co-ε-caprolactone)
PLDLA	Poly(L-lactide -Co-D,L-lactide acid)
PLGA	Poly(L,D-lactide-co-glycolide acid)
PLLA	Poly(L-lactide acid)
PLLA-AC	Poly(L-lactide-co-acryloyl carbonate)
PMGI	Poly(methyl glutarimide)
PU	Polyurethane
PUUR	Poly(urethane urea)
RADA	Self-assembled peptide
RGD	Arg-Gly-Asp
STG	Semitendinosus and gracilis
TGF	Transferring growth factor-β.
β-TCP	β tricalcium phosphate

10.1 Introduction

Ligaments perform essential roles in body stabilization via maintaining bone alignments and guiding motions. They provide bone-to-bone connections in various parts of the body such as the knee. Anterior cruciate ligament (ACL) is a critical ligament in the knee joint that connects the femur to the tibia. It is the most vulnerable ligament as a consequence of twisting or bending of the knee (Yates et al. 2012). Annually, more than 350,000 Americans suffer from ACL injuries (Ma et al. 2011). Young athletes who play vigorous sports such as soccer, rugby and ski are more susceptible to ACL injuries. The acute ACL injuries further lead to instability and degenerative diseases including osteoarthritis, meniscus, and knee cartilage tears (Petrigliano et al. 2006). Lacking significant vascularization and enveloping by synovial tissue cause poor intrinsic healing capacity for ACL (Murray et al. 2000). Surgical treatments are, therefore, crucial to substitute the symptomatic unstable ligament with various grafts (Rice et al. 2012). Different biological and synthetic grafts have been attempted for reconstruction of the injured ligament (Crawford et al. 2005; George et al. 2006). The performance of these grafts is promising. However, their long-term drawbacks have an impact on their applications. The proliferation of appropriate cell type on biocompatible and resorbable structures is a new strategy for ligament reconstruction (Rong-Mei 2011; Dunn et al. 2006). This technique is still its infancy, and no clinical application has been published to date. Here, we describe the main aspects of ligament structure, orientation and injuries. Afterwards, the various therapeutic methods for ACL regeneration and reconstruction are compared. The new approaches that have been undertaken to

fabricate biomimetic scaffolds for ACL tissue engineering are also discussed.

10.2 Anterior Cruciate Ligament

ACL of an adult human) is an intra-articular tissue with 22–41 mm length and cross-section of 44.4–57.5 mm^2 (Amis and Dawkins 1991; Gentleman et al. 2004). It originates from femoral condyle, twists approximately 180° to form anteromedial and posterolateral bundles, and inserts into the tibial plateau (Woo et al. 2005). ACL is mainly composed of water (>65 %) and thus classified as a soft tissue (Rodrigues et al. 2013). The dried ACL consists of mainly 70–80 % fibrous collagens with the ratio of 9:1 of collagen type I to III (Riechert et al. 2001). Collagen fibrils discriminate unique crimp patterns in every 45–60 μm to passively guide the motion. This crimp helps ACL to sustain a high range of loads throughout the motion of knee (Vunjak-Novakovic et al. 2004; Platzer et al. 2003). Any defects in the structure of ACL, therefore, lead to movement disability. The mechanisms of ligament injuries are multi-factorial and depend on both intrinsic and extrinsic factors. Contact or direct trauma, structural vulnerability, and rapid growth are some intrinsic factors that lead to ACL injuries, subsequent instability, abnormal loading patterns, and the development of osteoarthritis (Rodrigues et al. 2013).

The hypovascular and hypocellular nature of ligaments retards their self-regeneration capacities. Following ACL injury, disruption of the thin synovial sheath of ACL causes blood dissipation in the synovial fluid. This dissipation hardens formation of localized hematoma and further provision of cytokines, growth factors and reparative cells at the injury site (Murray et al. 2000). These cells, however, exhibit low mobility, proliferation, metabolic activities and matrix production tendency (Bray et al. 2002). The higher matrix metalloproteinase activities and poor adhesive strength of these cells descend ligament self-healing (Zhou et al. 2005). The inability of ACL to regenerate itself makes surgical interventions inevitable. The injured ACL, therefore, is typically reconstructed using different biological or synthetic grafts. The long-term performance of a substituted graft depends upon its structure, operation procedure, initial tension, and graft fixation. Furthermore, type of graft and its long-term viability, morbidity, and quick returning to the full activity of a patient are key aspects for clinicians in their graft selection (Mayr et al. 2012). The applications of different approaches in ACL replacement are discussed in the following section.

10.3 ACL Replacements

ACL reconstruction (ACLR) is the standard care procedure with more than 200,000 operations performed in the US in 2011 (Shelton and Fagan 2011). These reconstructions increase the health care cost to more than $1 billion (Leiter et al. 2014). The alternative graft is passed through the tunnels made in the femur and tibia and is secured using fixatives (Bach et al. 2012). The tendons of the same patient (autograft) or donor tissues (allograft), non-degradable synthetic grafts are alternative approaches that have been attempted for ACLR (Goldblatt et al. 2005). Here, various types of autogeneic, allogeneic, and synthetic grafts for ACLR are reviewed by comparing their advantage and disadvantages. Moreover, we briefly discuss different graft fixation methods due to their role in weight bearing.

10.3.1 Autograft

The first attempt to reconstruct the torn ACL using tethered fascia lata graft was performed in 1917 (Hey Groves 1917). Autologous grafts remain the most popular method for ACLR and are considered as "gold standard" because of the high rate of success (85–90 %) of their long-term clinical outcomes (Leong et al. 2014). In this method, the injured ligament is substituted with a tendon of own patient. Patella tendon, hamstring tendons (e.g. semitendinosus and gracilis (STG)), and quadriceps are commonly used as an alternative tissue (Haut Donahue et al. 2002). It significantly promotes bone-to-bone healing, initial fixation securing and returning to pre-injury

activities (Forster and Forster 2005). Donor site morbidity including anterior knee pain, kneeling pain, patellar fracture, higher graft failure rates, and patellofemoral pain are its main complications (Laboute et al. 2010). Hamstring tendon (HT) is another alternative in autogeneic grafts. Their mechanical properties including ultimate tensile yield strength, stiffness, and stress relaxation curve mimic the native ACL (Bogunovic et al. 2013). The widening of femoral tunnel and weakness of the hamstring muscles after the operation, however, are considered as their potential adverse effect (Struewer et al. 2013). The long-term performance of bone-patellar tendon-bone (BPTB) and HT grafts are compared in Table 10.1. ACLR are favorable due to the higher rate of satisfaction and a lower degree of osteoarthritis (OA) in HT grafts. The rate of satisfaction was reported based on International Knee Documentation Committee Subjective Knee Evaluation Form (IKDC).

10.3.2 Allograft

Substitution of cadaver tissues instead of damaged ACL eliminates the issue of donor site morbidity. The use of allografts shorten the operation period and reduce the size of incisions (Caborn and Selby 2002). Further advantages of allograft include no size limitation appropriate for revision surgery, and lower incidence of postoperative arthrofibrosis and knee stiffness (Beasley et al. 2005; Amendola and Stolley 2009). The most applicable allograft materials include patellar ligament, and Achilles tendon (Sherman and Banffy 2004). Other tissues such as anterior or posterior tibialis and peroneus longus have also been used for allograft ACLR (Pearsall et al. 2003).

The 5-year follow-up on the ACL reconstruction revealed that the allograft leads to less pain and better short-term function compared to autograft (Poehling et al. 2005). The main drawback

Table 10.1 Long-term follow-up of autogenic graft in ACLR

Graft	Case (n)	Follow-up (year)	Degree of OA[a] (%)	IKDC satisfaction score (%)	Ref
BPTB	117	10.3	40	88.7	Ahn et al. (2012)
BPTB	63	16.1	62	78	Gerhard et al. (2013)
BPTB	109	11.1	47	71	Kessler et al. (2008)
BPTB	90	15	51	65	Leys et al. (2012)
BPTB	72	7	25	51	Lidén et al. (2008)
BPTB	18.5	33	16.5	80	Mihelic et al. (2011)
BPTB	100	24.5	27	46	Pernin et al. (2010)
BPTB	59	7	45	85	Roe et al. (2005)
BPTB	25	11	84	72	Sajovic et al. (2011)
BPTB	73	13.5	54.2	75	Struewer et al. (2012)
BPTB	28	8.8	N/A	84	Wipfler et al. (2011)
BPTB	99	5.5	N/A	80	Brandsson et al. (2001)
HT	90	15	39	77	Leys et al. (2012)
HT	41	7	20	63	Lidén et al. (2008)
HT	61	7	14	89	Roe et al. (2005)
HT	25	8.8	N/A	94.4	Wipfler et al. (2011)
HT	68	14.6	45	75	Leiter et al. (2013)
HT	52	9.9	24.9	71.1	Struewer et al. (2013)
HT	271	6.8	28	33	Asik et al. (2007)

[a]*OA* Osteoarthritis, *IKDC* International Knee Documentation Committee Subjective Knee Evaluation Form, *BPTB* Bone-Patellar Tendon-Bone, *HT* Hamstring Tendon

of these grafts is the significant risk of disease transmission that can be minimized by sterilization of graft prior to reconstruction. Moreover, an allograft may lead to immune rejection, delayed incorporation (Jackson et al. 1993) and bone tunnel enlargement (Fahey et al. 1994) that enhances the rate of failure. Besides, the remodeling activity of allograft tissue is slower compared to autograft due to the lower cellularity and no revascularization of allograft (Jackson et al. 1993). The mechanical properties of allografts for ligament are lower than autografts in terms of knee laxity, stress to failure, and postoperative traumatic rupture rate (Nagda et al. 2010; Scheffler et al. 2008).

10.3.3 Xenograft

Replacement of torn ligamentwith animal tissue, xenograft, has been attempted in less than 10 % of ACLR (Marx et al. 2003). The xenograft tissue is rigorously treated prior to implantation to reduce the immunologic side effects. Decellularization, enzymatic cleavage, glutaraldehyde cross-linking, and terminal sterilization by irradiation are the optimized treatments for xenograft reconstruction (Stone et al. 2007). These therapeutic techniques have the same advantages and disadvantages of allograft reconstruction. Immunological rejection and diseases transmission, however, are the serious drawbacks of xenograft replacement (Ekwueme et al. 2011).

10.3.4 Synthetic Ligament Implants

The high risk of donor site morbidity and disease transmission lead to the need to develop biocompatible and mechanically strong prostheses. Synthetic ligaments have been applied for ACLR since the 1970s. The initial satisfactions of synthetic prostheses are due to their lack of harvest site pathology, availability, accelerated rehabilitation period and significant strength (Legnani et al. 2010). Moreover, the less surgical operation is required, which is more convenient for the patient.

The artificial ligaments are categorized into three distinct groups: (1) prosthetic, (2) augmentation, and (3) scaffold. The prosthesis functions as a permanent implant due to their high mechanical properties. The augmentation device is bioresorbable and acts as a temporary stent to increase the initial strength of the graft (Mascarenhas and MacDonald 2008). The scaffold consists of a porous structure that stimulates tissue ingrowth and transforming loads to neotissue (Nishimoto et al. 2012).

Despite the merit performance of the grafts in their initial stages, osteoarthritis, synovitis, and rupture are their main complications post-implantation (Miller et al. 2006). Several types of ligament prostheses, therefore, have been withdrawn from the market due to their long-term drawbacks. The augmentation devices, on the other hand, did not show significant complications in early stages (Muren et al. 2003). Scaffolds are the third generation of implants, and their long-term drawbacks have not yet been studied completely.

The mechanical properties of various grafts applicable for ACL reconstruction are presented in Table 10.2. Biological grafts and an augmentation device (Leeds-Keio) have similar ultimate tensile strength compared to the human ACL. This resemblance in mechanical properties led to a lower rate of complications compared to other synthetic implants. The stability and risk of failure of these grafts for ACLR rely not only on the source and mechanical properties of grafts but also on their fixation method. In addition, the biological or synthetic graft is secured within the bone tunnel in femur and tibia. The attachment site, therefore, plays a significant role in graft performance.

10.4 Graft Fixation

Reproducing the mechanical and biological properties of the natural ligament is a critical issue in ACLR. The attachment sites, however, are more important especially after reconstruction. Native ligament as a soft tissue with high tensile strength attaches to the bone optimized for compressive

Table 10.2 Mechanical properties of various grafts in ACLR

Graft	Ultimate tensile stress (N)	Stiffness (N/mm)	Ultimate strain (%)	Ref
Human ACL[a]	2,160	242	11.6	Woo et al. (1991)
Human PT	1,784	210	–	Wilson et al. (1999)
Human STG	2,422	238	–	Wilson et al. (1999)
Gore-Tex™	5,300	322	9	Weitzel et al. (2002)
Dacron®	3,631	39	18.7	Hsu et al. (2007)
Leeds-Keio	2,200	200	35	Matsumoto and Fujikawa (2001)
Ligaid®	2,700	165	27	Christel (1994)
LAD®	1,730	56	22	Christel (1994)
Carbon fibre	660	230×10^9	1	Weitzel et al. (2002)
Trevira®	1,866	68.3	12.7	Letsch and Garcia-Schürmann (1993)

[a]*ACL* Anterior Cruciate Ligament, *PT* Patellar Tendon, *STG* Semitendinosus and gracilis

loadings. Their interface, known as enthesis, prevent the stress concentration, provide homeostasis and absorb load transmission (Woo et al. 1983). ACL enthesis is less than a centimeter (356–780 μm) and composed of gradient of cell phenotype, chemical composition and tissue organization (Wang et al. 2006). This gradient is diminished upon tunnel formation during ACL replacement operation. The attachment site, therefore, is contemplated as the weak link until graft-to-tunnel healing is obtained (Harvey et al. 2005). The postsurgical rehabilitation regimens require the immediate ability to regain the full range of motion, re-establishment of neuromuscular function, and weight bearing ability (Brand et al. 2000). Facilitating graft-tunnel healing and mimicking the bone-to-ligament interface, therefore, are the main objectives of graft fixation.

Following surgery, most of the constraints are applied to graft attachment site. Therefore, initial stability of reconstruction depends upon enthesis rather than graft structure (Beasley et al. 2005). The current fixation methods in ACL reconstruction are categorized into two distinct groups. An indirect fixation methods, such as Endo-buttons (Bartlett et al. 2001) and cross-pin fixation (Zantop et al. 2004) (Fig. 10.1a, c) suspends the graft within the bone tunnel in femur. In the direct fixation, on the other hand, the graft is compressed against the outer surface of the bone or the wall of bone tunnel (Hapa and Barber 2009). The interference screws, for instance, directly fix the graft to the femoral and/or tibial attachment sites (Fig. 10.1b, d, e). The proper stress distribution and total displacement of these screws have significant impacts on the graft fixation (Abdullah et al. 2012). Different metallic (Bourke et al. 2013; Drogset et al. 2010) and biodegradable polymers (Oh et al. 2006; Macarini et al. 2008; Cox et al. 2010) were used to fabricate an interference screw as a graft fixative. The bioabsorbable screws are comprised from composites of polymer and calcium phosphate particles. Rigidfix® and BioScrew®, for instance, were comprised from poly($_L$-lactic acid) (PLLA) and hydroxyapatite (Oh et al. 2006; Macarini et al. 2008). The mechanical properties of these fixative screws are comparable with their metal counterparts, within the range of 150–450 N of repetitive forces (Adam et al. 2004). Moreover, the application of polymeric screws reduces the risk of graft laceration or bone plug fracture and have an improved compatibility with magnetic resonance imaging without compromising the future revision surgery necessitated for removing of metal screws (Brand et al. 2000). The hydrolytic degradation profile of these screws, however, leads to the swelling issues in the insertion site due to local pH drop. The intrinsic non-degradable nature of the metallic fixation devices

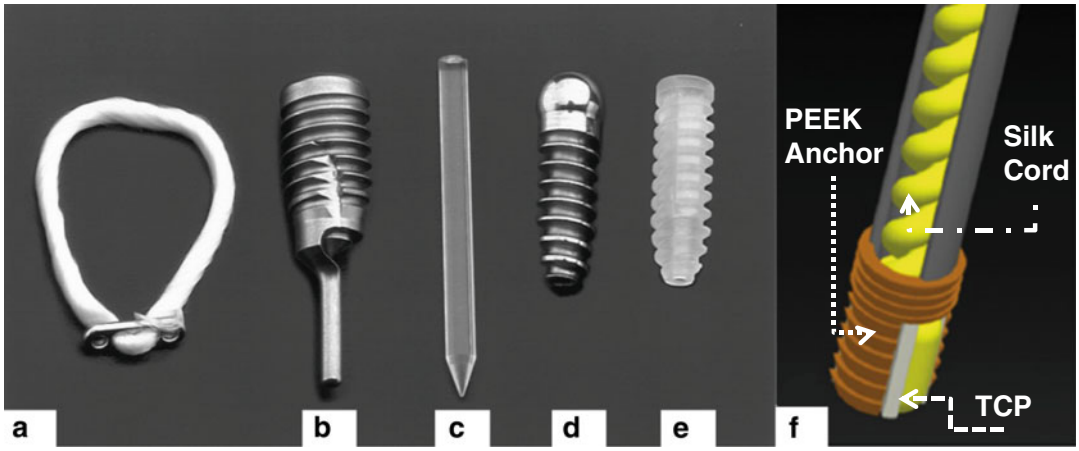

Fig. 10.1 The current fixation devices for ACLR: (**a**) EndoButton CL, (**b**) Bone Mulch™ Screw, (**c**) Rigidfix®, (**d**) BioRCI® screw, (**e**) Bioscrew® and (**f**) tricalcium phosphate (TCP) anchor

also leads to the over-time osteoporosis. These drawbacks are some of the factors for the low success rate of ACLR.

More recently, a novel off-the-shelf construct was fabricated for ligament enthesis. This acellular construct featured a silk cord enlaced upon a bioactive anchor (Li et al. 2014). The hierarchical structure of silk cord mimicked the mechanical performance of ligament (Li and Snedeker 2013). As shown in Fig. 10.1b this scaffold is then hooked around the hybrid anchor comprised of porous, H-shaped structure of tricalcium phosphate (TCP) housed with polyether ether ketone (PEEK). While TCP enhanced the osteoinductivity of the construct, PEEK housing protected it from mechanical damage. The teeth anchoring topography of this implant completely fitted within femoral bone tunnel during ACL reconstruction. In vitro assessment of mechanical properties revealed that there are no significant differences in graft elongation, slippage and pull-out strength of this implant with commercially available interference screws (Li et al. 2014). Three months *in vivo* studies in pig, moreover, verified the proper incorporation of the graft within bone tunnel and regeneration of four regions of the ligament to bone enthesis. Long-term *in vivo* assessments and preclinical evaluation of ligament regeneration still need to be accomplished prior to clinical trial and application for human ACL repair. The non-biodegradation nature of PEEK in this implant maybe leads to osteoporosis and limit its clinical application.

Despite the promising initial outcomes of ACL replacement, there are issues and complications entangled with commercially available grafts and fixations. The donor site morbidity and/or immune-rejection nature of biological grafts, and inadequate fatigue and creep properties of synthetic grafts are their main complications. The insufficient mechanical stability of graft fixations and formation of non-mineralized soft tissue within bone tunnels, moreover, are obstacles to full graft-to-bone integration. These limitations encourage scientists to find new methods to regenerate the ligament and its interference with bone. Tissue engineering is considered as a new approach, which might overcome some of the abovementioned issues. In the following session, we describe attempts towards engineering ligament tissue.

10.5 Tissue Engineering of Ligaments

Tissue engineering integrates proper cells and biological and/or mechanical stimulations, and scaffolds to regenerate the ligament (Altman et al. 2002a). The state-of-the-art of tissue engineering was entirely reviewed in many review

articles (Palsson and Bhatia 2004; Vacanti 2012; Mooney and Vacanti 1993; Chapekar 2000; Shieh and Vacanti 2005). This approach could regenerate the ligament tissue and facilitate its integration within the bone tunnel. The application of stem cell biology, materials science, the development of a dynamic *in vitro* culture system, recent progress and challenges in ligament tissue engineering are reviewed in this section.

10.5.1 Cell Sources

Cells play crucial roles in tissue regeneration and repair. They populate the scaffold and replicate native tissues. Their survival, phenotype, proliferation, differentiation, interaction with other cells, and generation of native ECM underline the success in tissue engineering (Ge et al. 2005a). The proper cells for ligament regeneration should meet some requirements including availability, rapid proliferation, differentiation into *in situ* cells, and vitality within the intra-articular environment of the knee (Arnsdorf et al. 2009). Primary fibroblasts from different origins, embryonic and adult stem cells were harvested and added to the scaffold to engineer ligament (Bellincampi et al. 1998). Fibroblasts, immunologically relevant cells, are frequently extracted from the skin, anterior cruciate and medial collateral ligaments (Cooper et al. 2006; Brune et al. 2007; Liu et al. 2008a). Mesenchymal stem cells derived from bone marrow (bMSC) (Pittenger et al. 2002), synovium (Liu et al. 2015), and ligament (Cheng et al. 2010) are adult cells with high rate of proliferation and collagen deposition for ligament tissue engineering. Therefore, they are recognized as a preferable cell source for engineering the ligament (Arthur et al. 2009). Their proliferation and differentiation, however, depend upon matrix composition, mechanical forces, cytokines, and growth factors (Engler et al. 2006; Marklein and Burdick 2010; Kloxin et al. 2010). The combination of MSC and primary ACL cells has also been attempted for ligament tissue engineering. For instance, Canseco et al. revealed that co-culturing of 50:50 ratio of MSC and primary ACL cells ratio the highest collagen I and tenascin-C expression and collagen I/III ratio (Canseco et al. 2012).

10.5.2 Growth Factors

Growth factorspromote sequential stages of inflammation, proliferation and remodeling (Moreau et al. 2005). Basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) have a significant influence on growth and differentiation of ligamentous cells (Moreau et al. 2006). Other growth factors including transforming growth factor (TGF-β1), bone morphogenetic protein (BMP) and insulin-like growth factor (IGF-1) stimulate collagen and proteoglycans synthesis and cellular proliferation (Hagerty et al. 2012).

The presence of growth factors at the site of injured ACL modifies matrix synthesis and cell proliferation, migration and differentiation. For instance, EGF enhances proliferation of epithelial and mesenchymal cells (Jason et al. 2004). PDGF, moreover, stimulates angiogenesis, cell proliferation and fibroblast mitogenesis that ultimately enhance ligament mechanical properties (Hee et al. 2012). The presence of angiogenic growth factors such as IGF-1 and bFGF promotes the proliferation of fibroblasts and myoblasts (Molloy et al. 2003). Despite the significant impacts of growth factors on cell differentiation, their application are limited due to their rapid inactivation and extremely short plasma half-life (Chan et al. 2000). Therefore, they are incorporated into scaffolds to prolong the duration of their activity (Whitaker et al. 2001).

10.5.3 Scaffolds

Scaffold is a temporary structure and logistic template for tissue engineering. It serves as "informational templates" to the cells, by patterning implementation, binding ligands and sustained release of cytokines (Ferreira et al. 2007). The ideal scaffold has a three-dimensional (3D) structure composed of biocompatible materials

with a controllable degradation profile. The degradation rate needs to be commensurate with neotissue formation while still maintaining the mechanical properties during the degradation period and tissue regeneration. The slow rate of ligament healing necessitates sufficient mechanical support for at least 3 months. Therefore, it is ideal to design a scaffold that retains half of its strength for a period of 6 months. The presence of interconnected pores with an average diameter in the range of 150–200 μm is critical for ligament repair (Goh et al. 2013). This interconnectivity enables sufficient mass transfer for nutrients and waste products and provides an appropriate environment for cell adhesion, proliferation and differentiation (Vieira et al. 2009). Several methods have been assessed to design biomimetic scaffolds for engineering of the ligament (Chen et al. 2003; Kardestuncer et al. 2006; Feng et al. 2007; Liu et al. 2009). Hydrogels, for instance, mimic the viscoelastic feature of ligament due to water absorption and releasing it under stress (Slaughter et al. 2009). However, the low mechanical properties and fast degradation profiles of hydrogels are not favorable for ligament tissue engineering (Nicodemus and Bryant 2008). This section provides an overview of biomaterials that have been assessed for ligament regeneration. Computer-based modeling of scaffolds to investigate their application for ligament engineering is beyond our scope and can be found in other papers (Laurent et al. 2011, 2012; Vieira et al. 2012; Naghashzargar et al. 2014). The various constructs designed for ligament engineering are presented in Table 10.3 with respect to their fabrication process, cell types, bioactive substrate and possible animal model. Most of these studies were based on *in vitro* or *in vivo* tests and have not yet approached to the stage of clinical trials.

10.5.3.1 Collagenous Structures

Fibrous collagen is the main component of dried ligament that has been used for ligament engineering (Goh et al. 2003; Freeman 2009a; Kwansa et al. 2010). In the 1990s, fibroblasts were harvested into crosslinked collagens in parallel for both *in vitro* and *in vivo* studies (Bellincampi et al. 1998; Cavallaro et al. 1994; Dunn et al. 1992, 1995). The fibroblast remained viable for prolonged periods and promoted neoligament remodeling (Bellincampi et al. 1998). However, the parallel arrangement of fibers with stress direction may cause a long-term failure due to fatigue, creep and abrasive wear (Dunn et al. 1995).

Composite formation of collagen fibers is an alternative strategy in ACL engineering. Dunn et al. were the pioneers to embed the collagen fibers within PLA or collagen matrix to find neoligament ingrowth (Dunn et al. 1997). Embedding collagen fibers into the collagen hydrogel was a further attempt to mimic native ligament (Gentleman et al. 2006). Despite significant improvement in mechanical properties of collagen-based composites, *in vivo* investigation in rabbit ACL led to approximately 50 % ruptures (Dunn et al. 1997). Moreover, batch-to-batch variability in combination with loosening the mechanical strength are the main drawbacks of collagenous scaffolds (Chvapil et al. 1993).

10.5.3.2 Silk Based Scaffolds

Silk is a biocompatible, fibrous biopolymer with intrinsic mechanical properties and slow degradation profile (Vepari and Kaplan 2007; Lu et al. 2011). Despite these superior properties, silk is covered with the self-assembled highly crystalline structure of sericin (Jiang et al. 2006; Wang et al. 2008). This protein, however, elicits immune response activity and must be removed from silk fibers for the biomedical application (Meinel et al. 2005; Panilaitis et al. 2003; Teuschl et al. 2014). Silk-based scaffold for engineered-ligament were fabricated in various forms (Horan et al. 2006). Braided or knitted silk fabrics were widely used for this application. The initial cell proliferation in braided or embroidered fabrics were successful (Seo et al. 2007). Their long-term applications, however, were limited due to poor tissue integration, and their inadequate abrasion and fatigue profile (Yahia 1997). The knitted scaffolds, on the contrary, possess suitable internal porosity for cell integration and proliferation (Wang et al. 2011). In this structure, cells were seeded within the gel system comprised of fibrin or collagen, which physically covered the knitted

Table 10.3 Application of different materials in ligament tissue engineering

Composition	Fabrication process[a;b;c]	Ref
Ligament application		
Cellulose	Partial dissolution[1;-;-]	Mathew et al. (2012a)
Cellulose-PAAm	Double-network[-;-;-]	Hagiwara et al. (2010)
Cellulose-collagen	Crosslinking[1,2;-;-]	Mathew et al. (2012b, 2013)
Collagen	Braiding-twisting[1;-;-], braiding-crosslinking[-;-;*I*], crosslinking[1,3,4;A,B;*II*], fibrous[5;-;-], fibrous-crosslinking1[,2,6;-;*III*], knitting[-;-;*IV*],lyophilisation[1;-;-],multilamellar[2;-;-]	Bellincampi et al. (1998), Walters et al. (2012), Robayo et al. (2011), Gigante et al. (2009), Cavallaro et al. (1994), Caruso and Dunn (2004, 2005), Dunn et al. (1992, 1995), Gentleman et al. (2003, 2006), Weadock et al. (1995), Enea et al. (2011, 2013), Nöth et al. (2005), Haddad-Weber et al. (2010), Henshaw et al. (2006), Chvapil et al. (1993), and Qiu et al. (2014)
Collagen-elastin	Electrospinning[1,7;-;-]	Mizutani et al. (2014)
Collagen-PLA	Crosslinking-matrix[-;-;*II*]	Dunn et al. (1997)
Collagen-PRP	Neutralisation[1;C;-], sponge[1;-;*I,V,VI*]	Murray et al. (2006a, b, 2007a, b), Fleming et al. (2009), Joshi et al. (2009), and Spindler et al. (2009)
PRP	Filling[-;B;*VI,VII*]	Valentí Nin et al. (2009) and Murray et al. (2009)
Fibronectin-TCPS	Coating[1,8;-;-]	Hannafin et al. (2006)
Silk	Fibrous[3;-;*III*], twisting[3;D;*I*], knitting[1,3;-;-], knitting-electrospinning[3;-;-], knitting-sponge[3,9;-;*III,VI*]	Li and Snedeker (2013), Liu et al. (2007, 2008a, b, 2015), Altman et al. (2002b), Horan et al. (2009a), Teh et al. (2011), and Fan et al. (2008a, 2009)
Silk-collagen	Knitting-sponge[3;-;*III*], fibrous[2;-;*III*]	Chen et al. (2008a), Panas et al. (2009), and Panas-Perez et al. (2013)
Silk-collagen-HA	Knitting-sponge[1,10;-;*V*]	Seo et al. (2009)
Silk-gelatin	Knitting-sponge[3;-;-], braiding-hydrogel[3;-;-]	Fan et al. (2008b, c)
Silk-RADA	Knitting-sponge-coating[3;-;-]	Chen et al. (2012)
Silk-RGD	Film[1,3;-;-], twisting[3;D,E,F;-]	Moreau et al. (2006, 2008) and Chen et al. (2003, 2006)
Silk-HA	Lyophilisation[3;-;-]	Garcia-Fuentes et al. (2009)
Silk-HAp	Knitting-sponge-coating[3,11,12;F;-]	He et al. (2012, 2013)
Silk-PLGA	Knitting-electrospinning[3;-;-],knitting-coating[3;D;-]	Toh et al. (2006) and Sahoo et al. (2010a, b)
Silk-PLGA-PLCL	Knitting-electrospinning[3;-;-]	Vaquette et al. (2010)
PEUUR	Electrospinning[11;-;-]	Cardwell et al. (2014)
PEUUR-PEGDA	Electrospinning-electrospraying[5;-;-]	Thayer et al. (2013)
Poly(α-hydroxy acids)	Electrospinning[1,3,5;D;-], electrospinning-braiding3[-;-], braiding[1,2,3,4,8,13;G;*III*], braiding-twisting[-;-;-], twisting[1,2,3;-;-], knitting-wrapping[3;-;*III*], knitting[-;-;-], knitting-electrospinning[3;-;-]	Cooper et al. (2005, 2006, 2007), Surrao et al. (2012a), Sahoo et al. (2006, 2010c), Barber et al. (2013), Van Eijk et al. (2004, 2008), Lu et al. (2005), Freeman et al. (2007), Heckmann et al. (2007), Kreja et al. (2012), Ge et al. (2005b, c), and Subramony et al. (2014)
PLLA-AC	Electrospinning[14;-;-]	Chen et al. (2014)
PLLA-collagen	Braiding-wrapping[-;D;*III*]	Kimura et al. (2008)
PLGA-collagen	Electrospinning[2;-;-], knitting-coating[3;-;-]	Full et al. (2015), and Sahoo et al. (2007)
PDTDDD-collagen	Braiding[-;-;*II*]	Tovar et al. (2012)
PLLA-PEGDA	Braiding-twisting-hydrogel[4;H;-]	Freeman et al. (2011)
PLA-chitosan	Braiding-coating[1;-;-]	Sarukawa et al. (2011)

(continued)

Table 10.3 (continued)

Composition	Fabrication process[a;b;c]	Ref
PCLDLLA-chitosan	Electrospinning-hydrogel[1;-;-]	Hayami et al. (2010)
PE-chitosan	Nonwoven-coating[-;-;III]	Kawai et al. (2010)
Chitosan-alginate	Wet spinning[4;-;-]	Majima et al. (2005)
Chitosan-HA	Wet spinning[4,13;-;III,IV]	Funakoshi et al. (2005), Majima et al. (2007), and Irie et al. (2011)
PDTEC	Fibrous tightening[2;-;-]	Bourke et al. (2004)
HYAFF-11®	Knitting[3;-;-]	Cristino et al. (2005)
PU	Electrospinning[1;-;-]	Lee et al. (2005)
Interface		
Chondroitin sulfate-collagen	Lyophilisation-crosslinking[1;D,E,F,I;-]	Murray et al. (2003)
PEGDA-HAp-RGD	Crosslinking (PC)[11;-;-]	Paxton et al. (2009)
Brushite	Anchor formation[13;-;-]	Paxton et al. (2010a)
Brunshite-fibrinogen	Anchor formation[15;-;-]	Paxton et al. (2010b)
PCL-β-TCP	Extrusion-electrospinning[16;-;-]	Erisken et al. (2008, 2010)
PCL-HAP-PEU	Electrospinning[16;-;-]	Samavedi et al. (2011)
PLCL	Extrusion-salt leaching[4;A;IV]	Lee et al. (2011)
PLGA-PG	Electrospinning-coating[16;-;-]	Li et al. (2009)
Collagen-PLL	Hydrogel-coating[11;J;IV]	Phillips et al. (2008)
PLGA/silk-alginate	Incorporating in hydrogel[3;D,K;-]	Wang et al. (2009)
PMGI	Electrospinning[14;B;-]	Shi et al. (2010)
HAP-PCL-PUUR	Co-electrospinning[3;-;-]	Samavedi et al. (2012)
PCL-ACP	Co-electrospinning[16;-;-]	Ramalingam et al. (2012)
PLGA-45S5 BG	Strata[1,17,18,19;-;IV]	Lu et al. (2003), Spalazzi et al. (2006, 2008a, b), and Wang et al. (2007)
PDMS-PEGDA	Strata-crosslinking[20;-;-]	Munoz-Pinto et al. (2010)
Augmentations		
PUUR	Woven band[-;-;III,VI], wet spinning[-;-;-]	Liljensten et al. (2002) and Gisselfält et al. (2002)
PLLA-PLGA	Braiding[-;-;II]	Dürselen et al. (2006)

[a]Cell Types: **1**. *Anterior Cruciate Ligament Fibroblast*; **2**. *Dermal Fibroblast*; **3**. *bone Marrow Stem Cell*; **4**. *Patellar Tendon Fibroblast*; **5**. *Mesenchymal Stem Cell*; **6**. *CRL-1213*; **7**. *Periodontal Ligament Fibroblast*; **8**. *Medial Collateral Ligament Fibroblast*; **9**. *Synovium Marrow Stem Cell*; **10**. *T-Lymphocyte*; **11**. *Primary Fibroblast*; **12**. *Primary Osteoblast*; **13**. *Achilles Tendon Fibroblast*; **14**. *NIH3T3*; **15**. *Embryonic Tendon Fibroblast*; **16**. *MC3T3*; **17**. *Humeral Trabecular Bone Osteoblast*; **18**. *Cortical Bone Osteoblast*; **19**. *SaoS-2*; **20**. *Calvarial Osteoblast*

[b]Bioactive Substrates: **A**. *Bone Morphogenetic Protein*; **B**. *Growth and Differentiation Factors*; **C**. *Thrombin*; **D**. *Basic Fibroblast Growth Factor*; **E**. *Epidermal Growth Factor*; **F**. *Transferring Growth Factor-β*; **G**. *Fibronectin*; **H**. *Bovine Serum Albumin*; **I**. *Platelet Derived Growth Factor*; **J**. *Runx2/Cbfa7*; **K**. *Insulin-like Growth Factor*

[c]Animal Model: **I**. *Goat*; **II**. *Sheep*; **III**. *Rabbit*; **IV**. *Rat*; **V**. *Dog*; **VI**. *Pig*; **VII**. *Human*

scaffold (Jockenhoevel et al. 2001). The dissociation of covered gel from scaffold under dynamic loads (Ouyang et al. 2005) and nutrient transmission issues (Charles et al. 1998) are the main drawbacks of these scaffolds for ligament tissue engineering. In a series of studies supervised by Goh, this issue was overcome via rolling microporous fiber silk membrane around knitted silk (Peh et al. 2007; Teh et al. 2009). The microporous structure filled up the openings of knitted scaffold and formed a 3D architecture suitable for ACL engineering (Liu et al. 2008b). The 24 weeks implantation of MSCs-seeded scaffolds into rabbits (Fan et al. 2008a) and pigs (Fan et al. 2009) revealed direct and indirect entheses, respectively. This microporous structure, how-

ever, presents several limitations. It neither possesses osteoinductive features in ligament-to-bone interfaces, nor it mimics the ligament microenvironment. In order to elaborate osteoconductivity and osteoinductivity of these structures, it was attempted to deposit hydroxyapatite on silk hybrid gels (He et al. 2013). Further trilineage co-culturing of osteoblast, bMSC and fibroblast cells on different sections of this hybrid scaffold resulted in the formaiton of ligament-to-bone interface (He et al. 2012). In this hybrid scaffold, the knitted silk scaffold withstood mechanical loads, and hydroxyapatite coating might have been responsible for bone-to-bone healing. The cellular gradient of ligament enthesis was mimicked upon trilineage co-culturing of MSC (He et al. 2012). This approach is still under investigation, and no *in vivo* evaluation has been assessed.

A composite of silk and collagen/gelatin matrices with enhanced biocompatibility have also been prepared for ligament engineering (Hardy and Scheibel 2010; Hardy et al. 2008). Immersion of knitted silk into collagen sponge regulates collagen expression and fibril assembly in engineered-ligament of rabbit (Chen et al. 2008a). Furthermore, the proliferation of human ligament fibroblasts was investigated upon collagen-silk composite (Seo et al. 2009; Fan et al. 2008b, c). This hybrid was composed of knitted silk coated with collagen membrane. The collagen membrane consisted of hyaluronan and chondroitin-6-sulfate in its structure. The *in vivo* study in dog models revealed enhancement of angiogenicity and cell migration.

The novel design strategy for engineered-ligament relies upon mimicking ACL geometry and kinematics. ACL equally distributes the applied load through individual fiber bundles and stabilizes the knee joint. At the same time, its fiber bundles keep their own isometric conditions (Woo et al. 2005). This structure was resembled by incorporation of bFGF into the fibrous scaffold to design SeriACL™ graft.

10.5.3.3 Seri-ACL™

The SeriACL™ (Serica Technologies, Medford, MA, USA) graft is a transitory scaffold fabricated from silk fibroin (Horan et al. 2009a). Bundles of silk fibers were wound into strands and cords to form the matrix. This structure resembles collagen fiber hierarchy to form fascicles and ligament (Silver et al. 2003). The twisted fiber architecture comprised of 208 individual bundles with a diameter less than 350 μm to overcome mass transport limitations. In this system, nutrient and metabolites can passively diffuse into fibers. These 208 bundles displaced equally across the cross-section of each tunnel. The SeriACL™ organizes four-bundle macrostructure when it is folded about its longitudinal and transverse axes. Moreover, it provides adequate void volume for cell and tissue infiltration and fits within an 8-mm diameter bone tunnel.

The graft withstands the yield of over 1840 N and ultimate tensile strength of 3960 N. In addition, its stiffness is high enough to stabilize the knee yet low enough for preventing stress shielding. Moreover, it resembled the previously reported properties of ACL (Laurencin and Freeman 2005). The immune response of small and large animals to the SeriACL™ graft revealed that it did not induce a hypersensitivity reaction and synovitis after 12 months (Horan et al. 2009b). In addition, it did not trigger acute inflammation based on assessments of pain and swelling of the knee. The neoligament formation, moreover, was evidenced by the inability to identify the original silk matrix after a year of post-operation (Altman et al. 2008). The SeriACL™ graft is currently under clinical trial investigation in Europe. However, no data has been released for the clinical studies yet.

10.5.3.4 Sugar-Based Scaffolds

Cellulose and other sugar-based polymers have been contemplated for regeneration of ligament (Silva et al. 2010). Incorporation of cellulose into biological or synthetic hydrogels elevates their mechanical properties for ligament tissue engineering application (Mathew et al. 2013). For instance, cellulose/polyacrylamide hydrogel formed upon double-network technique sustains both high elongation and compression (Hagiwara et al. 2010). This hydrogel revealed similar mechanical properties compared to the natural

ligament. However, its biological performance has not been evaluated yet. The combination of cellulose nanofiber and collagen fibrils followed by crosslinking of collagen solution have the promising mechanical performance for ligament (Mathew et al. 2012b). For instance, crosslinking of nanocomposites contained 50 % cellulose nanofiber increased the strength of collagen fibrils form 56.2 ± 12.8 MPa to 150.6 ± 12.3 MPa. The nanocomposite was highly reinforced and stabilized due to strong physical entanglement between biopolymers as well as the chemical interaction of collagen matrix and fibrils (Mathew et al. 2012b). In addition to enhancement of mechanical properties upon composite formation, application of non-toxic crosslinking agent such as genipin increased biocompatibility of engineered ligament (Mathew et al. 2013).

Polysaccharides such as hyaluronan and alginate have a similar chemical structure with glycosaminoglycan presented in ligament (Jianqi et al. 2002). The implantation of braided scaffolds comprised of these biopolymers in small animals revealed significant enhancement in collagen synthesis and improvement in mechanical properties (Majima et al. 2007; Irie et al. 2011). However, it is necessary to regulate their fabrication process to increase fibroblast invasion and angiogenicity to assess biodegradability and biocompatibility of scaffolds (Irie et al. 2011).

10.5.3.5 Synthetic Structures

Synthetic polymers with biodegradable and biocompatible features are commercially available. Their tailorable mechanical properties introduce them as appealing materials for scaffold fabrication. These synthetic scaffolds present plastic deformation and resemble intrinsic mechanical properties of the ligament. Therefore, they seem as promising candidates for engineered ligament. Several scaffolds have been fabricated from PLLA, poly(glycolic acid) (PGA) and their copolymers, and poly(ester urethane urea) (PUUR) upon various fabrication techniques including electrospinning (Surrao et al. 2012a; Full et al. 2015; Hayami et al. 2010), braiding (Cooper et al. 2005; Sarukawa et al. 2011) and knitting (Ge et al. 2005b, c).

Electrospun nanofibers mimic ECM structure upon their high surface area to volume ratio (Ashammakhi et al. 2012), which promotes cell attachment and proliferation. Besides, crimp pattern on their surface resulted in acquiring similar magnitudes of mechanical strength for toe region in native ACL (Surrao et al. 2012b). Therefore, electrospun fabrics were assessed as feasible scaffolds for engineering ligament (Cardwell et al. 2014; Surrao et al. 2012a). Different orientation of electrospun mats were fabricated from PUUR in various concentration and seeded via murine MSC line (Cardwell et al. 2014). This variation in concentration led to the production of fibers with different diameters and consequently affected the MSC differentiation into ligament lineage. After 2 weeks, the amount of expressed collagen and decorin were significantly higher within larger-sized fibers. Fiber alignment, on the other hand, did not have a significant effect on cell differentiation (Cardwell et al. 2014). In addition to application of plain electrospun fabrics for engineering ligament, embedding the electrospun fibers of poly(ε-caprolactone-*co*-$_{\text{L,D-lactide}}$) (PCLDLLA) into methacrylated glycol chitosan hydrogel replicated geometric microenvironment of ligament (Hayami et al. 2010). While electrospun fibers withstood mechanical loads, the chitosan-based hydrogel facilitated cell distribution and proliferation.

The aligned electrospun fibers of PLLA were braided to form a template for enhancing the proliferation and tenogenic differentiation of human MSC (Barber et al. 2011). In other attempts, yarns of PLLA or PLGA were braided to comprise three regions of bone attachment ends and intra-articular zone (Cooper et al. 2005; Lu et al. 2005). This heterogeneous structure enhanced cell proliferation and tissue ingrowth through rabbit's ACL (Cooper et al. 2007). The architectural, mechanical and biological properties of braided scaffolds are significantly relied upon the amount of fibers and number of bundles (Barber et al. 2011; Cooper et al. 2005). In another study, it is shown that the braiding angle has an impact on pore size, surface area, and tissue integration (Freeman 2009b). The higher braiding angle at the insertion points leads to small pore size and

low porosity that resist wear and increase tissue integration.

The braided scaffold has robust mechanical properties and enhances scaffold handling (Tamayol et al. 2013) while the twisted structure replicates the crimped pattern of the native ligament (Freeman et al. 2009). Therefore, the combination of these fabrication techniques may lead to a scaffold with promising mechanical properties resembling ligament architecture (Freeman et al. 2011). In order to fabricate the braided-twisted scaffold, extruded PLLA fibers were collected as nine groups of six fibers. Each group was twisted in a counter-clockwise direction to form a fiber bundle. Afterwards, three bundles were twisted in a counter-clockwise direction around one another to form a yarn. These three yarns were then braided together to form a scaffold (Freeman et al. 2007). Recently, extruded collagen fibers were braided-twisted to improve its mechanical properties. The ductility modification of collagenous scaffolds, however, is inevitable for its preclinical tests (Walters et al. 2012). Despite appealing outcomes of twisting and braiding techniques, the ideal fiber materials, diameters, spacing and angles still remain unresolved (Kuo et al. 2010).

Knitting the microfibers is another strategy for the fabrication of interconnected porous scaffold applicable for ligament repair. For instance, knitting of PLLA-PLGA yarns developed a construct with reliable mechanical properties within 20 weeks *in vitro* degradation (Ge et al. 2005c). The scaffold was further seeded via MSC and wrapped using fascia. Upon 20 weeks implantation into rabbit model, collagen synthesis was significantly promoted, and stronger construct were produced (Ge et al. 2005b). Despite high interconnectivity of knitted scaffolds, synthesis of collagen was imperative to promote cell attachment and proliferation. The application of knitted scaffolds, however, is still infancy and has not yet been approved for clinical trials.

10.5.3.6 Interface Tissue Engineering

The integration of engineered ligament into bone tunnels is still concerned as a major issue. The current fixation methods do not provide the biomimetic properties of enthesis. Tissue engineering approaches have been attempted to fabricate structures with a gradient in chemical composition and cellular contents. Two separate paradigms were established to engineer ligament enthesis. In the first approach, the insertion site was constructed upon stratified structures cultured via separate cell types (Spalazzi et al. 2006). These different cells would further initiate cell-mediated metaplasia and lead to natural enthesis-like tissue (Wang et al. 2007). The second paradigm is regenerating of enthesis upon biochemically and/or mechanically stimulation of MSC to promote local cell differentiation (Wang et al. 2009; Shi et al. 2010).

The strata constructs were composed form three phases cultured with various mature cells. The knitted mesh composed from PLGA (10:90) was cultured via fibroblast cells to mimic ligament region. The bony side, on the other hands, was constructed from sintered PLGA (85:15) and 45S5 Bioglass® microspheres followed by culturing with osteoblasts. The intermediate region, however, was comprised from sintered PLGA microspheres and used as an arid region (Spalazzi et al. 2006). This triphasic stratum enhanced proliferation of osteoblast and fibroblast and their migration into interface phase. The desired fibrocartilaginous region, however, was not recognized through this phase (Spalazzi et al. 2006). This drawback was further addressed upon incorporation of chondrocytes within interface phase to regenerate fibrocartilage region within strata (Spalazzi et al. 2008a). This fibrocartilage region, however, was not fully localized to the intended middle phase of strata.

Biochemical stimuli of MSC for promoting local differentiation was accomplished upon continuously graded scaffolds (Phillips et al. 2008). These gradients were fabricated upon incorporation of calcium phosphate components into electrospun fibers (Erisken et al. 2008, 2010; Li et al. 2009; Zhang et al. 2012). For instance, the nanohydroxyapatite (nHAP) particles were dispersed into poly(ε-caprolactone) solution and went through co-electrospinning with poly(ester urethane urea). The electrospun fibers were then treated with simulated body fluid to fabricate

Table 10.4 The comparison of different components of various bioreactor

Component	Advantages	Disadvantages	Ref
Actuator			
Pneumatic	Ease of maintenance, cleanliness, low cost, high power-to-weight ratio	High friction, low sensitivity slow response to input signal, low accuracy (~ ±0.1 mm)	van Varseveld and Bone (1997)
Linear motor	High speed, high accuracy (~ ±1 μm)	High cost	Van Den Braembussche et al. (1996)
Step motor-ball screws	Adequate accuracy (~ ±5 μm), high loading capacity, suitable for multichamber-shared loading system		Altman et al. (2002c)
Culture chamber			
Integrated	Culturing multiple samples	High risk of contamination	Butler et al. (2009a)
Separated	Reduced cross-contamination, control of independent environment	Complex design, expensive manufacturing	Parent et al. (2011)
Air exchange			
Hydrophobic filter leak	Adequate gas exchange	High risk of contamination	Webb et al. (2006)
Labyrinth channel	Elimination of contamination	Dependency on medium circulation	Parent et al. (2011)

mineral gradient (Samavedi et al. 2011). Upon proliferation of bMSC cells on the graded scaffold, the formation of phenotypic gradient promoted the regeneration of ligament enthesis (Samavedi et al. 2012). Despite mimicking gradient compositions of ligament interface, these structures are still in their infancy, and more investigations are required to prior to the preclinical application.

10.5.4 Bioreactors

The in vitro cell culturing of engineered scaffolds is usually performed in two-dimensional (2D) cultivation systems. They are disposable, operator-friendly and economically effective. However, they endure from low cell seeding efficiencies (Martin et al. 2004), operator-dependent nature and non-uniform cell distribution (Wendt et al. 2003). Moreover, the lack of controls on the physiological environment such as pH and oxygen is their main drawbacks and make severe restrictions (Martin and Vermette 2005). These restrictions can be addressed upon providing an *in vitro* dynamic environment and regulating the biological parameters (Freed et al. 2006). Bioreactor creates the microenvironment experienced by cells *in vivo* while allowing cellular proliferation, differentiation and matrix production in an in vitro mechanical environment (Oragui et al. 2011).

Various types of bioreactors such as spinner flasks, flow perfusion, pulsatile flow reactor, and rotating wall vessel enhanced the proliferation of different cell types (Wendt et al. 2009). Despite the type of bioreactors, they are mainly constructed from actuator and culture chamber to provide various mechanical stimulation and controllable microenvironment, respectively (Naveen Kumar et al. 2011). The advantage and disadvantages of different components are compared in Table 10.4. Other parts of bioreactors include incubator, medium circulation, monitoring, feedback, and medium analysis systems, depending on the operational requirements.

The mechanical stimulation of cell-cultured constructs regulates cellular processes including cell alignment, proliferation and ECM synthesis (Butler et al. 2009b). In the case of engineered ligament, multidimensional strains were applied to the scaffold for mimicking the physiological movement of ACL and enhancing cell proliferation (Kahn et al. 2008). This mechanical stimulus

further induces cell differentiation, proliferation (Chen et al. 2008b) and remodeling of ACL fibroblast cells (Tetsunaga et al. 2009). These regulations, however, are highly dependent upon the type, magnitude and duration of mechanical stimulation (Benhardt and Cosgriff-Hernandez 2009). For instance, upon application of static loads at different times, various cell responses were detected. The braided PLGA went through mechanical stimuli during seeding, immediately after seeding, and 2 days post-seeding (van Eijk et al. 2008). The applied loads significantly enhanced cell differentiation and matrix production in a short period of times. The highest amount of collagen production and DNA content, however, were found in unloaded scaffolds in 21-days post-seeding due to their higher cell densities.

Despite promising outcomes of bioreactors in pre-clinical evaluations, only two systems have been commercialized for ligament engineering. The Bose ElectroForce® BioDynamic® test instrument (Bose®) and LigaGen system are applicable for ligament and other orthopedics tissue engineering. Based upon information revealed by manufacturers, the Bose® instrument (Minnesota, MN, USA) provides a programmable uniaxial stretching stimulation with a controllable circulation environment for engineered ligament (Bose Electro Force Bio Dynamic Instrument). Distinguishing the real-time force-displacement curve of the scaffold is another benefit of this system. The LigaGen system (Minnetonka, MN, USA), on the other hands, is an incubator-compatible bioreactor and provides a controllable physiological support system for cell growth under multidimensional strains (LigaGen Ligament Tendon Bioreactor). Real-time monitoring of the mechanical properties of scaffold during culture period is the main advantage of this bioreactor. For instance, in a study conducted by Angelidis, acellularized tendons were cultured by MSC and incubated in LigaGen instrument (Angelidis et al. 2010). Five days application of cyclic strains on these constructs significantly elevated their tensile strength and elastic modulus (Angelidis et al. 2010). Despite the promising effects of commercial bioreactors on mechanical properties and cellular proliferation of engineered ligament, their high cost and the burden of providing rotational and tensile loadings are major issues for their limited applications (Wang et al. 2012).

10.5.5 Animal Models

The ultimate aim of tissue engineering is a regeneration of human tissue. Upon physicomechanical characterization of the fabricated scaffold, their biocompatibility will be assessed *in vitro* (Doroski et al. 2007). The proper scaffold with promising *in vitro* outcomes them be implanted into animal models. Rabbit, pig, dog, goat, and sheep are selected animals, which have been hosted for ACL constructs (Carpenter and Hankenson 2004). These animals possess compatible anatomic structures and simulate the pathologic conditions of human ACL (Jackson et al. 1987a). The outcomes of animal studies, however, are highly species dependent and do not entirely resemble human performance (Jackson et al. 1987b; Xerogeanes et al. 1998).

10.6 Conclusions

The anterior cruciate ligament is a vulnerable tissue in the knee joint. The poor intrinsic healing capacity of ACL leads to surgical treatment. Currently, there is no ideal graft for ACLR without any complications. The standard graft should reproduce biomechanics of native ACL with reliable initial fixation. Possession of low risk of disease transmission and donor site morbidity are the most intrinsic parameters for this graft. Substitution of torn ACL with biological graft (i.e. autogenic or allogenic), however, is currently the most successful technique in ACLR. Their success rates of autogenous grafts are more than 85 % in long-term clinical studies. The donor site morbidity and feasibility of disease transmission, however, are the main drawbacks of biological grafts. Moreover, most of the patients do not return to pre-injury level of activity, due to structural differences between the

graft and native ACL. Implantation of non-degradable prostheses is significantly hindered as a consequence of their high rate of failure and synovitis.

Tissue engineering is in its infancy to restore, maintain and improve ACL function. Fibrous scaffolds composed of natural or synthetic polymers were cultured via ligament cells to resemble the performance of native ACL. Some of these constructs have been implanted into animal models and shown promising regeneration of ACL. Their clinical evaluation, however, has not been assessed for these engineered ligaments. The *in vitro* outcomes of braided constructs fabricated from synthetic or natural polymer, approaches undertaken to address the issue of bone interface and integration, and advances in specific bioreactor design for *in situ* mechanical stimulation of engineered ligament are promising. Further development in these domains may resolve the issue of ACL by complete regeneration of this tissue.

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Hard-Soft Tissue Interface Engineering

11

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Abstract

The musculoskeletal system is comprised of three distinct tissue categories: structural mineralized tissues, actuating muscular soft tissues, and connective tissues. Where connective tissues – ligament, tendon and cartilage – meet with bones, a graded interface in mechanical properties occurs that allows the transmission of load without creating stress concentrations that would cause tissue damage. This interface typically occurs over less than 1 mm and contains a three order of magnitude difference in elastic stiffness, in addition to changes in cell type and growth factor concentrations among others. Like all engineered tissues, the replication of these interfaces requires the production of scaffolds that will provide chemical and mechanical cues, resulting in biologically accurate cellular differentiation. For interface tissues however, the scaffold must provide spatially graded chemical and mechanical cues over sub millimetre length scales. Naturally, this complicates the manufacture of the scaffolds and every stage of their subsequent cell seeding and growth, as each region has different optimal conditions. Given the higher degree of difficulty associated with replicating interface tissues compared to surrounding homogeneous tissues, it is likely that the development of complex musculoskeletal tissue systems will continue to be limited by the engineering of connective tissues interfaces with bone.

Keywords

Interface engineering • Ligament • Tendon • Tissue engineering • Scaffold • Mechanical properties

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11.1 Introduction

The interface between bone and soft tissues such as tendon, ligament or cartilage is characterized by a multiphasic interface containing spatial gradients in mechanical properties, structure and cell type. These gradients exist over a distance of less than 1 mm and serve to mediate load transfer between highly dissimilar tissues by minimizing stress concentrations. As mechanical mediators in the musculoskeletal system, the insertion sites of ligaments or tendons with bone, or the bone-cartilage (osteochondral) interface, have high incidences of acute and long-term injury respectively (United States Bone & Joint Initiative 2011). Principal sites of failure are the rotator cuff tendon (600,000 surgeries in the US per year (Yamaguchi 2011)), the anterior cruciate ligament (ACL) (100,000 surgeries in the US per year (AAOS 2014)), and osteoarthritis of the hip joint (580,000 surgeries in the US in 2013 (OECD 2013)). Additionally, current surgical repair techniques for many of these tissues are largely unsatisfactory as they do not regenerate the complex structure of the natural interface (Rodeo et al. 1993). Given the variety and prevalence of failures, repair or regeneration of interface tissues remains a significant clinical challenge.

This chapter begins by discussing the anatomy and materials involved in two distinct types of natural interface tissues: the ligament or tendon insertion site and the osteochondral interface. This will include a discussion of the challenges associated with determining the mechanical properties of these highly heterogeneous tissues. The bulk of the chapter contains an outline of specific strategies and methods employed when engineering interface tissues. Firstly, scaffold specific methods concerning manufacture, mechanics and composition will be considered. Secondly, exogenous factors in the system such as growth factors will be discussed. Thirdly, cellular factors concerning cell type and cell differentiation will be reviewed. Overall, the methods required and problems associated with generating biomimetic gradients will be considered for each of the factors influencing the tissue replication process.

11.2 Structure of Natural Interface Tissues

Connective tissues in the musculoskeletal system are subdivided into tendons (bone-muscle interactions in tension), ligaments (bone-bone interactions in tension) and cartilage (bone-bone interactions in compression). In this section we will discuss these tissues and their interface with mineralized tissue, primarily bone.

11.2.1 Ligament and Tendon Insertions

Bone is a stiff rigid connective tissue consisting of a highly organized extra cellular matrix (ECM) of type I collagen fibers, interconnected with stiffening hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) mineral deposits. Bone mineral is highly impure, containing 3 % by weight structural water (Yoder et al. 2012), and around 15 % substitution of carbonate (CO_3^{2-}) ions for either OH^- or PO^{4+} groups (Elliott 1994). This composite is maintained by an extensive vascular and nervous system along with the "osteo" group of cells: osteoclasts, osteoblasts and osteocytes. Bone's primary function is to support and protect the soft tissues surrounding it, while the central marrow maintains and produces populations of the blood cells. Ligaments and tendons are compliant, hydrated, connective tissues whose primary function is to transmit tensile forces between either two bones, or a bone and a muscle respectively. They consist of 86 wt% (dry) highly aligned type I collagen, assembled into a hierarchical structure of fibrils (Amiel et al. 1984). The remainder of the ligament or tendon is a proteoglycan matrix surrounding rows of fibroblast cells. The insertion site (enthesis) of the ligament or tendon into bone can be between 100 μm and 1 mm across (Evans et al. 1990; Genin et al. 2009) with a structure that can vary from ligament to ligament and even between two ends of the same ligament. From an anatomical perspective the insertion of ligaments or tendons into SCB) is subdivided into two broad designations: direct (fibrocartilagenous) entheses and indirect (fibrous) entheses.

The medial collateral ligament (MCL) insertion into the femur and the supraspinatus tendon insertion into the humerus are both typical examples of a direct, fibrocartilaginous enthesis. At direct insertions the deep fibers of the ligament or tendon join perpendicular to the bone surface through a multi-tissue fibrocartilagenous transition of four distinct yet continuous zones (Fig. 11.1). These are pure ligament or tendon, unmineralized fibrocartilage, mineralized fibrocartilage and subchondral bone (Yahia and Newman 1970; Benjamin et al. 1986).

Zone 1, ligament or tendon, consists of highly aligned collagen fibrils supporting fibroblasts with minimal vascularization and nerve supply as it approaches the enthesis. In zone 2, the tissue transforms into unmineralized fibrocartilage, where the cells become more rounded chondrocytes and the degree of collagen alignment lowers to 8° deviation from the tendon alignment (Genin et al. 2009). Zone 3, known as mineralized fibrocartilage, is histologically separated from zone 2 by the mineralization tidemark (Benjamin et al. 1986) but contains similar chondrocytic cell populations as in zone 2. Zone 4 is bone proper and hence is characterized by significant intrafibrillar mineral content, vascularization, a neural network, and the presence of the osteo group of cells to conduct bone remodelling.

The degree and location of mineralization across the two fibrocartilaginous regions of the enthesis is not completely conclusive. The classical view, stemming from histological staining, is that the mineral content increases abruptly at the clear visual mineralization tidemark. This is supported by SEM energy dispersive x-ray analysis (EDAX) of the bovine patellar tendon (Moffat et al. 2008), along with Fourier transform infrared spectroscopy imaging (FTIR-I) of the bovine (ACL) (Spalazzi et al. 2007). Conversely, Raman spectroscopy of the rat supraspinatus tendon insertion has shown a linearly increasing phosphate ion content, and hence mineral content, across the combined width of both fibrocartilaginous regions (Wopenka et al. 2008; Genin et al. 2009). Hence, a single rule governing mineral distribution across all entheses is not yet known, and the specific microstructure and composition must be considered on a tissue-by-tissue basis.

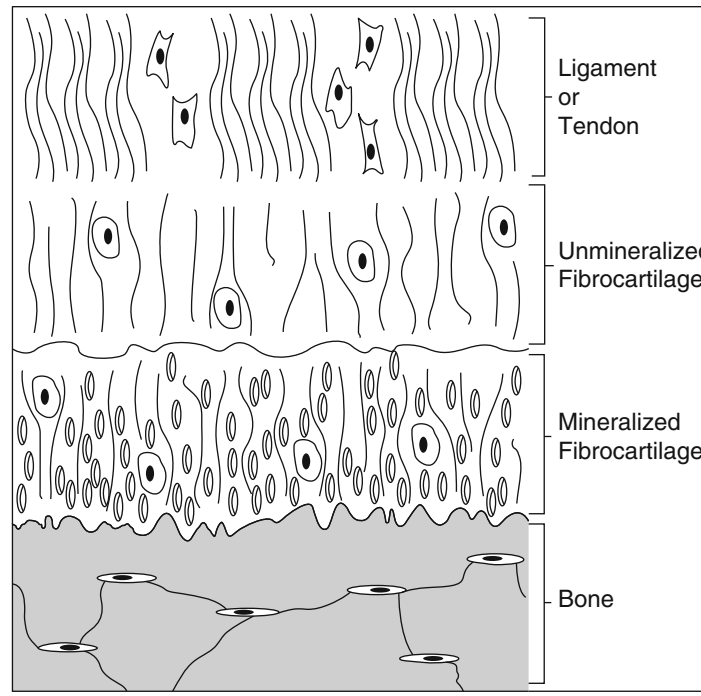

Fig. 11.1 The structure of a direct insertion of a ligament or tendon enthesis with bone. Ligament and tendon contain highly aligned, hierarchical collagen fibrils containing elongated fibroblast cells. In the unmineralized fibrocartilage the degree of collagen organization and alignment goes down and the cells become rounder chondrocytes. In the mineralized fibrocartilage the organic structure remains unchanged but hydroxyapatite crystals begin to fill the interfibrillar space. Finally, the mineralized fibrocartilage forms an interdigitated interface with subchondral bone containing mature osteocytes and the canaliculi that interconnect them

Indirect entheses differ from direct insertions in that the ligament or tendon fibres approach the bone surface at an acute angle where the outer fibers blend into the periosteum. The deep fibers penetrate directly into the outer SCB) via Sharpey's fibers (Lui et al. 2010) with little to no fibrocartilaginous transition and often just a mineralization tidemark separating the two tissues under microscopy (Walsh 2007). This results in a characteristically less mechanically robust interface for indirect insertions (Benjamin and Ralphs 1998). An example of an indirect insertion would include the tibial insertion of the MCL.

11.2.2 The Osteochondral Interface

Cartilage is an avascular, aneural tissue that lines joints to aid in low friction movement and serves to resist compressive forces in the joint. Cartilage consists of a network of type II collagen, filled with a proteoglycan containing fluid phase, with a large internal osmotic pressure. Volume constraints imposed by the collagen network on the proteins that occupy the fluid filled space cause the tissue to swell and load the collagen fibrils in tension (Mow et al. 1992).

The bone-cartilage junction is characterized by a zone of calcified cartilage (ZCC) approximately 100–200 μm across (Hunziker et al. 2002). Thus giving an interface structure that is similar in size to, but less differentiated than, the four region ligament insertions. The ZCC forms during postnatal development via calcification of the basal layer of the overlaying hyaline cartilage. The calcification front is known as the tidemark and can be clearly seen on histological sections as an undulating line (Fig. 11.2). Types II and V collagen that make up hyaline cartilage, extend across the ZCC and remain highly hydrated in this region and hence less densely packed compared to the neighbouring subchondral bone (SCB). It is the hydration, proteoglycans, collagen type and presence of hypertrophic chondrocytes that makes this region a calcified cartilage as opposed to bone that is lacking osteo group cells. Mineral particles in the ZCC are of a similar composition size and distribution to that found in bone (Zizak et al. 2003). The mineral content across the ZCC varies greatly from tissue to tissue. Because of the cartilage-like structure of this region, there is a large proportion of fluid filled interfibrillar space. When mineral is secreted from the SCB, some proportion of this fluid is displaced, allowing for large variations in the degree of mineralization, depending on the quantity of mineral secreted from SCB). Many studies have shown that the mineral concentration increases across the ZCC as you progress from the bone towards the tidemark with cartilage proper (Reid and Boyde 1987; Ferguson et al. 2003; Gupta et al. 2005). These studies should be considered as a rebuttal to the commonly held belief that the mineral concentration

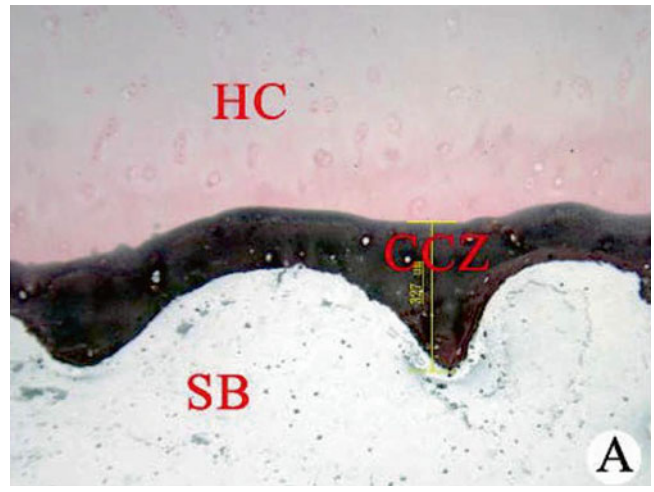

Fig. 11.2 The osteochondral interface occurring at the medial femoral condyle of a human knee. The three regions of the osteochondral interface: Hyaline Cartilage (*HC*), Calcified Cartilage Zone (*CCZ*) and the Subchondral Bone (*SB*) can be seen. Von Kossa staining of this tissue shows the mineralization in each region of the interface (scale bar in CCZ region: 327 μm) (Reproduced with permission from Zhang (2012))

forms a functional gradient across the ZCC. The width of the ZCC is limited on the bone side by the vascular front, beyond which bone remodelling by the group of osteo cells is limited, and on the cartilage side by the progression of the mineral tidemark into the unmineralized cartilage.

11.2.3 Mechanical Properties of Interface Tissues

Ligament or tendon entheses, or the osteochondral junction, serve to join two tissues with multiple orders of magnitude differences in mechanical properties. Ligament, tendon and cartilage have indentation moduli in the region 1–100 MPa (Hauch et al. 2009; McKee et al. 2011). Whereas the SCB) they anchor to has an indentation modulus three orders of magnitude larger, approximately 10 GPa (Bembey et al. 2006).

The challenge of mechanical integration of two materials with vastly disparate moduli arises in many engineering contexts. A common solution to this is to use functional gradients in microstructure and composition that give rise to similar functional gradients in mechanical properties. Thus, alleviating problems related to strength, stiffness and fracture at the material interface (Suresh and Mortenson 1998). Hence, a fundamental understanding of the structure-function relationships, and the mechanical properties they give rise to in the native tissue, is essential for successful tissue engineering solutions. Yet, direct measurement of the mechanical properties of either ligament or tendon entheses, or the osteochondral junction, is difficult due to their highly heterogeneous nature coupled with the small length scales involved.

Many of the issues associated with direct measurement of the interface have been bypassed with bulk compression or tensile experiments, where microscopic video analysis, or ultrasound scanning, was used to determine local strain across the different zones of the interface. Bulk compression of the enthesis of the bovine ACL showed a smooth and gradual increase in strain from the bone, through the mineralized and unmineralized fibrocartilage, to the pure ligament, suggesting a corresponding decrease in modulus over the same region (Moffat et al. 2008). Similar observations of the local strain distribution in the bovine ACL enthesis were obtained from ultrasound during cyclic tensile testing of a whole femur-ACL-tibia complex (Spalazzi et al. 2006b).

Direct measurement of mechanical properties of interface tissues at severely dehydrated conditions can be conducted using nanoindentation. Dehydrating the osteochondral region of human patellae (Gupta et al. 2005) caused the soft tissue to stiffen so significantly that nanoindentation mapping with sharp indenters (as would be conducted on a weld metal or ceramic composite) could be conducted. Their nanoindentation results, alongside quantitative backscattering SEM, revealed that the morphology of the gradients in mechanical properties closely match that of the mineral distribution. This is in accordance with the idea that the elastic properties of mineralized tissues can be predicted from the mineral volume fraction and will follow particulate composite bounds regardless of length scale (Oyen and Ko 2008; Ferguson 2009). Yet, the mechanism by which mineral concentration gives rise to tissue stiffening can be considered more complex than just a volume fraction composite bounds problem. It has also been suggested by theoretical analysis in Oyen et al. and simulations by Genin et al. that significant tissue stiffening occurs when the mineral concentration becomes sufficient that a mechanically continuous network of crystals has formed within the collagen fibers (Oyen et al. 2008; Genin et al. 2009).

In contrast to severely dehydrated tissues, direct measurement of mechanical properties at biologically relevant conditions is more challenging. Both Hauch et al. (2009) and Abraham and Haut Donahue (2013) conducted nanoindentation of hydrated meniscal attachment sites using a 150 μm radius spherical tip. Their line mapping suggests that the mechanical transition occurs over a wide 200 μm region spanning the mineralization tidemark. For this tissue, this is equivalent to approximately half way through the unmineralized fibrocartilage to half way through the mineralized fibrocartilage. However, these experimental conditions give an indenter-sample

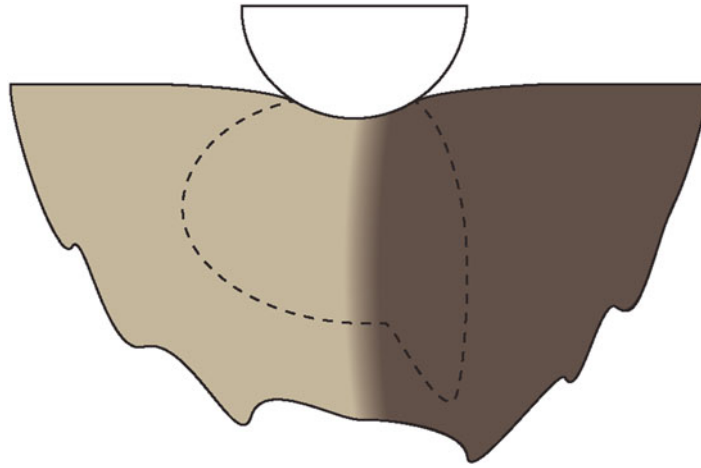

Fig. 11.3 The stress field occuring during indentation over a material containing a sharp transition in elastic modulus. The *dark right hand side* signifies the stiff material and the *lighter left hand side* the more compliant material. It can be seen that the stiffer side deforms out into the more compliant side during indentation. The *dashed line* represents a typical stress field under the indenter. It can be seen that if the contact patch is similar in size, or larger than the interface region, that the stress field extends outside the interface range being tested in a non-uniform manner

contact patch of 25–40 μm in diameter at SCB) and soft tissue respectively, thus inducing indentation specific artefacts in the results. Finite element simulations by this author show that indentation at length scales equivalent to material heterogeneities results in the transition region of modulus appearing wider than it actually is (Armitage and Oyen 2015). In Fig. 11.3, the dashed line shows an approximation of the stress field during indentation, for a material with a sharp transition in modulus. From this it can be seen that any indent close to the interface gives a modulus that is a non-uniform average of a region bridging the interface. Hence, when the indenter contact patch is of a similar size to, or larger than the material heterogeneity, the width of the transition region in the observed function of modulus appears much wider than the true function of modulus. As such, the true nature of the transition in mechanical properties of the ligament or tendon enthesis, or the osteochondral interface, remains inconclusive. This is due to a combination of lack of direct testing at native conditions and issues associated with testing heterogeneous materials with a probe of a similar size to the material heterogeneities.

11.3 Engineering of Tissue Interfaces

As discussed above, the interfaces between ligaments and tendons with bone, or cartilage with bone, are a key site of injury or long-term degradation in the musculoskeletal system. Additionally, these interfaces are some of the most complex tissues to regenerate due to their small size and high degree of heterogeneity in mechanical properties, microstructure, composition and cell phenotype. Tissue engineering methods have already been implemented for replication of many of the homogenous tissues involved in these interfaces such as bone (Amini et al. 2012), ligament (Yilgor et al. 2012), tendon (Butler et al. 2008) and cartilage (Moutos and Guilak 2008). Yet, in the current state of the field, biomimetic fixation to bone of the homogeneous tissue engineered ligament or tendon graft is a key obstacle to successful clinical implementation. Natively, the variety of distinct tissues that are present at the interface, interact with one another to provide the physiological function of mediating load transfer between vastly different parts of the musculoskeletal system. Thus the

aim of tissue engineering in this field is to replicate the structure-function relationships of the native tissue interfaces, and allow the engineered tissues to replicate the same physiological function. In this section we will discuss the strategies associated with replicating the natural structure-function relationships that allow this to happen. This includes material specific scaffold properties, cellular influences, and the application of external stimuli such as growth factors.

11.3.1 Scaffolds

Scaffolds in tissue engineering replace the function of the natural ECM and hence provide the medium through which cells, soluble proteins, mechanical cues and other external factors are delivered to the growing tissue. Additionally, the chemical, mechanical and topographical properties of the scaffold on a microscopic level, constitute a significant proportion of signalling in the cellular microenvironment, and hence have a large degree of control over cellular differentiation and growth. For the reproduction of an interface tissue the scaffold needs to mimic the gradient in structural organisation, biological factors and mechanical properties that are present in the natural tissue. There are two main approaches to the construction of scaffolds aiming to replicate the graduated properties of a natural interface tissue. The first is to create a number of discrete phases that each mimic a single region of the natural tissue, these can then be either chemically or mechanically joined to create an interface scaffold. The second option is to produce a single mass of scaffold material that is graded during manufacture to replicate an interface tissue.

Discrete scaffolds, made of a number of separate homogeneous scaffolds that are then joined together, are the easiest and most obvious option for multiphase scaffolds to replicate interface tissues. Examples of this for osteochondral interface reproduction include: (Schaefer et al. 2000), where seeded and cultured bone and cartilage scaffolds were sutured together to create an interface tissue; or (Niederauer et al. 2000) where polymer bone and cartilage scaffolds were glued together prior to culture. Yet, if significant care is not taken over the joining method then issues arise with scaffolds created in this way. The microstructure of the interface between regions of discretely joined scaffolds can result in a lack of interconnectivity between the two porous networks, inhibiting cellular infiltration, nutrient transport and cell-cell signalling between the two populations. Signalling between the cell populations in different regions of the growing tissue is known to be important for regulating tissue growth and viability in the ligament enthesis (Jiang et al. 2005; Wang et al. 2007). Furthermore, the primary *in situ* function of interface tissues is to mediate the joining of highly dissimilar materials. Hence, mechanical or chemical joining of discrete tissues in an abrupt manner results in a lack of mechanical integration at the interface, that is unlikely to adequately replace the natural biomechanical function in this region.

A gold standard example of a ligament enthesis scaffold, where distinct regions have been interconnected to give a graded structure, can be found in (Spalazzi et al. 2006a, 2008). In this work, three phases were individually synthesized as follows: a fibrous poly(lactic-co-glycolic acid) (PLGA) 10:90 knitted mesh for ligament fibroblast culture; a sintered network of PLGA 85:15 microspheres for chondrocyte culture of the fibrocartilaginous regions; and a sintered network of PLGA 85:15 and bioactive glass microspheres for osteoblast culture of a bony region. These three phases were sintered together to maintain a degree of similarity and structural continuity throughout the scaffold (PLA continuity at the ligament-fibrocartilaginous interface and PLGA continuity at the fibrocartilaginous-osseous interface). Subsequently the scaffold was tri-cultured in a subcutaneous rat model with fibroblast, chondrocyte and osteoblast cell lines pre-seeded in each region respectively. This resulted in mineralization localized to the osseous scaffold phase, and a distribution of collagen types that mimicked those found in natural tissue (Wang et al. 2006).

Continuously graded scaffolds, or those consisting of distinct regions that have been fully interconnected and integrated with one another prior to cell seeding, are more complex to produce but alleviate many of the issues with discrete scaffolds. Within these scaffolds gradients in pore size, mechanical properties and scaffold chemistry can all be used to influence region specific cell proliferation, or differentiation, to induce distinct localized tissue types. Yet, all of these factors are highly interdependent. Microscopic matrix stiffness, as felt in the immediate cellular environment is dependent on polymer choice, which affects surface chemistry. Pore size will affect macroscopic material properties and permeability, and hence influence fluid flow and cell-cell signalling within the scaffold. Hence, researchers must take significant care during scaffold design, as many symbiotic parameters must be prioritized simultaneously. Fabrication methods for continuously graded scaffolds will be discussed later.

11.3.1.1 Scaffold Properties

Pore size has been shown to be important for cell proliferation, with each of the primary cell types involved in interface tissues having an ideal range of pore sizes for optimal tissue growth. In general, fibroblasts and chondrocytes favour smaller pores compared to osteo group cells that favour larger pores. Specifically, fibroblasts and chondrocytes proliferate well on the surface of substrates with pores on the order of 10 μm but struggle to penetrate the bulk of the scaffold (Nehrer et al. 1997; Bhardwaj et al. 2001; Lowery et al. 2010). Scaffolds with pores in the range 70–120 μm have been observed to promote both chondrocyte and fibroblast proliferation throughout the scaffold pore structure (Salem et al. 2002; Griffon et al. 2006). In bone scaffolds the minimum pore size as determined by cell sizes is approximately 100 μm. Yet, pore size being approximately equal to cell size results in a lack of vascularization and hypoxic conditions, resulting in many papers concluding that osteogenesis is best served by pore sizes greater than 300 μm (Karageorgiou and Kaplan 2005; Murphy et al. 2010). However, an upper functional limit is also placed on pore size by the mechanical integrity of the scaffold, which is often desired to be stiffest in bony regions. Gradients in pore size through an interface scaffold can be achieved using a number of techniques such as freeze drying, graded hydrogels or functionally graded electrospinning, all of which will be discussed herein.

Mechanical properties of interface tissue engineering scaffolds are important factors that help to regulate cell phenotype expression (stem cells) or culture viability (bi or tri-culture of pre-differentiated cell lines). Scaffold mechanical properties can be considered on two length scales. On a microscopic scale (tens of microns in the immediate cellular microenvironment) the wall stiffness of foam-like porous scaffolds, or the individual fiber stiffness of fibrous scaffolds, heavily influences phenotype expression or culture viability. Cells attach to the ECM via mechanosensitive focal adhesion protein complexes (Chen 2008). These attachments allow them to probe their surroundings by actively pulling on the protein tethers, and subsequently sensing the resistance to the induced deformations up to a depth of 5 μm (Buxboim et al. 2010). For stem cells the mechanical response that they sense in this region is a determining factor regarding their differentiation into a range of cell types (Engler et al. 2006; Trappmann et al. 2012). On a macroscopic scale, the bulk mechanical properties of a scaffold must be sufficiently robust for pre-implantation culture and handling during surgery. While this is important for any tissue engineered scaffold, it provides additional complications for the higher loads and more aggressive surgical techniques associated with orthopaedic surgery. Moreover, once implanted, scaffolds must match the macroscale mechanical properties of the tissues surrounding them; implanted scaffolds are required to replace biomechanical function of the lost native tissue from the point of implantation, through to the completion of tissue remodelling. Scaffolds for interface tissues are hence required to simultaneously mimic the macroscale mechanical properties of the natural tissue, in both magnitude and distribution, whilst containing a gradient in microscale material stiffness to direct cellular differentiation. This has historically

proven difficult, as scaffolds focused on replicating macroscale properties of bone or cartilage have failed to provide the high porosity or chemical cues required for successful tissue growth, causing them to fail *in vivo* (O'Brien 2011). An example of a scaffold that successfully recreated many of the macroscale mechanical properties of the ACL can be found in (Cooper et al. 2005; Freeman et al. 2007). These papers detail a fibrous scaffold produced from a combination of braided and twisted poly l-lactic acid (PLLA) where the tensile mechanical properties mimicked both the initial toeing in region of load (characteristic of collagen fiber uncrimping) followed by a linear region of approximately the correct stiffness. This fibrous scaffold was also shown to be biocompatible. An *in vitro* study and an *in vivo* rabbit model showed that the fibrous structure presented sufficient porosity for cellular ingrowth to the centre of the tissue engineered ligament by 12 weeks (Lu et al. 2005; Cooper et al. 2007). Yet, this scaffold was not designed to regenerate the multiphasic ligament-bone interface and was implanted by merely suturing into a bone tunnel. As such, this scaffold was observed to sufficiently replicate mechanical tensile function of the natural ligament in the bulk region but not regenerate the graded tissue interface characteristic of the ligament bone junction. A recent work (Chung et al. 2014) used a similarly braided PLL scaffold to replace tendon function but also integrated their braided scaffold into regions of porous HA nanocomposite bone scaffold at either end. This work showed good scaffold uptake in a rabbit model, attributed to the enhanced healing possibility of the bone scaffold to bone rather than a tendon scaffold to bone. This work did not seek to generate a multiphasic interface between the braided tendon scaffold and the nanocomposite bone scaffold and it remains to be seen if the lack of an enthesis like region will cause increased incidence of failure in scaffolds of this type.

The focal adhesion protein complexes that cells utilize to sense their mechanical microenvironment are composed of integrins, trans membrane receptor proteins that facilitate cell-ECM binding interactions (Chen 2008). Integrins bind with a family of molecules on the ECM, ligands, and the specificity of the pairing mediates many cell processes including gene expression, cell proliferation and cell differentiation (Langholz et al. 1995; Zandstra et al. 2000; Lutolf and Hubbell 2005). There are over 20 known integrin proteins (Ruoslahti 1996), each of which can bind with a number of ligand molecules. Yet, integrin specificity for the ligands present in the native ECM has been observed to be important in culture viability, while the misregulation of integrin-ligand binding has been linked with serious cellular diseases such as cancer metastasis (Logsdon et al. 2003). Hence, it has been concluded that both the density and type of integrin-ligand bonds that form between a cellular population and its ECM mediate much of the cells behaviour (Massia and Hubbell 1991; Asthagiri et al. 1999). Ligands occur naturally within the sequences of proteins that make up the natural ECM including collagen, fibronectin, hyaluronic acid and proteoglycans. This makes these molecules attractive options for tissue engineered scaffolds, as they will continue to present biologically relevant types and densities of ligands for cell-ECM binding. Conversely, synthetic polymers such as poly(esters), or natural ones occurring outside of the body, such as silk or alginate, need be functionalized with ligands to promote cell-ECM binding. For instance (Paxton et al. 2009), incorporated the RGD ligand into a poly ethylene glycol (PEG) hydrogel, showing increased attachment of primary tendon fibroblasts over unfunctionalized PEG gels. Given the important role of integrin-ligand binding in all aspects of the cellular life, it can be an important tool when designing interface tissue engineered scaffolds, where zonal differentiation is required. Use of ligands to control stem cell differentiation is discussed in (Saha et al. 2007) for neural, mesenchymal, epithelial and embryonic stem cells. While these principles have not as of yet been applied to gradient scaffolds for interface tissues, their potential for influencing localized differentiation is clear. Additionally, methods of gradient generation in ligand concentration or type have been developed in hydrogels for studying cell attachment or motility as a function of ligand density (Lühmann and Hall 2009; He et al. 2010;

Sarvestani 2010). These gradient generation methods, alongside knowledge of specific integrin-ligand pairings that induce specific cell phenotypes, have the ability to assist guided differentiation of stem cells for interface tissue scaffolds.

Inorganic inclusions such as calcium phosphate, calcium carbonate and silicon dioxide have all been shown to promote osteoinduction in tissue engineered scaffolds for bone regeneration (Yuan et al. 1998; Xynos et al. 2001; Ripamonti et al. 2009; Poologasundarampillai et al. 2011). The mechanism by which inorganic mineral deposits promote osteoinduction is multifaceted. Firstly, the elastic stiffness of these materials is in the gigapascal range, promoting differentiation into osteoprogenitor cell types that naturally occupy stiff tissue environments. Secondly, these inorganic inclusions undergo cellular resorption and remodelling via ionic exchange at the mineral-cell boundary. This interaction promotes osteoblast proliferation and attachment by regulating cellular signalling pathways (Barrère et al. 2006). Additionally, during remodelling, the resorption of calcium phosphate, calcium carbonate or silicon dioxide inclusions does not induce abnormal or harmful levels of ions in the body (LeGeros 1993; Barrère et al. 2006; Rahaman et al. 2011). Their controllable deposition, and natural biocompatibility, makes inorganic inclusions a good option for promoting growth of bony tissue over fibrous tissue in regions of an enthesis or osteochondral scaffold. Multiple studies have employed this strategy, including: injecting calcium phosphate into a tendon graft to improve osteoinduction (Mutsuzaki et al. 2004); a 45S5 bioactive glass phase to promote a bony region in an ACL enthesis scaffold (Spalazzi et al. 2008); using calcium phosphate bone cement to simultaneously fixate a tendon graft in the bone tunnel and promote osteoinduction (Tien et al. 2004); inclusion of HA in a PEG hydrogel to assess interface formation with bone (Paxton et al. 2009); inclusion of HA particles within an alginate hydrogel to observe chondrocyte behaviour with different mineral concentrations (Khanarian et al. 2012); and graded deposition of HA using a varied incubation time in simulated body fluid (Li et al. 2009).

11.3.1.2 Scaffold Manufacturing Techniques

Freeze-drying is a widely used technique for the manufacture of highly interconnected porous scaffolds for tissue engineering. A range of materials can be freeze dried, including both organic and inorganic polymers such as collagen or polycaprolactone (Whang and Healy 2002; Maquet et al. 2003; Haugh et al. 2010) or stiff minerals such as HA (Macchetta et al. 2009). As a technique, freeze-drying offers a high degree of control over scaffold pore size, making it particularly apt for musculoskeletal scaffolds. Additionally, compared to other scaffold production techniques, freeze-drying eliminates the need for porogens that would later need to be leached away from the scaffold material. Graded structures of pore size or composition can also be manufactured. Gradients in pore size can be achieved by manipulating the thermodynamic conditions for ice crystal nucleation across the scaffold (Macchetta et al. 2009). Compositional gradients can be achieved by use of a graded proportion of two different precursor slurries (Weisgerber et al. 2013). A known disadvantage of freeze-dried scaffolds is cell encapsulation. Due to the inhospitable conditions associated with the freeze and sublimate steps, cells cannot be encapsulated within the scaffold during manufacture and must be infiltrated postproduction. In comparison to hydrogels this means that cells mount to the sidewalls of the foam rather than get encapsulated on all sides by the matrix, this often reduces the viability of their scaffold attachment. A freeze dried scaffold for osteochondral interface tissue engineering was described over a series of three papers (Harley et al. 2010a, b; Lynn et al. 2010). This scaffold was made from a collagen – glycosaminoglycan (collagen-GAG or CG) slurry and contained a gradient in both pore size and chemical composition. SEM images of this scaffold can be seen in Fig. 11.4 and show the graded transition from a dense cartilaginous scaffold to a more porous and mineralized osseous scaffold.

Hydrogels are hydrophilic polymer networks that swell to many times their dry weight when exposed to water. In this way hydrogels mimic the ECM of cartilaginous tissues and hence have

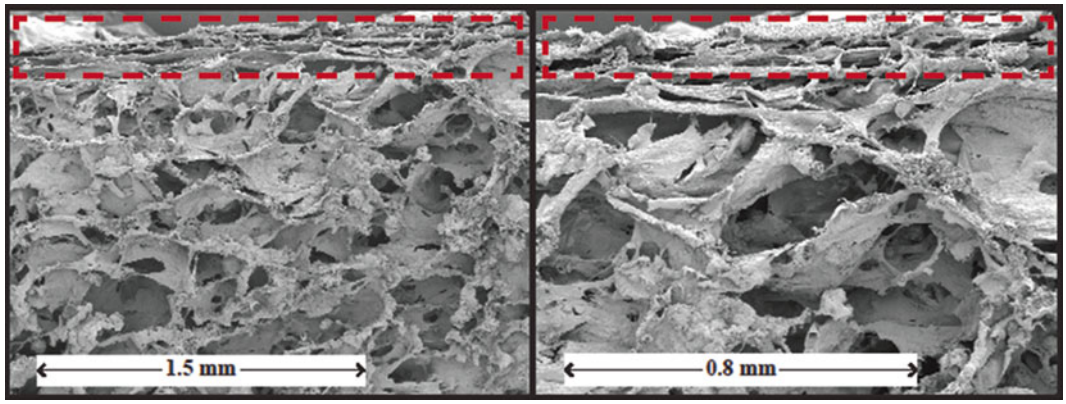

Fig. 11.4 An SEM image of a CG scaffold designed for replication of an osteochondral interface tissue. *Left* and *right* images are the same scaffold at different magnifications. The *red dashed line* indicates the dense cartilaginous scaffold while the more porous area below is the osseous scaffold (Adapted with thanks and permission from Harley et al. (2010b))

been considered as possible scaffolds for engineering of cartilage, among others (Suh and Matthew 2000). Hydrogels are prepared by either physical entanglement or chemical crosslinking of polymer chains. Polymer chain interconnectivity is brought about by either thermal settings or reversible bridging reactions in the case of physical entanglements, or by covalent bonding or photopolymerization in the case of chemical crosslinking (Oyen 2014). Additionally, it is simple to create interpenetrating double networks of two independent hydrogels. Double networks can be formed by mixing separate polymer solutions that are then cross-linked either in series or parallel, depending on the crosslinking techniques required for each network. Moreover, it is relatively simple to manufacture gradients in mechanical properties, pore size and chemistry within hydrogels. By generating a changing ratio of two polymers solutions the gel then contains a gradient in properties from that of purely one phase, to the properties of purely the other phase. For example, a mechanically and chemically graded collagen hydrogel was created using a microfluidics system for the study of matrix effects on hematopoietic stem cells (HSCs) (Mahadik et al. 2014). Alternatively, a homogenous polymer solution can be cross-linked in a graded manner to create a gradient in properties dependent on cross-linking density. Figure 11.5 shows examples of each of these methods of creating gradient hydrogels.

Electrospinning produces a mat of nanofibrous mesh from a large variety of organic and inorganic polymers. Electrospinning produces a fundamentally different structured scaffold compared to the open cell foam structures produced by freeze drying and other porogen based methods. By manipulating the process parameters of the experiment, fiber diameter, pore size and specimen thickness can be controlled. Furthermore, the morphology of the nanofibrous structure can be manipulated in a large variety of ways. Highly aligned fibers can be produced by spinning onto a rotating collector (Fennessey and Farris 2004), while a less dense and more 3D mesh can be produced by spinning into a fluid bath (Cai et al. 2013). Intricate collector geometries can also be used to produce complex patterned geometries of fibers (Zhang and Chang 2007). In addition to manipulating mesh morphology the fiber composition can be adapted to suit scaffold specific needs. Copolymer nanofibers can be formed by mixing two polymers in the precursor solution (Ma et al. 2005), or interpenetrating meshes of multiple distinct fibers can be formed from simultaneous spinning onto a single collector (Ifkovits et al. 2009). Furthermore, HA and bioactive glass inclusions can be incorporated into the fibers via sol-gel processes (Song et al. 2008; Poologasundarampillai et al. 2011).

Graded electrospun scaffolds with functional gradients in any of the parameters discussed above

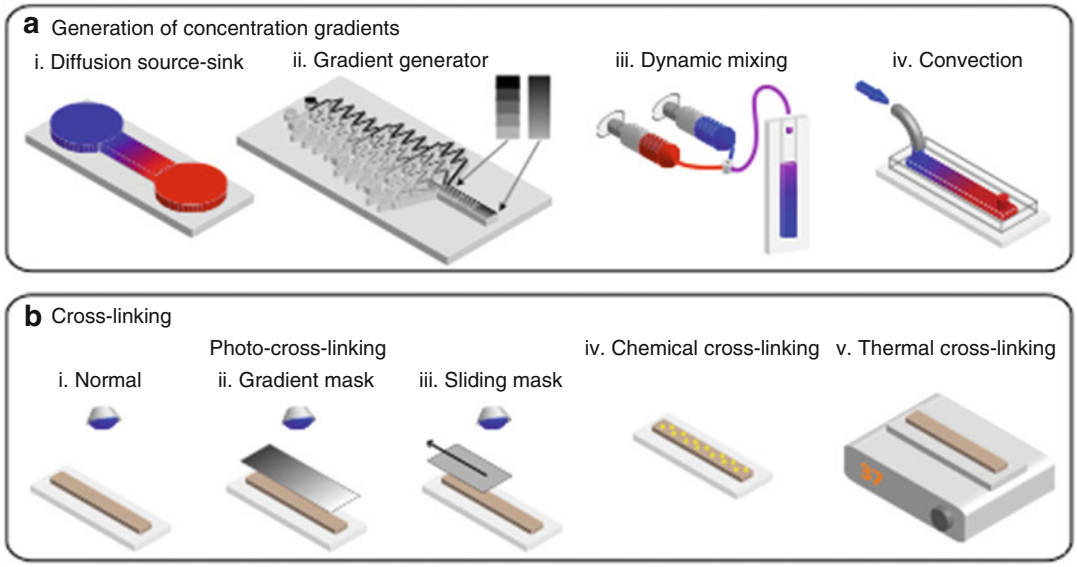

Fig. 11.5 Methods of generating gradients in hydrogel properties for scaffold production. (**a**) Methods of mixing two solutions of different gel precursors solutions to generate a graded solid. (**b**) Methods of grading the crosslinking density of a gel precursor to produce graded solids (Figure reproduced with permission from Sant et al. (2010))

can hence be produced by temporal manipulation of the relevant experimental parameters. A hyaluronic acid mesh was electrospun containing both a mechanical gradient, by changing crosslinking density, and a chemical gradient, by changing RGD peptide concentration. Subsequent culture with avian aortic cells investigated the effect of the mechanical and chemical gradients on cell attachment and proliferation (Sundararaghavan and Burdick 2011). However, graded electrospun scaffolds have not as of yet been employed for regeneration of either the osteochondral interface or the bone-tendon enthesis.

11.3.2 External Factors

Gradients in concentration of signalling molecules in the cellular microenvironment affect all aspects of cell behaviour. Growth factors are one such class of signalling molecules and many growth factors (TGF-β, PDGF, bFGF and IGF-1) have been shown to have a positive role in culture viability for homogeneous cartilage, tendon or ligament tissues. This occurs by upregulation of both fibroblast proliferation and ECM production (Molloy et al. 2003; Thomopoulos et al. 2013). In a large number of studies, cocktails of these growth factors have been delivered with varying doses and release profiles into scaffolds, promoting tissue growth. Additionally, growth factor BMP-2 has been used to successfully promote healing of the tendon-bone interface within the bone tunnel after surgery (Chen et al. 2008). In one study, delivery of BMP-2 via a retroviral factor induced a juxtaposed tendon-bone replacement for the ACL to generate a graded, enthesis-like structure, 8 weeks after surgery (Martinek et al. 2002).

When considering scaffolds for graded interface tissues the different growth factor requirements of each tissue region must be considered. Additionally, the spatial arrangement, temporal release profile and interactions with one another must all be taken into account. Across the native tendon-bone interface the type of growth factors found varies greatly. For example, growth factors bFGF and GDF-5 are found in the native tendon, TGF-β3 and IGF-1 in the fibrocartilagenous transition and BMP-2 and VEGF in the bone (Galatz et al. 2006; Liu et al. 2008). However, the large numbers of growth factors present in situ, along with their relative quantities and spatial

pattern within the complex enthesis tissue structure, means that an optimal distribution for tissue engineering has not yet been laid out. In one study, opposing gradients of TGF-β1 and BMP-2 growth factors were shown to induce patterning of cell phenotype differentiation, and subsequent tissue specific ECM composition (Dormer et al. 2010). Tactics like this, along with the knowledge of native growth factor distributions can allow for directed differentiation into the spatial distribution of cell phenotypes found *in situ*.

11.3.3 Cells

For interface tissue scaffolds the method of cell culture can be classified in two primary groups. Firstly, co-culture of two or more distinct cell lines where each population is characteristic of a distinct tissue region. Secondly, stem cell culture, where the mechanical, morphological and chemical properties of the scaffold direct stem cell differentiation into a range of phenotypes. In co-culture systems cell-cell signalling between populations is a key factor in influencing cell behaviour. A co-culture system consisting of a mass of bovine chondrocytes, covered in a monolayer of bovine osteoblasts, was used to investigate the co-culture effects for an osteochondral interface scaffold. Cross-sectioned staining from this study is shown in Fig. 11.6. This study showed that the co-culture method damped both the chondrocyte GAG expression compared to a chondrocyte control, and osteoblast mineralization compared to an osteoblast control (Jiang et al. 2005). In a further 3D study the co-culture

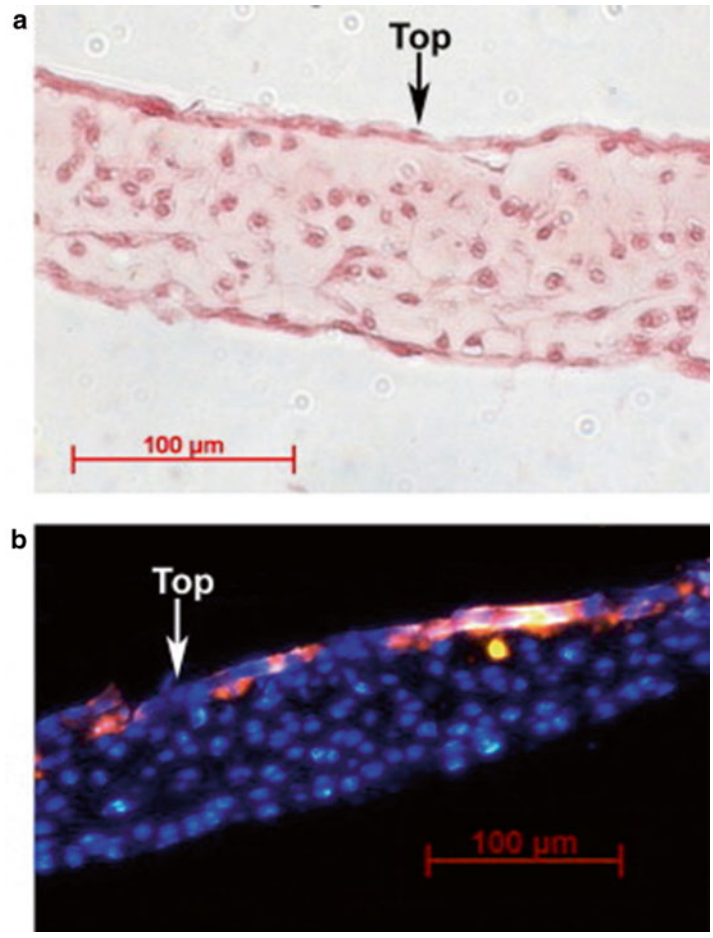

Fig. 11.6 Cross sectioned images of osteoblast and chondrocyte co-culture with different staining. The "*top*" in both images is a monolayer of osteoblasts covering the mass of chondrocyte cells below it. (**a**) H&E staining of cell mass distribution showing how cells at the interface between populations became elongated compared to the more spherical cells in the centre of the mass. (**b**) Osteoblasts stained with CM-DiI (*orange*) and nuclei of both populations stained with DAPI (*blue*) (Figure reused with permission from Jiang et al. (2005))

system promoted formation of an interface region, similar to that of the ZCC, within a 3D osteochondral interface scaffold (Jiang et al. 2010). A different co-culture study cultured fibroblasts and osteoblasts in tissue wells split with a permeable hydrogel divider. Similar results regarding the damping of both fibroblast GAG production and osteoblast mineralization were observed (Wang et al. 2007). These studies show that co-culture may facilitate the formation of a fibrocartilaginous interface region by damping the characteristics that arise in mono-culture of either of the cell populations involved.

A promising alternative to co-culture systems is to use multipotent or pluripotent progenitor stem cells, that are then directed to differentiate into the appropriate cell types for each region of the interface. Differentiation is directed by the graded mechanical, morphological and chemical factors of the scaffold that are discussed previously. Mesanchymal stem cells (MSCs) are a promising option for enthesis or osteochondral tissue engineering. MSCs can be harvested from adult bone marrow and have the ability to differentiate into osteoblasts, chondrocytes and fibroblasts, among others (Caplan 1991). A study to recreate the osteochondral interface using guided differentiation of MSCs has found recent success (Cheng et al. 2011). In this work MSCs were cultured within collagen microspheres into two sub populations: a chondrogenic population (Hui et al. 2008), and an osteogenic population (Chan et al. 2010). A layer of undifferentiated cells in collagen microspheres was placed between layers of the chondrogenic and osteogenic populations. The chemical cues received from the differentiated populations on either side, then prompted differentiation of the central layer into an interface region characterized by hypertrophic chondrocytes, as found in native tissue. In a different study, primary fibroblast cells, that have the ability to differentiate into osteoblasts, were cultured on collagen scaffolds in the presence of the osteogenic transcription factor Runx2/Cbfa1. The transcription factor was localized using immobilized retroviral deposits to create a gradient in transcription factor concentration. This resulted in a spatial gradient in differentiation of the primary fibroblasts into osteocytic and fibrocytic cell types, creating a corresponding gradient in cell-deposited matrix mineralization (Phillips et al. 2008).

11.4 Conclusions

Injuries to the soft tissues that connect the musculoskeletal system together are common. They include, sport-related acute tears of ligaments or tendons and age-related osteoarthritis of cartilage. Injuries such as these are significant problems clinically with largely unsatisfactory repair techniques. Tissue engineering has proven to be promising strategy for the reproduction of many homogeneous orthopaedic tissues such as bone, ligament and cartilage. However, even if fully biomimetic cartilage, ligament or tendon is produced then integrative clinical implantation is still challenging. Since, merely juxtaposing either cartilage with bone, or ligament with bone, does not result in the regeneration of the stratified natural interface. Consequently, tissue engineering efforts should focus on replication of the whole interface, from the soft tissue through to bone, and hence allow for a simpler bone-bone repair to occur post surgery.

Ligament or tendon entheses, and osteochondral interfaces, have unique and complex structures that are optimized to fulfil a niche biological role. Moreover, the exact distribution of some factors within the enthesis, including mineral content and mechanical response in each region, is under dispute. In order for tissue engineering efforts to be successful, these fundamental properties of the natural tissue must be understood in order for them to be recreated in scaffolds.

Scaffolds for interface tissue replication can be produced by either, joining a number of discrete homogeneous scaffolds, or, by fabrication of a single graded scaffold. Discrete scaffolds are easier to produce but significant care must be taken for full integration to be achieved at the interfaces. Regardless of the manufacturing method chosen, a number of scaffold properties must be controlled through the thickness. This is in order to ensure that either stem cell differentiation is appropriately

directed, or, co-cultured distinct cell lines have the optimal properties for their viability. Pore size has a significant effect on different cell line viabilities, but also affects macroscale mechanical properties, and is itself dependent on the scaffold material and manufacturing method. Microscale mechanical properties of the scaffold are key in stem cell differentiation, while the macroscale mechanical properties allow the scaffold to replicate biomechanical function once implanted. Chemical properties of the scaffold such as: the availability of relevant ligands; degradation properties of the polymer backbone; and any external growth factors that are added, combine to control cellular activity. In summary, for successful interface tissue engineering this interdependent set of properties must be simultaneously controlled to achieve biomimetic, region specific, tissue types.

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Part III

Applied Strategies for Tissue Engineering: Dentin, Enamel, Cementum and PDL

Cementum and Periodontal Ligament Regeneration

12

Danijela Menicanin, K. Hynes, J. Han, S. Gronthos, and P.M. Bartold

Abstract

The unique anatomy and composition of the periodontium make periodontal tissue healing and regeneration a complex process. Periodontal regeneration aims to recapitulate the crucial stages of wound healing associated with periodontal development in order to restore lost tissues to their original form and function and for regeneration to occur, healing events must progress in an ordered and programmed sequence both temporally and spatially, replicating key developmental events. A number of procedures have been employed to promote true and predictable regeneration of the periodontium. Principally, the approaches are based on the use of graft materials to compensate for the bone loss incurred as a result of periodontal disease, use of barrier membranes for guided tissue regeneration and use of bioactive molecules. More recently, the concept of tissue engineering has been integrated into research and applications of regenerative dentistry, including periodontics, to aim to manage damaged and lost oral tissues, through reconstruction and regeneration of the periodontium and alleviate the shortcomings of more conventional therapeutic options. The essential components for generating effective cellular based therapeutic strategies include a population of multi-potential progenitor cells, presence of signalling molecules/inductive morphogenic signals and a

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conductive extracellular matrix scaffold or appropriate delivery system. Mesenchymal stem cells are considered suitable candidates for cell-based tissue engineering strategies owing to their extensive expansion rate and potential to differentiate into cells of multiple organs and systems. Mesenchymal stem cells derived from multiple tissue sources have been investigated in pre-clinical animal studies and clinical settings for the treatment and regeneration of the periodontium.

Keywords
Periodontal regeneration • Cementum regeneration • Tissue engineering • Dental stem cells • iPSc • Tissue engineering

12.1 Introduction

Tissue regeneration presents as a significant therapeutic advancement in treatment of periodontal tissues damaged or lost due to injury, trauma or disease. As a concept, it focuses on replenishment of the affected areas with progenitor cell populations able to differentiate into a multitude of different functional cells, reestablishment of desired architectural and morphological tissue structure, reinstatement of vasculature and nutrient supply resulting in continued maintenance of regenerated healthy tissue and installed preventative measures of disease reoccurrence. In this chapter we present challenges associated with the regeneration of the periodontium, a complex, highly organised, load bearing structure constituted of diverse and unique tissue types. We further discuss current findings and advancements in stem cell based tissue engineering applications and introduce potential future prospects and direction in periodontal regenerative therapies.

Tooth formation is a complex, stringently regulated process involving interplay of dental epithelium and neural crest cell derived ectomesenchyme (Jernvall and Thesleff 2000; Pispa and Thesleff 2003; Thesleff and Sharpe 1997; Thesleff et al. 1991, 1995). In order to define developmental and morphological changes that occur during odontogenesis, the process has been subdivided into distinguished sections of progress, termed bud, cap, bell and crown stage. It is during the latter phases of development, namely the bell stage, that the tooth germs form into three distinct structures, the enamel organ, dental papilla and dental follicle (Nanci 2003). The dental follicle is a fibrocellular envelope that lines the enamel organ and the dental papilla and further gives rise to the supporting tissues of the tooth (Nanci 2003). Development of the periodontium starts with the formation of the Hertwig's epithelial root sheath, initiated by the proliferation of dental epithelial cells from the cervical loop of the enamel organ. The formed sheath acts to separate dental follicle cells from cells of the dental papilla which then differentiate into dentine forming odontoblasts. This process is followed by secretion of a hyaline layer onto the dentine surface by root sheath cells (Slavkin et al. 1989). It has been postulated that, following fragmentation of Hertwig's root sheath, this matrix of proteins subsequently provides an appropriate attachment substrate to support differentiation of cementoblasts from ectomesenchymal progenitors of the dental follicle (Hammarstrom et al. 1996; MacNeil and Thomas 1993). Cementum deposition continues with the apical development of the root and this allows for integration of Sharpey fibre collagen bundles, into the newly generated cementum.

Periodontal ligament formation is initiated by activation of dental follicle-derived fibroblasts (Yamamoto et al. 1994; Yamamoto and Hinrichsen 1993). Furthermore, osteoblasts, also derived from the dental follicle, are at this stage activated to synthesize mineralised matrix of the alveolar bone lining the tooth socket. The development of the periodontium is finalised as the Sharpey's fibres, already attached to the cementum lining the tooth root, insert into the

newly-generated bone and, with that, complete and stabilise the architectural structure and composition of the attachment complex (Cate 1975). The coordinated progress of events including formation of cementum, periodontal ligament and alveolar bone coupled with epithelial transformation to give rise to junctional epithelium during tooth eruption, result in development of the periodontium (Boskey 1996). Biochemical composition and functional role of each component of the tooth support structure are briefly outlined below.

12.2 The Periodontal Complex

12.2.1 Cementum

Cementum is an avascular, mineralised connective tissue composed of a cellular and an acellular component. It lines the root of the tooth and firmly connects to the dentine covering the root (Bosshardt and Selvig 1997). Primary cementum, the acellular constituent, lines the cervical portion of the root and plays a critical role in the anchoring of PDL fibres to the tooth. On the other hand, the cellular component, otherwise termed secondary cementum, covers the apical portion of the root and in its adaptive nature provides a cushioning buffer to absorb/support mechanical load bearing of this tissue and allow for orthodontic tooth movement (Nanci and Bosshardt 2006). It is termed cellular due the presence of cementoblasts, cementum forming cells, and cementocytes, cells engulfed in lacunae resembling osteocytes in bone. Approximately 45–50 % of cementum is composed of inorganic hydroxyapatite whilst organic collagen and non-collagenous matrix proteins constitute the remaining 50 %. Collagen Type I constitutes the majority (90 %) of the organic component of cementum and the remainder of the organic collagens are composed of collagen types III and XII (Nanci and Bosshardt 2006). The non-collagenous, matrix proteins, which constitute 50 % of cementum tissue include several proteoglycans, bone sialoprotein, osteonectin, osteopontin, and osteocalcin, all associated with formation of bone tissue (Bosshardt et al. 1998; Matias et al. 2003).

12.2.2 Periodontal Ligament (PDL)

PDL is a mechano-responsive tissue located between the tooth root and its supporting alveolar bone. It holds a pivotal role in maintenance of mechanical resistance and support of mechanical load exerted by the forces of mastication. This malleable structure has the ability to conform to load bearing by altering its width by up to 50 %, without compromising its architecture and function. It further plays a regulatory role in proprioception processes governing mastication (Nanci and Bosshardt 2006; Beertsen et al. 1997).

This specialised connective tissue features rapid matrix turnover, supported by ample vasculature, which allows for dynamicity within its cellular and extracellular compartments (Nanci 2003). The PDL cellular constituents include fibroblasts, as well as osteoblasts/osteoclasts and cementoblasts/cementocytes, populations contained within the ligament but functionally associated with bone/cementum respectively. Other cell types present within PDL include epithelial cell rests of Malassez, macrophages, nerve cells, endothelial cells, epithelial cells and mesenchymal progenitor populations (Han et al. 2003). The extracellular component is mainly formed of fibrous element, predominantly populated by defined bundles of Collagen Type I, II and XII fibres. Surrounding the collagens is an extracellular non-fibrous matrix rich in proteoglycans, hyaluronic acid, glycoproteins, glycolipids, minerals, growth factors and water (Nanci 2003).

The ability of periodontal ligament to remodel and allow for tooth movement is particularly important in maintenance of the periodontium (Berkovitz 1990).

12.2.3 Alveolar Bone

The alveolar bone, otherwise termed the alveolar process, serves to provide sockets for teeth to embed into and is connected to the basal bone of the jaw. It further houses nerves and vasculature for the dental and periodontal structure support. The inner component of the alveolar process, also referred to as bundle bone, directly lines the tooth socket and provides matrix for the integration

and attachment of PDL collagen fibre bundles. Alveolar bone undergoes continuous tissue remodelling to support its normal function and to sustain increased mechanical loading (Nanci and Bosshardt 2006; Saffar et al. 1997).

12.2.4 The Oral Mucosa

The oral mucosa is comprised of two parts, a superficial stratified squamous epithelial layer and an underlying fibrous connective tissue layer, lamina propria. The oral epithelium is composed of four layers, ranging in levels of cell maturity, with the most immature cells housed in the basal layer in close proximity to the lamina propria and the most outer, keratinised layer serving to endure forces of mastication and protect its underlying tissue from other traumatic forces exerted as part of normal oral function. Keratinocytes, the most widely distributed cell type of oral epithelium, located at the epithelial surface, are continually sacrificed and undergo cell death to, in turn, be replaced by the progressive maturation of proliferating and differentiating cells from the underlying layers. Other cell types, constituting the epithelium of oral mucosa, include Langerhans' cells, Merkel cells, melanocytes and inflammatory cells. The lamina propria is composed of loose irregular connective tissue that houses collagen and elastin fibres, vasculature and lymphatic vessels, and neural elements as well as its cellular components, including fibroblasts, immune cells and endothelial cells (Nanci 2003).

The gingiva attaches to the neck of the tooth by a specialised epithelial structure at the junction of the epithelium known as the junctional epithelium, which provides a seal between the soft tissues and the hard tissues of the tooth (Nanci and Bosshardt 2006).

12.3 Dental Disease

12.3.1 Periodontitis

Periodontitis is a chronic inflammatory disease of the periodontium. This disease presents with irreversible destruction of tooth support structures including gingiva, periodontal ligament and alveolar bone. The chronic, untreated state of this disease results in progressive loss of tooth attachment and its surrounding bone. When untreated, the disease may lead to premature tooth loss and hence an aesthetically and functionally compromised dentition (Pihlstrom et al. 2005). The pathogenesis of periodontal disease is initiated by formation of microbial biofilms in the subgingival sulcus. In response to this microbial challenge, the host immune system drives an inflammatory reaction resulting in host tissue damage. A number of modifying host factors including underlying associated conditions potentially associated with compromised immunity, tobacco smoking and genetic susceptibility play a role in the pathogenesis of this disease (Bergstrom 2004; Michalowicz et al. 2000).

Periodontitis has been associated with multiple systemic disorders including cardiovascular disease (Scannapieco et al. 2003), diabetes (Soskolne and Klinger 2001), and rheumatoid arthritis (Bartold et al. 2005). Periodontitis is prevalent in adult human populations worldwide, with an estimated 22.9 % of Australian population suffering from either moderate or severe periodontitis (Roberts-Thomson and Do 2007). The health system carries a heavy burden in terms of the economic cost associated with treatment and maintenance of chronic periodontal disease and a broad range of consequential implications on patient's quality of life. Effective and sustainable therapies aimed at periodontal regeneration rely on stimulating the reconstitution of all constituents of the periodontium. To restore the original architecture and uncompromised function, gingiva, cementum, periodontal ligament and alveolar bone require complete restoration by neogenesis. The reinstatement of tooth attachment to it supporting structures, i.e. formation of Sharpey's fibres is essential for the PDL to embed into the newly generated cementum and alveolar bone (Narayanan and Bartold 1996).

Considering the complexity of complete periodontal regeneration, it is not surprising that

current therapeutic approaches show limited clinical outcomes and the future of successful treatment will rely on greater understanding of dental structure biology coupled with current advancements in biomedical research.

12.3.2 Periodontal Wound Healing

Periodontal wound healing is an elaborate process, requiring a detailed understanding of the complexity of the anatomy and composition of the periodontium (Melcher 1976).

Currently, a number of surgical and non-surgical approaches focus on periodontal tissue repair and mainly involve open flab debridement, resective methods and scaling and root planning. The objective of these procedures is to arrest progression of periodontal disease, by removal of the diseased tissue, and minimize pathogen colonization. Commonly, these processes lead to the formation of a long junctional epithelial attachment, which is non-physiological in nature and results in a poor connection between the roots and the alveolar bone (Caton et al. 1980). In addition, minimal regeneration of other structures, including cementum, ligament and bone is achieved with such procedures, further highlighting the limitations of these approaches (Narayanan and Bartold 1996).

The typical outcomes of these processes are outlined in Table 12.1.

Table 12.1 Periodontal wound healing responses by repair and regeneration

Repair	Regeneration
Control of inflammation	Formation of new functional attachment, including formation of cementum, periodontal ligament and alveolar bone
Formation of long junctional epithelium	
Re-attachment of connective tissue to adjacent root surface	
New bone separation from the root surface by long functional epithelium, accompanied by root resorption, and/or ankylosis	

Adapted from reference Narayanan and Bartold (1996)

12.4 Current Treatment Approaches

Numerous procedures have been trialled and subsequently introduced as part of clinical practice to try to achieve predictable and sustainable regeneration of the periodontium. In line with research findings, utilization of graft materials has been utilized, to substitute the incurred bone loss (Aichelmann-Reidy and Yukna 1998). In addition, the use of bioactive stimulants (Esposito et al. 2003; Sculean et al. 2008a; Bosshardt 2008) and use of barrier membranes for guided tissue regeneration has been introduced (Gottlow et al. 1984, 1986).

12.4.1 Bone Grafts

Bone grafts have been investigated extensively to assess their ability to replace lost structures and stimulate formation of mineralized tissues. Implantation of numerous grafting materials in the form of autografts, allografts, xenografts and alloplastic materials has been reported to result in some gain in clinical attachment levels and displays evidence of bone replacement. On the other hand, histological assessment following implantation of such biomaterials has illustrated that osteoinductive and cementogenic capacity of grafting materials is minimal, and that in most cases the implant became encased in fibrous tissue structures (Bartold et al. 2006; Garraway et al. 1998).

A systematic review conducted in 2003 demonstrated that there were no differences observed in clinical outcomes across implantation of different graft materials (Reynolds et al. 2003) and that administration of alloplastic grafts into periodontal sites resulted in periodontal repair rather than regeneration of functional tissue (Reynolds et al. 2003).

Inconsistent findings, conflicting reports and insufficient data on their clinical and therapeutic effectiveness have led to limited application and utilization of bone grafts in regeneration of periodontal tissues (Ivanovski 2009).

12.4.2 Guided Tissue Regeneration

Guided tissue regeneration (GTR), a commonly applied clinical procedure, is currently considered the "gold standard" of periodontal regenerative therapies (Karring et al. 1993; Nakae et al. 1991). In 1976, Melcher hypothesized that "first cells to populate a wound will dictate the nature and quality of tissue that forms there" (Melcher 1976). Guided by this proposal Nyman et al. set out to apply guided tissue regeneration to damaged periodontal structures in experimental animal models and in humans (Nyman et al. 1982a, b). GTR involves the use of a membrane to act as tissue barrier and selectively promote repopulation of the site by periodontal ligament progenitor populations (Ivanovski 2009). The procedure relies on guided migration and localisation of periodontal ligament, cementogenic and osteogenic progenitor cells into the wound site (Nyman et al. 1982a, b). Initial GTR approaches, reported in 1982, utilized membranes produced from cellulose acetate (Nyman et al. 1982b), which were subsequently superseded by, non-resorbable membranes made of expanded polytetrafluroethelene (ePTFE) (Becker et al. 1988; Cortellini et al. 1990) Implantation of non-resorbable membranes required their removal and re-entry into the defect site, which led to multiple postoperative complications and compromised clinical outcomes for the patients (Murphy 1995; Selvig et al. 1992).

Subsequent introduction of resorbable membranes, most commonly constituted of collagen and copolymers of polylactic/polyglycolic acid, circumvented the above-mentioned issue. However, membrane exposure still presents a major limitation to the therapeutic application of GTR in periodontal healing and tissue regeneration (Sculean et al. 2007; Tonetti et al. 2004). Moreover, resorbable membranes are often administered in combination with grafting materials due to their lack of supportive structural integrity (Ivanovski 2009).

Despite its conceptual validity, limited efficacy and highly variable clinical outcomes have been reported for this technique sensitive procedure (Bratthall et al. 1998; Pontoriero and Lindhe 1995; Wallace et al. 1994; Tonetti et al. 1996, 1998). These results have been assigned to the lack of desired mechanical properties and biocompatibility of composite materials used in GTR membranes, which would impact upon their crucial role of wound stabilisation and space maintenance.

12.4.3 Delivery of Bioactive Materials

Direct delivery of bioactive molecules to sites of periodontal destruction has been trialled to stimulate wound healing and regeneration of damaged and lost periodontal structures. Past research, conducted in canine and primate models of periodontal disease, has identified and characterised a potential role for platelet-derived growth factor (PDGF) and insulin-like growth factor-I (IGF-I) in enhancement of tissue regeneration (Lynch et al. 1991; Rutherford et al. 1993). Additionally, bone morphogenetic proteins (BMP) have also displayed propensity to stimulate bone and cementum regeneration (Ripamonti and Reddi 1994).

A large number of inductive factors with potential bioactive and regenerative capacities have been investigated to date. One of the most widely investigated factors is an enamel matrix derivative (EMD). EMD is associated with regulatory mechanism underlying initial tooth formation, specifically during the stages of epithelial and mesenchymal interactions. Whilst their mode of action is not fully understood yet, enamel matrix proteins play a critical role in the formation of cementum and periodontal ligament (Bosshardt 2008). As such, a biological-based product, Emdogain™ (Straumann AG, Basel, Switzerland), that is extracted from developing porcine teeth, has been developed. In addition to its main constituents, amelogenins and a mixture of other enamel matrix proteins, Emdogain™ has been supplemented with TGF- β and BMP. These additional factors have been added to promote tissue regeneration of intrabony defects by biomimicking the microenvironment of initial formation of tooth attachment structure (Esposito et al. 2003;

Sculean et al. 2008a; Bosshardt 2008). Furthermore, it has been demonstrated that therapeutic effectiveness of EMD, in regeneration of intrabony defects, is enhanced by coupled application and combined engraftment with bone grafting materials (Trombelli and Farina 2008). The observed benefits of combined implantation could be attributed to the structural reinforcement of EMD, a gel like material of non-supportive consistency, which lacks constitutional rigidity and spatial integrity (Ivanovski 2009).

The findings obtained from a number of clinical trials, where EMD has been used to treat periodontal related bone loss, display significant heterogeneity, but have demonstrated consistent pocket depth reduction and improved probing attachment levels. It has been further determined that treatment with Emdogain™ results in comparable clinical outcomes to those noted for GTR, (Esposito et al. 2003; Jepsen et al. 2004), with one clinical trial study reporting a significant decrease in horizonatal furcation depth and reduced postoperative pain and swelling observed in patients treated with EMD (Jepsen et al. 2004). EMD has shown clinical success in treatment of patients presenting with severe attachment loss (Chen et al. 2010), has demonstrated long-term stability of therapeutic outcomes (Sculean et al. 2008b) and presents with low levels of immunogenicity (Nikolopoulos et al. 2002; Zetterstrom et al. 1997). As such, EMD is the only commercially available product that has shown clinically significant results in periodontal regeneration (Hirooka 1998).

In addition, clinical application of these procedures is limited to specific cases that present with vertical bone loss, generally surrounding single teeth and or between furcation lesions. As such, this eliminates utilization in patients where a horizontal patterned loss of periodontal attachment is present throughout the oral cavity.

As outlined above, the periodontium is a complex organ consisting of epithelial tissue and both soft and mineralised connective tissues. It comprises the gingiva, underlying fibrous periodontal ligament, alveolar bone of the jaws, and cementum which lines the root of the tooth (Ivanovski 2009). Current regenerative procedure aim to mimic the physiological and biological stages of periodontal wound healing and recapitulate the processed involved in periodontal development to reinstate the structure and function of lost tissues (MacNeil and Somerman 1999). Hence, an understanding of underlying processes determinative to the development of the periodontium is pivotal in order to decipher the cellular and molecular regulators of periodontal regeneration.

For tissue regeneration to eventuate, stages of healing must progress in a regulated and programmed manner, both temporally and spatially, mimicking the key events in periodontal development (Bartold et al. 2000). Currently, it is understood that initial migration of the progenitor cell populations to the wound site, followed by their attachment to the denuded root surface is required for their proliferation and differentiation into mature, functional cells of the periodontium. The complete and successful recruitment, expansion and maturation of progenitor cells into tissue components is dependent on the presence of inductive signals, growth factors and a stimulative microenvironment (Aukhil 2000). Alternatively, in a non-inductive environment that lacks cellular and molecular signalling factors, healing may be limited and result in tissue repair and formation of scar tissue as opposed to tissue regeneration. The presence and involvement of progenitor cells is fundamental to periodontal regeneration, as these populations are the undifferentiated subsets of cells comprising the, and responsible for the restoration of lost periodontal tissues encompassing bone, cementum and ligament. Based on these observations and identification of stem-like cells derived from dental tissues, current research trends have focused on innovation and development of cellular-based techniques to treat degenerated periodontium.

12.5 Cell-Based Tissue Engineering

Considering the limitations and shortcomings of currently available therapeutic approaches for the treatment of periodontal disease, research has begun to focus towards tissue engineering

(Srisuwan et al. 2006). This alternative approach, encompasses the concepts of cell and development biology as well as biotechnology and biomaterials science to overcome the inadequacies and downfalls of conventional treatment modalities (Srisuwan et al. 2006). Effective and pertinent utilization of tissue engineering requires integration of three essential components; a population of multipotential progenitor cells, signalling molecules/inductive morphogenic signals and a conductive extracellular matrix scaffold (Srisuwan et al. 2006; Nakashima and Reddi 2003; Nakashima 2005).

Considerable benefits are evident from research and application of tissue engineering concepts to the field of regenerative dentistry. Restoration and management of damaged or lost oral tissues is envisaged through reconstruction and regeneration techniques facilitated by cell based tissue engineering therapies (Bartold et al. 2000; Nakashima and Reddi 2003; Nakahara et al. 2003; Owen et al. 2005). In the context of periodontal regenerative therapy, a potential tissue engineering approach involves incorporation of progenitor cells in a prefabricated three-dimensional construct, which is subsequently implanted into the defect site (Bartold et al. 2000). This strategy would alleviate some of the limitations associated with current regenerative procedures as it allows direct placement of growth factors and progenitor cells into the defect site, circumventing the delay in recruitment of endogenous progenitor cells to the wound (Lin et al. 2008). As mentioned above, desired outcomes of periodontal tissue engineering entail alliance of the following pivotal factors: a potent population of progenitor cells with high proliferative capacity and potential to mature into functional, tissue-forming osteoblasts, cementoblasts and fibroblasts; the inductive biomolecules to stimulate cellular process and tissue growth and development and the structurally and molecularly supportive matrix scaffold (Hughes et al. 2010). Furthermore, the presence of angiogenic signals and formation of associated vasculature is pivotal for successful tissue regeneration, as generation of vascular networks provides nutritional support to developing tissues and ensures homeostatic maintenance. In addition, appropriate mechanical loading is required for the development of highly organised, functional PDL fibres. Lastly, antimicrobial strategies are required to eradicate infection and inflammation and hence to minimise the host immune response (Taba et al. 2005).

Biomaterial scaffolds have multiple purposes including cell housing and delivery, and further support cell attachment, determine space retention and the morphology of the implanted matrix and support angiogenesis and vasculature formation to the site (Nakashima and Reddi 2003; Nakashima and Akamine 2005). Manufacturing and selection of optimal scaffold materials requires consideration of numerous aspects including porosity, tissue conductivity, biocompatibility, resorption rate, and potential versatility as a delivery vehicle for different cell populations (Duailibi et al. 2006; Murphy and Mooney 1999).

Considering the complex nature of the periodontium and structural interplay of multiple soft and hard tissues, numerous issues need to be considered when contemplating tissue engineering based-regenerative protocols. To achieve restoration of original architecture and functionality, current advanced approaches require utilization of sophisticated biomaterial scaffolds in conjunction with the implantation of optimal progenitor cellular subtypes.

12.5.1 Challenges and Limitations Associated with Tissue Engineering Based Approaches to Periodontal Therapy

Multiple biological and technical challenges and limitations associated with recapitulation of functional periodontal tissues cause significant concerns when examining potential clinical therapies.

The difficulty in achieving complete and functional tissue regeneration can be attributed to the complexity of the native tissue structure and the essential requirement to strategically bio-mimic its wound healing process, in a spatiotemporal manner (Ivanovski et al. 2014).

Therapeutic modalities utilizing barrier membranes and bioactive molecules have displayed shortcomings in response to the abovementioned issues and resulted in varied and limited reconstitution of the periodontium and as such their application remains clinically unpredictable (Ivanovski 2009; Ivanovski et al. 2014).

For a scaffold to be suitable for implantation into damaged or disease periodontium it should posses the ability to withstand the physiologic loading of the tissue whilst supporting its other functionalities. Therefore the biomaterial should present with suitable morphological features and a gradient of mechanical and bio/physiochemical properties to achieve functional regeneration and integration of bone, ligament, cartilage and tendon with one another and within the host environment (Ivanovski et al. 2014; Dormer et al. 2010).

12.5.2 Use of Biomaterials in Regeneration of Dental Tissues

As part of tissue engineering, biomaterial matrices perform multiple roles and as such are required to display multiple properties to support the multifold functionality (Ivanovski et al. 2014; Zhang et al. 2014). Initially, biomaterial scaffolds act to provide structural support and a homing platform for cells implanted into the defected site and subsequently aid to replicate the anatomy of lost structures. In parallel, they aim to actively promote integration of implanted cells and materials into the host tissue and further support cell migration, colonization, growth, survival and differentiation. Additionally, biomaterials hold an inductive role in the initiation of tissue regeneration and in stimulation of angiogenesis and vascularization of the newly formed tissues, as part of their support for ongoing maturation and subsequent remodelling of developed structures (Ivanovski et al. 2014; Zhang et al. 2014). Finally the implanted scaffold plays a part in determent of adhesion and colonization of microbial pathogens to minimize post-treatment infections (Zhang et al. 2014).

To meet these essential criteria the biomaterial should be constructed as a highly porous interconnected network with desirable surface feature and demonstrate biocompatibility and timely and controlled biodegradability (Hutmacher and Cool 2007). It is fundamental that the degradation of the scaffold temporally aligns with the remodelling and maturation of the regenerated tissue (Lam et al. 2009).

An array of materials suitable for regeneration of ostegoenic/periodontal related tissues have been investigated to date and a summery is outlined in Table 12.2.

Reciprocal interactions between the cellular material and scaffold structure are pivotal to gain functional tissue regeneration, as is highlighted by vast investigation and manipulation of various aspects and constituents of utilized biomaterials. Factors taken into account, include surface roughness, microtopography, nanotopography, porosity and surface energy of the biomaterial (reviewed in Zhang et al. 2014).

Roughness of the surface and the associated microtopography affect the adhesion and differentiation of cells (Zhang et al. 2014). Similarly, surface energy (also termed surface wettability) portrays a directly proportional relationship to cellular adhesion and differentiation, i.e. increased surface energy (hydrophilic charge) results in enhanced attachment, proliferation and expression of differentiation progression markers in osteogenic progenitors (Lim et al. 2008; Lai et al. 2010; Qu et al. 2007). Surface porosity is critical in cellular integration and ingrowth processes and is involved in establishment and reinforcement of the host tissue-implant interface (Zhang et al. 2014).

Furthermore, employment of biologically active coatings of scaffold matrices has been extensively investigated to illustrate potential benefits involved in regeneration of tissues requiring osteointegration (Table 12.2) (Zhang et al. 2014). Some of the materials used to date include calcium-phosphate based bioceramics, extracellular matrix molecules (ECM) components, biological peptides, metal ion incorporated coatings, titanium nanotubes, growth factors, bisphosphonates, strontium, and anti-infection coatings (reviewed in Zhang et al. 2014).

Table 12.2 Pre-clinical and clinically applicable matrices used in periodontal regeneration

Biomaterial	Summary	Examples	References
Bioactive ceramics	Bioactive ceramics have been considered the best choice to date for bone-grafting materials in periodontal pockets because of their osteoinductive and osteoconductive properties	Hydroxyapatite (HA)	Dorozhkin (2010)
		Beta-tricalcium phosphate (β-TCP)	Li et al. (2009b)
		Calcium phosphate (CaP) -coated surface	Shue et al. (2012)
Degradable polymers	Degradable polymers have been predominantly used in guided tissue and guided bone regeneration membranes where a degree of flexibility is required	Natural	
		Collagen	Sachar et al. (2014)
		Gelatin	Kaigler et al. (2013)
		Chitosan	Suh and Matthew (2000)
		Synthetic	
		Polycaprolactone (PCL)	Liu and Ma (2004)
		Polyglycolide (PGA)	Liu and Ma (2004)
		Polylactide (PLA)	Liu and Ma (2004)
		Polycaprolactone/beta-tricalcium phosphate (PCL/β-TCP)	Costa et al. (2014)
Composites	Composites refer to the combination of different material sources, such as a bioactive ceramic and a degradable polymer. The advantage of using a composite is that the polymer is able to provide the flexibility required while the bioactive ceramics provide the stiffness required	Numerous combinations have been investigated however most studied composite to date is collagen/HA	Lickorish et al. (2004) and Perez and Ginebra (2013)
Bioactive molecules	Bioactive molecules work through enhancing wound healing, promoting tissue regeneration of both soft and hard tissues and regulate cell activities	Natural	
		Platelet-rich plasma (PRP)	Creeper et al. (2009)
		Platelet-rich fibrin (PRF)	Thorat et al. (2011)
		Synthetic	
		Enamel matrix derivative (EMD)	Ribeiro et al. (2011)

Current research advances and clinical applications are focused on biomimetic approaches utilizing stem-cell-based tissue engineering approaches. Moreover, significant emphasis is placed on development of sophisticated biomaterials and cultivation of optimal progenitor populations to achieve predictable, functional and sustainable levels of periodontal regeneration.

As discussed earlier, identification, characterisation, isolation and propagation of the optimal cell population are of fundamental importance to tissue engineering. A significant part of research efforts, discussed in the following chapter, has focussed on isolating high-quality, readily-accessible postnatal stem cells to be utilized in tissue regenerative therapies.

12.6 Mesenchymal Stem Cells

Adult organs possess a limited capacity to self-regenerate and as such house a pool of undifferentiated stem cells ready to replenish cells damaged or lost due to injury and/or disease

(Blau et al. 2001). Since their initial discovery, stringent characterisation studies have been conducted to determine their full developmental potential and it has been demonstrated that beyond their ability to maintain homeostasis within their tissue niche of origin, they also have the potential to mature into other tissue lineages.

Mesenchymal stem cells (MSC) are a population of specialised postnatal stem cells, initially isolated from bone marrow and stromal components of thymus and spleen tissue (Friedenstein 1976; Friedenstein et al. 1966). Subsequent studies have demonstrated a widespread distribution of MSC-like cells in other organs and they have since been derived from trabecular bone, periosteum, articular cartilage, synovium, synovial fluid, skeletal muscle, adipose tissue (Zannettino et al. 2008), tendons, blood (Kuznetsov et al. 2001), blood vessels, umbilical cord vasculature (Erices et al. 2000), placental tissue (Portmann-Lanz et al. 2006; Soncini et al. 2007) fetal tissues, skin (Young et al. 2001) and dental tissues (Seo et al. 2004; Gronthos et al. 2000; Miura et al. 2003; Sonoyama et al. 2006, 2008; Guo et al. 2009, 2011; Li et al. 2011) . More specifically, the term MSC now denotes a heterogeneous mix of progenitor cells, where a subset population is capable of differentiating into cells of mesodermal (adipocytes, osteoblasts, chondrocytes, tenocytes, skeletal myocytes and visceral stromal cells) (Gronthos et al. 2003a; Horwitz et al. 1999; Jiang et al. 2002; Pereira et al. 1995; Pittenger et al. 1999; Smith et al. 2004), ectodermal (neurons, astrocytes) (Woodbury et al. 2000) and endodermal (hepatocytes) (Petersen et al. 1999) origins.

Whilst MSC exhibit a limited developmental capacity and a finite life span when compared to the pluripotent embryonic stem cells (ESC), they are not hindered by the associated ethical issues or tumor forming tendencies (Gronthos et al. 2003b). Additionally, MSC are reported to be hypoimmunogenic and possess immunomodulatory properties both of which are greatly beneficial and applicable to allogeneic cell transplantation (Iwata et al. 2009).

12.6.1 Utilisation and Efficacy of Bone Marrow Derived MSC (BMSC) in Regeneration of Dental Tissues

Bone forming capacity of BMSC has been well documented and thoroughly reviewed (Bruder et al. 1998; Derubeis and Cancedda 2004; Caplan 2005, 2007; Bianco et al. 2006; Arthur et al. 2008; Kode et al. 2009). Due to the demonstrated regenerative capacity of BMSC in treatment of bone defects, their involvement and regenerative potential in periodontal healing has been extensively studied. Outlined in Table 12.3 are major studies in which assessment of BMSC implantation into a range of periodontal defects was conducted in small and large animal models. These studies highlight the capacity of BMSC to support periodontal regeneration by promoting generation of alveolar bone, cementum and periodontal ligament and further contributed to neovascularisation of the defect area (Chen et al. 2008; Chung et al. 2011; Hasegawa et al. 2006; Kawaguchi et al. 2004; Kim et al. 2009; Li et al. 2009a; Pieri et al. 2009; Simsek et al. 2010; Tan et al. 2009; Tsumanuma et al. 2011; Wei et al. 2010; Yamada et al. 2004, 2011; Yang et al. 2010). Although it has been shown that BMSC possess the capacity to facilitate periodontal regeneration, isolation and derivation of this cell population can be invasive and challenging, thus utilization of dental derived MSC-like stem cell populations, which can advantageously be obtained from patients in the dental clinic, has been investigated for use in regenerative dentistry. In developed dental structures, most periodontal and dental tissues possess varying degrees of self-regenerative or self-reparative potential (Duailibi et al. 2006). This phenomenon is attributed to the existence and activation of multipotent progenitors into functional mature cells (Beertsen et al. 1997; Gould et al. 1980; Boyko et al. 1981; Liu et al. 1997). In dental structures, MSC-like cells have been obtained from dental pulp (Gronthos et al. 2000, 2002), periodontal ligament (Seo et al. 2004), exfoliated deciduous teeth (Miura et al. 2003; Cordeiro et al. 2008; Sakai et al. 2010; Shi et al. 2005;

Table 12.3 Bone marrow MSCs implanted into dental defects

Defect	Carrier	Animal model	Cell association	Duration of implant	Outcomes	References
Bone marrow derived MSC						
Bone defects on both sides of the mandible	Platelet-rich plasma (PRP)	Hybrid dog	Autologous	2, 4 and 8 weeks	This investigation aimed to elucidate if PRP can increase osteogenesis of BMSC in bone defects. Results of this study determined that PRP in combination with BMSs elicit true bone regeneration with the formation of well-formed mature bone and neovascularisation	Yamada et al. (2004)
Class III furcation defect	Atelocollagen	Beagle dogs	Autologous	1 month	Auto-transplantation of bone marrow derived MSC into periodontal defects aids regeneration with the formation of cementum, periodontal ligament and alveolar bone	Kawaguchi et al. (2004)
Class III furcation defect	Atelocollagen	Beagle dogs	Not defined	4 weeks	In this study BMSC were engineered to express GFP in order to elucidate the behaviour of BMSC once transplanted into periodontal defects. Four weeks after transplantation, the periodontal defects were almost completely regenerated and contained GFP positive cementoblasts, osteoblasts, osteocytes, and fibroblasts of the regenerated periodontal tissue. These results suggest that transplanted BMSC survived within the defect and differentiated into cells that make up the periodontal tissues	Hasegawa et al. (2006)
Transgingival periodontal defect – alveolar bony defects	Pluronic F-127 (PF-127) gel	New Zealand white rabbit	Autologous	6 weeks	This investigation assessed whether BMSC, engineered to express BMP-2, enhanced periodontal alveolar bone regeneration. Implanted BMP-2 expressing BMSC generated significantly more bone than non-manipulated BMSC	Chen et al. (2008)

Periodontal fenestration defects	Collagen membrane	Beagle dogs	Autologous	8 weeks	Transplantation of BMSC resulted in the formation of cementum, alveolar bone, and periodontal ligament leading to significantly greater periodontal regeneration. Additionally this study demonstrated that cryopreserved BMSC have the same regenerative capacity as freshly isolated BM-MSC	Li et al. (2009a)
Cylindrical defects in the edentulous mandibular ridge	Cylindrical defects in the edentulous mandibular ridge	Miniature pig	Not defined	3 months	Implantation of BMSC with platelet-rich plasma using a flurohydroxyapatite scaffold leads to enhanced bone formation after 3 months	Pieri et al. (2009)
Class III root furcation defect	Calcium alginate gel	Beagle dogs	Not defined	6 weeks	The aim of this study was to determine if BMSC transfected with bFGF promote periodontal regeneration more effectively than non-manipulated BMSC. The BM-MSCs expressing bFGF promoted faster regeneration of the periodontal bone tissue	Tan et al. (2009)
Saddle-like through-and-through defects	Hydroxyapatite/β--tricalcium phosphate	Beagle dogs	Autologous	8 weeks and 16 weeks	Transplantation of BMSCs and PDLSCs into peri-implant defects resulted in enhanced bone regeneration. There was no significant difference in regenerative potential between BMSCs and PDLSCs	Kim et al. (2009)
Class III furcation defect	Calcium alginate gel	Beagle dogs	Autologous	6 weeks	Upon transplantation, BMSC migrated into the periodontal ligament, alveolar bone, cementum and blood vessels. In addition the transplanted BMSC differentiated into periodontal ligament fibroblasts and osteoblasts leading to the regeneration of periodontal tissues	Wei et al. (2010)
Periodontal defect on the buccal of the mandibular first molar	Gelatin beads	Rat	Allotransplantation	3 weeks	Transplantation of BMSC grown on gelatin beads supported and enhanced periodontal regeneration through the formation of significantly more appropriately orientated periodontal ligament fibres and enhanced bone formation	Yang et al. (2010)

(continued)

Table 12.3 (continued)

Defect	Carrier	Animal model	Cell association	Duration of implant	Outcomes	References
Class II furcation defect	Platelet-rich plasma and 10 % calcium chloride	Mongrel dogs	Autologous	8 weeks	Implantation of BMSC with platelet-rich plasma lead to complete filling of the furcation defect with cementum, alveolar bone and periodontal ligament	Simsek et al. (2010)
Bilateral mandibular alveolar and periodontal defects	Pluronic F-127 (PF-127) gel	Beagle dogs	Autologous	8 weeks	MSCs expressing BMP-2 enhanced bone and periodontal regeneration in mandibular periodontal defects. Implantation of the BMP-2 expressing MSCs resulted in the regeneration of larger volumes of bone than seen from the MSC alone cells without the negative effects of root ankylosis and resorption. Functional Sharpey's fibres and cementum were also generated	Chung et al. (2011)
Bone defects on both sides of the mandible	Platelet-rich plasma (PRP)	Hybrid dog	Allogeneic	2, 4 and 8 weeks	Implanted BMSCs generated well-formed mature bone with neovascularization when compared to controls	Yamada et al. (2011)
Bone marrow derived cell sheets						
One-wall intrabony defect	Hydroxyapatite/β--tricalcium phosphate and collagen	Beagle dogs	Autologous	8 weeks	The aim of this study was to ascertain if cell sheets derived from bone marrow cells and PDLCs could enhance periodontal regeneration. Both the PDLC and bone marrow derived cell sheets enhanced periodontal regeneration with the formation of new cementum and well-oriented PDL fibres	Tsumanuma et al. (2011)

Wang et al. 2010), apical papilla (Sonoyama et al. 2006, 2008), and dental follicle (Guo et al. 2009, 2011; Li et al. 2011).

Characterisation of dental pulp and periodontal ligament derived cells demonstrated comparable growth and developmental profile to previously investigated BMSC (Seo et al. 2004; Gronthos et al. 2000; Miura et al. 2003). Furthermore, dental pulp derived cells exhibit the capacity to form dentin-pulp-like structures in vivo (Gronthos et al. 2000, 2002), whilst cells derived from the periodontal ligament exhibited the potential to generate cementum/PDL-like tissue (Seo et al. 2004). Based upon these findings the use of dental tissue derived stem cells has been stringently investigated in various tissue engineering approaches for the regeneration of dental structures.

12.6.2 Utilisation and Efficacy of Dental Pulp Derived MSC (DPSC) in Regeneration of Dental Tissues

Gronthos and colleagues demonstrated that dental pulp derived cells exhibited self-renewal and regenerative capacities, in terms of dental structures, superior to that of BMSC and as such this population was termed DPSC (Gronthos et al. 2000, 2002). Numerous studies have focused on DPSC implantation into an array of periodontal defects in dogs, however inconsistency across experimental settings impeded the significance of their collective findings (Yamada et al. 2011; d'Aquino et al. 2009; Ji et al. 2010; Park et al. 2011). These studies are outlined in Table 12.4. Two studies demonstrated enhanced tissue regeneration, with mature bone generation and the associated neovascularisation, by implantation of DPSC into surgically created defects (Yamada et al. 2011; Ji et al. 2010). Furthermore, it has been shown that combining calcium hydroxide with DPSC, stimulates the recruitment, migration, proliferation and mineralisation of implanted DPSC which results in enhanced levels of regeneration (Ji et al. 2010). Conversely, Park et al. compared the regenerative potential of PDLSC, periapical follicular stem cells (PAFSC) and DPSC and demonstrated negligible differences in regenerative outcomes of DPSC treated defects in comparison with non-treated controls, 8 weeks post implantation (Park et al. 2011). A clinical study (d'Aquino et al. 2009) investigated the treatment of alveolar bone defects, which commonly present as a result of impacted, unerupted or partially erupted wisdom tooth extraction. In order to prevent post-operative bone loss, previously isolated autologous DPSC, integrated into a collagen sponge scaffold, were implanted into the extracted tooth socket at the time of the extraction. The findings of this patient study demonstrated regeneration of autologous bone with evident repair of alveolar bone and reestablishment of periodontal tissue at the affected site (d'Aquino et al. 2009). These data suggest a role for DPSC in regeneration of the periodontium, particularly associated with bone and dentin restoration, however further characterisation is essential to standardise their utilization.

12.6.3 Utilisation and Efficacy of Periodontal Ligament Derived MSC (PDLSC) in Regeneration of Dental Tissues

Features unique to PDL, including rapid matrix turnover and continuous tissue remodelling (Berkovitz 1990; McCulloch et al. 2000) suggest that a pool of MSC exists within this structure. This concept is further supported by evidence of limited self-regeneration of PDL observed during the early stages of periodontal disease (Bartold and Narayanan 1998; Bartold 2006). In comparative analyses, where PDL derived progenitor populations were assessed alongside bone marrow and dental pulp derived stem cells, Seo et al. determined that this population of cells exhibited features both common to MSC and unique to PDLSC (Seo et al. 2004).

A mechanism has been postulated whereby following periodontal tissue damage, in order to restore the attachment complex between the tooth root and the adjacent alveolar bone,

Table 12.4 Dental derived stem cells implanted into dental defects

Defect	Carrier	Animal model	Cell association	Duration of implant	Outcomes	References
Dental pulp stem cells (DPSC)						
Oro-maxillo-facial bone defect	Collagen sponge	Humans	Autologous	Up to 1 year	Implantation of a biocomplex comprised of DPSC and a collagen sponge scaffold resulted in optimal bone repair resulting in complete regeneration of bone at the injury site	d'Aquino et al. (2009)
Half-moon class V cavities in the cervical area of premolars	Glass ionomer	Beagle dogs	Autologous	Not stated	This investigation assessed the effect calcium hydroxide has on the recruitment, proliferation, and mineralisation of DPSC and PDLSC. Calcium hydroxide enhanced regeneration by increasing the recruitment, migration, proliferation and mineralisation of DPSC	Ji et al. (2010)
Apical involvement defect	None mentioned	Beagle dogs	Autologous	8 weeks	The aim of this study was to compare the regenerative capacity of PDLSC, DPSC and PAFSC to see which cell population is the most appropriate for clinical applications. After 8 weeks of implantation the defects which received DPSC were very similar to those of the negative control indicating that DPSC did not promote regeneration. PDLSC were found to have the greatest regenerative capacity	Park et al. (2011)
Bone defects on both sides of the mandible	Platelet-rich plasma (PRP)	Hybrid dog	Allogeneic	2, 4 and 8 weeks	Implanted DPSC generated well-formed mature bone with neovascularization when compared to controls	Yamada et al. (2011)
Periodontal ligament stem cells (PDLSC)						
Periodontal defect surgically created on the buccal cortex of the mandibular molar	Hydroxyapatite/β-tricalcium phosphate particles	Immuno-deficient rats	Xeno-transplantation	6–8 weeks	Implanted PDLSC demonstrated the ability to form cementum/PDL-like structures and aid periodontal tissue repair	Seo et al. (2004)

Periodontal lesion of the maxilla and mandibular first molars	Hydroxyapatite and tricalcium phosphate	Miniature pig	Autologus	12 weeks	Transplanted GFP-labelled PDLSC had excellent capacity to form bone, cementum, and periodontal ligament when transplanted into a surgically created periodontal defect. GFP-labelled cells were identified in the newly formed periodontal bones suggesting that the transplanted cells contributed to new bone formation	Liu et al. (2008)
Saddle-like through-and-through defects	Hydroxyapatite/β-tricalcium phosphate	Beagle dogs	Autologous	8 weeks and 16 weeks	Transplantation of BMSC and PDLSC into peri-implant defects resulted in enhanced bone regeneration. There was no significant difference in regenerative potential between BMSC and PDLSC	Kim et al. (2009)
Half-moon class V cavities in the cervical area of premolars	Glass ionomer	Beagle dogs	Autologous	Not stated	This investigation assessed the effect calcium hydroxide has on the recruitment, proliferation and mineralisation of DPSC and PDLSC. Calcium hydroxide enhanced regeneration by increasing the recruitment, migration, proliferation and mineralisation of PDLSC	Ji et al. (2010)
Apical involvement defect	None mentioned	Beagle dogs	Autologous	8 weeks	The aim of this study was to compare the regenerative capacity of PDLSC, DPSC and PAFSC to see which cell population is the most appropriate for clinical applications. The autologous PDLSC generated new cementum, alveolar bone and Sharpey's fibres of periodontal ligament. PDLSC were found to have the greatest regenerative capacity	Park et al. (2011)
Periodontal ligament progenitor cells (PDLP)						
Deep intrabony defect	Hydroxyapatite/β-tricalcium phosphate	Human	Autologous	3–72 months	Demonstrated potential efficacy and safety of implanting autologous PDLP cells in treatment of human periodontitis with all three patients treated with PDLP cells displaying clinical benefits from the treatment	Feng et al. (2010)

(continued)

Table 12.4 (continued)

Defect	Carrier	Animal model	Cell association	Duration of implant	Outcomes	References
Periodontal ligament (PDL)						
Mucoperiosteal flaps raised between upper canines and premolars	Extracted autologous tooth roots	Miniature pig	Autologous	2, 4, and 12 weeks	Implanted PDL cells lead to the formation of connective tissue resembling periodontal ligament with orientated fibre bundles attached to the host bone and implanted root. There was minimal calcified tissue formed from the implanted PDL cells	Lang et al. (1995)
Furcation and interdental defects	ePTFE membrane	Miniature pig		10, 30 and 90 days	Some defects implanted with PDL cells resulted in extensive cementum and bone formation, this was however variable across different donor cells	Lang et al. (1998)
Periodontal window wounds	Collagen gel	Sprague-Dawley male rats	Autologous and xeno-transplantation	1 and 2 weeks	This study utilised two different methods for tracing implanted PDL cells including; collagen-coated fluorescent beads and cells expressing β-galactosidase. Transplanted PDL cells migrated to their tissue origin and promoted generation of new tissues	Lekic et al. (2001)
Surgically created bone cavities	Tooth root (+/– PDL cells)	Beagle dogs	Not defined	6 weeks	PDL cells had the capacity to form osteoblast lineage cells and contributed to regeneration of periodontal ligament through the formation of new cementum. However the PDL cells had minimal impact on alveolar bone formation	Isaka et al. (2001)
Periodontal ligament cell sheets (PDL cell sheets)						
Dehiscence defects in the bucal surface of the mesial root	Hyaluronic acid	Beagle dog	Autologous	8 weeks	Periodontal regeneration was observed in 3 out of 5 defects with the formation of new bone, periodontal ligament and cementum formation	Akizuki et al. (2005)
Mesial dehiscence defect	None stated	Athymic nude rat	Xeno-transplantation	1 and 4 weeks	Transplantation of HPDL cells resulted in the formation of periodontal ligament-like tissues which included an acellular cementum-like layer and fibrils anchoring into this layer. This work indicates that PDL cell sheets could be useful for periodontal regeneration	Hasegawa et al. (2005)

Periodontal defect in the mesial region of the maxilla and mandibular first molars	Hydroxyapatite and tricalcium phosphate	Miniature pig	Allogeneic	12 weeks	The aim of this study was to assess the immunogenicity and immunomodulation of PDLSC through allogeneic transplants of PDLSC sheets. Allogeneic transplantation of the PDLSC sheets enhanced periodontal tissue repair in a manner similar to that seen in autologous transplants. Additionally there was no evidence of rejection of the allogeneic cells	Ding et al. (2010)
One-wall intrabony defect	Hydroxyapatite/β-tricalcium phosphate and collagen	Beagle dogs	Autologous	8 weeks	The aim of this study was to ascertain if cell sheets derived from bone marrow cells and PDLC could enhance periodontal regeneration. Both the PDLC and bone marrow derived cell sheets enhanced periodontal regeneration with the formation of new cementum and well-oriented PDL fibres. PDL cell sheets had more regenerative capacity than the bone marrow sheets	Tsumanuma et al. (2011)
Periodontal ligament stem cells (PDLSC) and stem cells from apical papilla (SCAP)						
Cavity left after extraction of lower incisor	Root shaped HA/TCP block and gel foam	Miniature pig	Not defined	3 months	This study implanted PDLSC in combination with SCAP stem cells in an attempt to generate a root/periodontal complex capable of supporting a porcelain crown. Together, this strategy lead to the formation of bio-roots with significantly better compression strength than defects which did not receive stem cells	Sonoyama et al. (2006)
Periapical follicular stem cells (PAFSC)						
Apical involvement defect	None mentioned	Beagle dogs	Autologous	8 weeks	The aim of this study was compare the regenerative capacity of PDLSC, DPSC and PAFSC to see which cell population is the most appropriate for clinical applications. The autologous PAFSC generated new cementum, alveolar bone and Sharpey's fibres of periodontal ligament. However PDLSC were found to have the best regenerative capacity	Park et al. (2011)

(continued)

Table 12.4 (continued)

Defect	Carrier	Animal model	Cell association	Duration of implant	Outcomes	References
Dental bud cells (DBC)						
Alveolar sockets from tooth removal	Gelatin/ chondroitin-6-sulphate/hyaluronan tri-copolymer scaffold	Miniature pigs	Autologous	40 weeks	This study investigated the effect that bone marrow fluid has on DBC in terms of their ability to achieve tooth regeneration. Results identified that bone marrow fluid in combination with DBCs promoted tooth regeneration	Akizuki et al. (2005)
Stem cells from deciduous teeth (SHED)						
Critical sized bone defects in the parasymphyseal region of the mandible	Hydroxyapatite/β-tricalcium phosphate	Miniature pig	Autologus	2, 4 and 24 weeks	Implanted stem cells derived from miniature pig deciduous teeth (SPD) differentiated directly into new bone resulting in the formation of markedly more new bone formed in the defect site	Zheng et al. (2009)
Bone defects on both sides of the mandible	Platelet-rich plasma (PRP)	Hybrid dog	Allogeneic	2, 4 and 8 weeks	Implanted deciduous teeth stem cells (DTSCs) generated well-formed mature bone with neovascularization when compared to controls	Yamada et al. (2011)

mesenchymal progenitors are recruited and activated to proliferate and terminally differentiate into ligament forming cells or mineral secreting cementoblasts (Bartold and Narayanan 1998, 2006). Extensive studies have been conducted to assess the periodontal regenerative capacity of PDLSC in an array of animal models and two published human clinical studies. The findings of these studies are summarised in Table 12.4. Multiple studies, conducted in animal models, demonstrate the capacity of PDLSC to form periodontal ligament like structures with organised connective tissue resembling periodontal ligament and oriented fibre bundles attached to alveolar bone and tooth root, penetrating the bone and root surfaces resembling Sharpey's fibres (Sonoyama et al. 2006; Lang et al. 1995). In a proof-of-concept study of functional tooth regeneration, two distinct autologous stem cell populations (BMSC and PDLSC) in conjunction with artificial dental crowns, demonstrated regeneration of the root/periodontal structure (Kim et al. 2009). Moreover, this study resulted, not only in regeneration of both of the tissues, but the newly formed bio-roots displayed greater compressive strength when compared to previously tested HA/TCP carriers. The findings of this pilot study indicate that both populations of MSC (BMSC and PDLSC on HA/TCP) showed a comparable potential involvement in the regeneration of alveolar bone and were more effective than HA/TCP controls that did not contain MSC.

More recently, two human clinical pilot studies, reported increased function of damaged periodontal tissues in patients that received autologous ex vivo expanded PDL derived progenitor cells (Feng et al. 2010; Gault et al. 2010). Whilst assessment of tissue regeneration has been limited to indirect clinical observations, these preliminary findings illustrated safety and some efficacy associated with implantation of PDLSC in a clinical setting. In one of the studies, autologous progenitor cells derived from periodontal ligament, combined with a bone grafting substrate, were implanted into deep periodontal pockets, to reconstruct periodontal bony defects, and the patients were monitored over 72 months (Feng et al. 2010). This study illustrated that two out of three patients presented with reinstatement of healthy tissue. In accord, the third patient presented with increased tooth stability, reduced probing depth, and an overall improvement of attachment gain (Feng et al. 2010). The other clinical study demonstrated that implantation of apatite covered titanium cylinders coated by a bio-engineered periodontal ligament-like sheath, resulted in formation of a cementum like layer and bone tissue surrounding the implant (Gault et al. 2010). Organization of the newly formed structures indicated effective biological communication and integration of the implanted material and the host tissue (Gault et al. 2010). These trials demonstrate that the application of autologous PDL progenitors, as a part of cell based therapeutic modality for regenerative dentistry is effective in treatment of sites affected by periodontitis. To date PDLSC exhibit the greatest potential in regeneration of periodontium tissues and as such are currently most stringently investigated.

12.6.4 Utilisation and Efficacy of Stem Cells Derived from Human Exfoliated Deciduous Teeth (SHED) in Regeneration of Dental Tissues

SHED are a readily accessible source of dental derived progenitor cells as this population is obtained from the coronal pulp of exfoliated deciduous teeth. They are a multipotent stem cell population with differentiation capacity into mesenchymal and non-mesenchymal derived cells in vitro (Miura et al. 2003; Cordeiro et al. 2008; Sakai et al. 2010; Shi et al. 2005; Wang et al. 2010). In addition SHED display the potential to generate bone and dentin as well as induce formation of dental pulp-like complexes in vivo (Miura et al. 2003; Cordeiro et al. 2008; Sakai et al. 2010).

One study has assessed the capacity of transplanted allogeneic progenitor cells derived from

deciduous teeth of miniature pigs, termed SPD, to regenerate artificially created mandibular bone defects (Zheng et al. 2009). The findings illustrated direct contribution of SPD to the newly formed bone within the critical sized defect (Zheng et al. 2009). Similarly, in a canine model, dog derived allogeneic SHED-like cells were transplanted into mandibular defects to assess their regenerative capacity (Yamada et al. 2011). The progenitor populations were combined with platelet-rich plasma prior to implantation and demonstrated the ability to generate new bone and support neovascularisation of the tissue (Yamada et al. 2011).

Collectively, these data (reviewed in Table 12.4) suggest that SHED cells potentially present as an alternative progenitor population for therapeutic utilization in treatment of periodontal disease.

12.6.5 Utilisation and Efficacy of Stem Cells from Apical Papilla (SCAP) in Regeneration of Dental Tissues

Apical papilla provides an additional resource of dental derived stem cell populations (Sonoyama et al. 2006, 2008). Stem cells from apical papilla (SCAP) present a unique progenitor subtype as attainment of these cells is only possible during root development and prior to tooth eruption as they reside on the tips of growing immature permanent tooth roots (Sonoyama et al. 2006, 2008). It has been demonstrated that co-transplantation of SCAP alongside PDLSC on specially constructed bioscaffolds into tooth sockets of miniature-pigs, restores normal tooth function by generation of root/periodontal structures competently supporting a porcelain crown (Sonoyama et al. 2006). Currently, further characterisation is instrumental to define a potential role for utilization of SCAP as a treatment alternative for periodontal regeneration.

12.6.6 Utilisation and Efficacy of Dental Follicle Derived Stem Cells (DFC) in Regeneration of Dental Tissues

The dental follicle is a connective tissue sac surrounding the developing tooth germ preceding tooth eruption. It has been proposed that this structure, derived from the ectomesenchyme, contains cementoblast, osteoblast and PDL progenitor cells (Rutherford et al. 1993). It has previously been demonstrated that DFC, in the presence of a dentin matrix scaffold, display odontogenic differentiation capacity and contribute to dentin formation (Guo et al. 2009, 2011; Li et al. 2011). Furthermore, DFC also exhibit the ability to form periodontal ligament in vivo (Yokoi et al. 2007). In vitro differentiation studies have suggested that DFC can differentiate into cementum (Morsczeck et al. 2005) and it was later found that, when implanted into immunodeficient (SCID) mice on hydroxyapatite beads, DFC contributed to the formation of fibrous tissue and cementum-like matrix on the surface of the beads (Handa et al. 2002a, b).

Guo et al. assessed the ability of DFC to support and participate in tooth root formation (Guo et al. 2011; Chung et al. 2011). In this study DFC and a dentin matrix treated scaffold were implanted into the non-mineralised omental pocket, the highly mineralised skull and the inductive alveolar fossa of rats (Guo et al. 2011). The findings indicated that DFC contributed to dentin regeneration within the omental pockets and contributed to mineralized matrix formation in the skull defects. Moreover, within alveolar fossa, DFCs facilitated generation of root-like structures and pulp-dentin complexes with a layer of attachment tissue resembling PDL-cementum integration into the host alveolar bone (Guo et al. 2011). This study highlighted the capacity of DFC to regenerate the tooth root whilst it also demonstrated the role played by micro-environment during implantation (Guo et al. 2011).

It is evident that the application of MSC based tissue engineering concepts for periodontal regeneration has been widely investigated in a number of small and large animal models, including two clinical trials thus far (Hynes et al. 2012). The collective findings indicate that utilization of MSC presents a safe and efficacious therapeutic modality but considering current and potential future challenges associated with their attainment, population heterogeneity and ex-vivo manipulation, alternative sources of progenitors, circumventing these limitation, are currently being investigated.

12.6.7 Utilisation and Efficacy of Induced Pluripotent Stem (iPS) Cells in Regeneration of Dental Tissues

Despite the developmental potential of ESC, which are derived from the early mammalian embryo with extensive proliferative and all embryonic germ layer differentiation capacities, the use of embryos for procurement of human ESC lines is associated with considerable ethical concerns. These limitations encouraged and drove cellular reprogramming experiments whereby somatic cells are genetically manipulated to dedifferentiate to the state of pluripotency, which results in the generation of induced pluripotent stem (iPS) cells. iPS cells are derived by forced expression of four transcription factors (OCT4, SOX2, C-MYC and KLF4) in somatic cells (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Yu et al. 2007). iPSC possess features characteristic to embryonic stem cells, including proliferation and differentiation capacities, gene and protein expression, antigen presentation and others. Yet, their utilization does not raise concerns related to use of embryos. In this rapidly developing field, iPS cells have now been generated from numerous dental derived tissues including SHED (Yan et al. 2010), SCAP (Tamaoki et al. 2010), dental pulp (Beltrao-Braga et al. 2011), oral mucosa (Miyoshi et al. 2010), dental pulp of third molar mesenchymal stromal cells (Oda et al. 2010), gingival fibroblasts (Egusa et al. 2010; Wada et al. 2011) and periodontal ligament fibroblasts (Wada et al. 2011).

To date, one study has utilized iPSC for periodontal regeneration in a mouse periodontal fenestration defect model (Duan et al. 2011). In this study iPSC, combined with enamel matrix derivatives (EMD) or Emdogain®, were implanted on a silk fibrin scaffold gel into fenestration defects and enhanced generation of new cementum, alveolar bone and periodontal ligament (Duan et al. 2011). In vitro studies assessing the effect EMD has on iPS cells revealed that EMD promoted iPS cells to differentiate into osteogenic cells whilst inhibiting cell maturation and mineralization (Duan et al. 2011).

Although utilisation of iPS cells for periodontal regeneration appears to be promising, the required alterations to their genetic composition may result in uncontrolled cell growth and undirected development, which in turn minimises their behavioural predictability, an essential factor for clinical utilization. Furthermore, like ESC, undifferentiated iPSC possess tumorigenic tendencies, which present a significant limitation in therapeutic applications (Bongso et al. 2008; Lee et al. 2009). In light of this, induction of iPS toward a more differentiated, MSC-like state, prior to their utilization in patients, has been proposed as a measure to enable generation of sufficient cell numbers of homogenous progenitor populations but eliminate concerns related to uncontrolled cellular proliferation or differentiation (Hynes et al. 2013, 2014). Recent studies have demonstrated a reproducible method of generating MSC-like cells from different iPSC populations and have further illustrated their capacity to regenerate periodontal defects in mice (Hynes et al. 2013, 2014).

12.7 Future Prospects for Stem Cells Based Therapies in Periodontal Tissue Regeneration

Whilst stem cells present as an attractive candidate for therapeutic use, there are numerous challenges associated with the use of stem cells

and tissue engineering in cell based therapy. Their clinical application is uncertain due to lack of knowledge and understanding of critical process involved in maintaining the potency of these cells. The heterogeneity present within such cell populations accounts for their compromised and undefined profiles. This is manifested in terms of morphological variability, inconsistency in levels of proliferation and differentiation potentials, as well as differences in patterns of gene and protein expression profiles of the individual cell population subsets (Colter et al. 2001; Menicanin et al. 2010; Phinney 2007; Ho et al. 2008). The understanding of molecular mechanisms vital to proliferation, differentiation, engraftment and homing of stem cells is essential in order to expand their potential clinical applications. As such, some of the current limitations to their clinical utilization which may lead to lack of reproducibility and hence impair their therapeutic application need to be addressed. These include identification and isolation of appropriate precursor cell types, establishment of optimal growth and differentiation conditions in vitro and efficient modes of delivery for their successful transplantation (Gronthos et al. 2000). Furthermore, biotechnological engineering of biomaterial scaffolds to support, complement and enhance the regenerative capacity of integrated stem cells is still undergoing vast developmental advancements. Finally, in order for these cells to be used in humans, stringent preparation protocols according to the principles of Good Clinical Practice (GCP) and Good Manufacturing Practice (GMP) will be required. Both of these practices will require approval through appropriate government agencies.

Published findings of pre-clinical studies investigating stem cell based tissue regeneration reviewed here identify gaps in our current understanding and highlight obstacles that need to be addressed before cell-based regenerative therapy applications, for treatment of tissues lost due to periodontal disease, become available to practising dentists.

12.8 Conclusion

Cementum and periodontal ligament tissues form the attachment complex of the tooth and hold a fundamental part in proprioception, structural support, mechanical load bearing and physiological maintenance of homeostasis within the periodontium. Considering their significance, functionality of these tissue components is essential to a healthy and operative oral cavity.

Due to the complexity of the two periodontal tissues, complete regeneration may require implantation of more than one progenitor population and composition of carrier biomaterials needs to account for the anatomical differences between the structures within the periodontium (Bartold et al. 2006). A hypothetical approach, introduced in a review by Bartold et al. (2006), proposes that technological advances in tissue engineering could be directed towards development of a multi-surface matrix, housing cementoblasts on one side, facing the tooth root, and osteoblasts on the other, proximal to the alveolar bone. Such constructs would allow for the implanted cell carriers to guide tissue regeneration architecturally, morphologically and spatially analogous to the pre-existing structures. To accomplish complete regeneration of these two components of the periodontium using cell based therapy modalities, a number of essential factors need to be addressed. These include support of integration and long term survival of implanted cells, provision of appropriate induction and stimulation signals for cellular self-renewal, proliferation, differentiation and communication with the host microenvironment, resulting in recruitment of endogenous cells and supportive morphogenic molecules. With recent advances in characterisation of progenitor cell populations and elucidation of regulatory mechanisms involved in periodontal development, coupled with fast paced progress in generation of biocompatible scaffolds, we have the scope to optimise cell based tissue engineering protocols and apply them therapeutically to mimic the biological processes of the healing periodontium.

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Amelogenin in Enamel Tissue Engineering

13

Vuk Uskoković

Abstract

In this chapter the basic premises, the recent findings and the future challenges in the use of amelogenin for enamel tissue engineering are being discoursed on. Results emerging from the experiments performed to assess the fundamental physicochemical mechanisms of the interaction of amelogenin, the main protein of the enamel matrix, and the growing crystals of apatite, are mentioned, alongside a moderately comprehensive literature review of the subject at hand. The clinical importance of understanding this protein/mineral interaction at the nanoscale are highlighted as well as the potential for tooth enamel to act as an excellent model system for studying some of the essential aspects of biomineralization processes in general. The dominant paradigm stating that amelogenin directs the uniaxial growth of apatite crystals in enamel by slowing down the growth of (hk0) faces on which it adheres is being questioned based on the results demonstrating the ability of amelogenin to promote the nucleation and crystal growth of apatite under constant titration conditions designed to mimic those present in the developing enamel matrix. The role of numerous minor components of the enamel matrix is being highlighted as essential and impossible to compensate for by utilizing its more abundant ingredients only. It is concluded that the three major aspects of amelogenesis outlined hereby – (1) the assembly of amelogenin and other enamel matrix proteins, (2) the proteolytic activity, and (3) crystallization – need to be in precise synergy with each other in order for the grounds for the proper imitation of amelogenesis in the lab to be created.

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Keywords

Apatite • Bone • Calcium phosphate • Enamel • Tooth • Nanoparticle • Hard tissue engineering

13.1 Introduction

> Progress in the war against disease results from discoveries in remote and unexpected fields of medicine and the underlying sciences. (Bush 1945) A Report to the U.S. President by Vannevar Bush, Director of the Office of Scientific Research and Development, July 1945.

Understanding the interaction of organic and inorganic phases during biomineralization events at the atomic scale would present a milestone with repercussions for an array of biomedical fields revolving around teeth, bone and other organs composed of mineralized tissues. Thanks to its relative structural simplicity in the realm of mammalian hard tissues, tooth enamel presents an excellent model system for studying this interaction, even though reasonable concerns exist that the genesis of it may be governed by fundamentally different mechanisms compared to those present in bone. Namely, while nanoparticulate mineral particles comprising bone form by nucleation on the active surface of highly phosphorylated bone-specific proteins, exceptionally long crystals of the mineral phase form in enamel by the action of proteins with significantly lesser nucleation and growth potential.

As it is usually the case, fundamental insights of any nature create either an immediate or delayed effect on the way certain issues are practically solved and hopes exist that understanding the interaction between amelogenin, the main protein of the enamel matrix, and the crystals forming with a specific and highly defined structure from within its gelatinous volume, would revolutionize the clinical approach to dental restoration and, possibly, change the mainstream approach to orthopedic therapies too.

What follows is a short discourse on the current state of our knowledge on the interaction between these two species, amelogenin and apatite, in the course of which the strongest and, as it usually is, the most brittle of all mammalian tissues forms: dental enamel (Uskoković et al. 2010). Results of the recent studies set up to simultaneously yield an insight into the fundamental nature of this interaction and utilize it for the purpose of growing enamel in vitro will be mentioned too.

13.2 The Structure and Composition of Mature Enamel

Enamel is composed of 4–8 μm wide rod-shaped bundles of apatite fibers whose diameter is in the range of 40–60 nm and whose aspect ratio reaches up to $3 \cdot 10^4$ (Fig. 13.1). Apatite is the least soluble phase of calcium phosphates, with

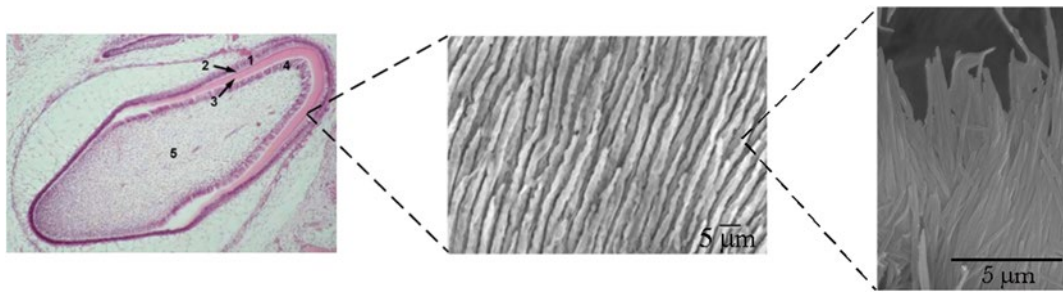

Fig. 13.1 Histological section of the developing human tooth in the maturation stage (*left*) and micrographs showing the parallel arrangement of enamel rods (*middle*) and the parallel arrangement of apatite nanofibers within each enamel rod (*right*). *1* ameloblasts, *2* enamel, *3* dentin, *4* odontoblasts, *5* pulp

the crystal structure adopting pseudo-hexagonal $P6_3/m$ space group. Owing to its structural flexibility, it allows for a moderate amount of substitution of its dominant, Ca^{2+} and $PO4^{3-}$ ions with a variety of biological microelements, so that its composition is most accurately given as $(Ca,Z)_{10}(PO_4,Y)_6(OH,X)_2$, where $Z = Na^+$, Mg^{2+}, K^+, Sr^{2+}, etc., $Y = CO_3^{2-}$, HPO_4^{2-}, and $X = Cl^-$, F^-. Some of these ions, such as Na^+ or Mg^{2+}, increase the solubility of the compound, while others, such as F^-, decrease it.

The great majority of enamel, 96–98 wt.%, is of mineral composition, which is more than in any other mammalian hard tissue. Water, fatty acids and various peptides account for the rest 2–4 wt.%. Discussions have been sparked recently about the nature of this miniscule amount of impurities. Namely, after it was found out that only 0.02 wt.% of glycoprotein in the spine of sea urchin (i.e., ~10 proteins per 10^6 unit cells) is enough to efficiently absorb the energy from propagating cracks and markedly increase the strength of the material (Stupp and Braun 1997), the long-lasting paradigm stating that these impurities present accidental remnants of incomplete proteolytic digestion of the enamel matrix has been questioned and challenged with a hypothesis that these peptides are purposefully left in the tissue so as to provide it with greater resistance to fracture under compression or shear.

Approximately one thousand apatite fibers are assembled in bundles within each enamel rod, 5–12 million of which are found lined up in rows per single tooth crown. The size and the packing density of the crystals of apatite comprising enamel are highly different from those comprising bone. Whereas bone consists of plate-shaped nanoscopic crystals with 20°×°10°×°2 nm in size on average (Eppell et al. 2001), the crystals of enamel, albeit of the same composition, are approximately 1,000 times longer along their [001], c-axis. In part, this has been made possible by the fact that enamel is a tissue that does not depend on intrinsic cellular proliferation in the course of its lifetime, the reason for which bone regeneration materials are nowadays designed to be porous so as to allow for the proliferation of bone cells across its volume (Cai et al. 2007). These structural dissimilarities between enamel and bone suggest that the mechanisms of their respective formation may be vastly different.

13.3 The Basic Model of Amelogenesis and a Question Mark Over It

The process of enamel growth, a.k.a. amelogenesis, is one of the slowest morphogenetic processes, taking more time to complete than it is needed for the embryo to form in utero, which speaks well in favor of its extraordinary complexity. Growing at the appositional rate of ~2–4 μm per day, enamel forms over a period of approximately 4 years in a process that involves a controlled crystal growth through gelatinous enamel matrix composed of a number of proteins at the overall concentration of 200–300 mg/ml, 90 % of which has been identified as a single protein: amelogenin. The remaining 10 % is comprised of other proteins: ameloblastin, enamelin, serum albumin, amelotin, and proteolytic enzymes. Together, they assemble into a scaffold that serves as a template for the uniaxial growth of apatite crystals.

The reigning model of enamel growth is built on the assumption that amelogenin self-assembles into narrowly disperse nanospheres with ~20 nm in diameter (Fig. 13.2a), which then align onto (hk0) faces of apatite crystals, blocking the adherence of the ionic growth units, Ca^{2+}, $H_xPO_4^{x-3}$ and OH^-, onto those faces and allowing for the crystal growth to occur only in the direction of [001] axis (Fig. 13.2b).

There are multiple grounds on which this paradigmatic explanation can be questioned. Firstly, recombinant amelogenin forms such nanospherical entities when suspended in water, but their existence in vivo has not been accurately pinpointed to this date. DNA molecules assemble into a variety of morphologies, from cubes to triangles to pentagons to hexagons to octahedrons (Aldaye et al. 2008) and could be used for the assembly of nanoparticles into superlattices (Young et al. 2014) and other sophisticated geometries that are otherwise difficult to obtain (Liu et al. 2013) wherefrom their use in organic electronics has begun to be intensely researched

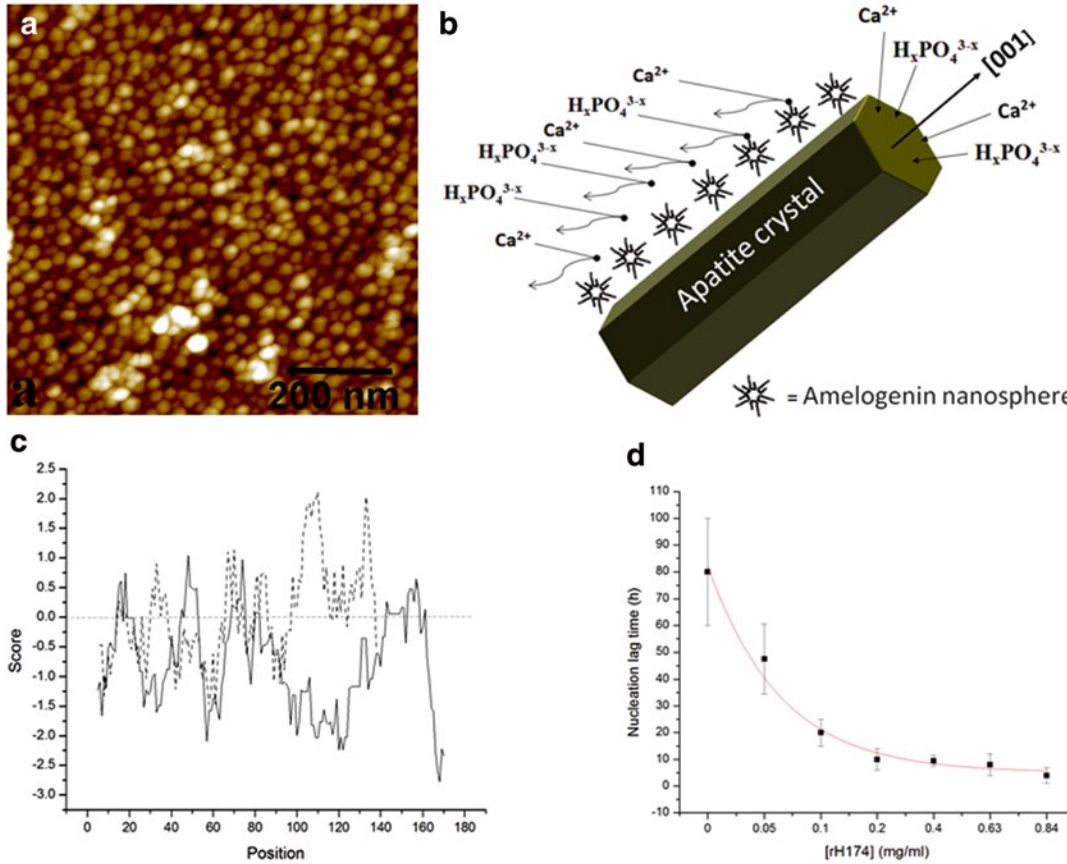

Fig. 13.2 (**a**) Monodisperse recombinant full-length human amelogenin nanospheres forming in water. Forty to sixty amelogenin molecules form a single nanospherical aggregate with 20–40 nm in size. (**b**) Schematic depiction of the crystal growth during amelogenesis according to the nowadays questionable dominant paradigm. (**c**) Hydrophobicity plots obtained using ExPASy ProtScale Kyte & Doolittle model (window size = 9; linear weight variation model) for human amelogenin (*straight line*) and human hemoglobin alpha chain (*dashed line*). The positive score on the diagram denotes hydrophobic sequences. (**d**) Nucleation lag time for the precipitation of apatite from aqueous suspensions of human recombinant full-length amelogenin in the concentration range of 0–840 μg/ml ($[KH_2PO_4] = 1.0$ M; $[CaCl_2] = 1.67$ M; pH = 7.4, T = 37 °C)

as well (Hamedi et al. 2012). None of these potentially practical potentials of DNA need be necessarily tied to its biological function as a storage place for the genetic content of the cell. Similarly, detection of morphologies adopted by amelogenin assemblies in vitro, be they nanospheres, nanobeads or nanofilaments (Martinez-Avila et al. 2012), may be irrelevant for explaining the biologically relevant forms and functions thereof.

Secondly, the abovementioned model of amelogenesis at the level of organic/inorganic interface assumes hydrophobicity of amelogenin, as the direct result of which it is supposed to act as an inhibitor rather than a promoter of crystallization of apatite. This common assumption is, however, incorrect, as amelogenin, like every other protein, contains alternately changing hydrophilic and hydrophobic sequences along its primary structure (Uskoković et al. 2011a). As shown in Fig. 13.2c, although amelogenin as a whole is still more hydrophobic than most proteins, it is, for example, more hydrophilic than human hemoglobin alpha chain. Moreover, the intensely hydrophilic 13-amino-acid-long segment towards the C-terminus of the protein

suggests its amphiphilicity, which may be crucial in endowing it with the ability to form nanospherical assemblies in water, similar in form to reverse micelles (Uskoković et al. 2005), with the hydrophilic ends exposed to the polar environment and the rest of the protein folded internally. In view of this, it is logical to expect that amelogenin is capable of promoting the nucleation of apatite in vitro. As shown in Fig. 13.2d, the nucleation lag time for precipitation of apatite from metastable solutions of KH_2PO_4 and $CaCl_2$ at the physiological pH decreases in direct proportion with the concentration of human recombinant full-length amelogenin (Uskoković et al. 2011b). Compared to recombinant human amelogenin used in these studies (rH174) (Uskoković et al. 2008), which lacks phosphorylation on ^{16}Ser residue, the biological variant of it is expected to have an even more pronounced propensity to stimulate the nucleation of apatite, considering the apatite-nucleation potential of abundantly phosphorylated extracellular bone and dentin matrix proteins. Other studies, having elucidated the conditions under which amelogenin can promote the nucleation of apatite (Wang et al. 2008; Tarasevich et al. 2007), came to a similar conclusion, thus implicitly questioning the correctness of the dominant paradigm in this field.

Another cue in terms of inverting the paradigm comes from the fact that adsorption of amelogenin onto a growing crystal surface appears to be the first step prior to the induction of surface-specific, controlled crystal growth (Uskoković et al. 2011c; Habelitz et al. 2004, 2005a). Figure 13.3a demonstrates a typical surface growth of apatite crystals immersed in amelogenin sols under low supersaturation ratios and a constant titration regimen. The overall process could be divided to three stages. In the first stage, amelogenin nanospheres from the solution adsorb onto the crystal growth substrates, forming miniature islands on them. In the second stage, the growth is observed to occur exclusively from inside of the amelogenin deposits, an effect that would be virtually impossible had it not been for the ability of amelogenin to promote apatite nucleation and growth. Finally, in the third stage, amelogenin deposits are fully replaced by the elongated crystals of apatite (Fig. 13.3a). The idea that adsorption of the protein implies the hindrance of the crystal growth on the binding sites is thus directly refuted. Osteocalcin, a protein involved in mineralization of bone, for example, does not constrain the growth of crystal planes, even though it binds to them (Robinson 2006).

Amelogenin may be thus said to act not as an inhibitor of crystal growth, but as a bridge between the ionic solutes or semisolid complexes and the crystalline surface that they are anchored to. One model based on hypothesized β-spirals formed by a series of β-turns in the secondary structure of folded amelogenin and their channeling of Ca^{2+} ions to the mineralization front was previously proposed (Renugopalakrishnan et al. 1989; Zheng et al. 1987). This view of amelogenin as an ion-channeling molecular entity also bears a resemblance to the model describing the formation of silicon nanowires in the so-called vapor-liquid-solid process, during which nanodroplets of gold deposited on top of silicon wafers attract silicon atoms from the vapor. Under sufficiently slowly increased supersaturation (Fig. 13.3c), the conditions for heterogeneous nucleation are approached without crossing the boundary for homogeneous nucleation too, leading to a highly specific growth from the underlying surface and, in this case, resulting in well-aligned nanowires oriented perpendicular to the substrate and perfectly parallel to each other. Nanowires obtained in one such process are shown in Fig. 13.3d.

Moreover, a model based on an analogy between (a) the role of amelogenin assemblies in channeling the controlled transfer of ions from the solution onto the growing faces, and (b) the ion-tunneling effect through the hydrophobic center of ion channel proteins located at the cell membrane (Murakami 1995), could thus be proposed. Namely, ionophores need to be hydrophobic in order to be soluble in the lipid membrane layers, whereas this internal hydrophobicity is also crucial in terms of enabling the ion-channeling effect through their core (Nelson and Cox 2004). The presence of hydrophobic domains

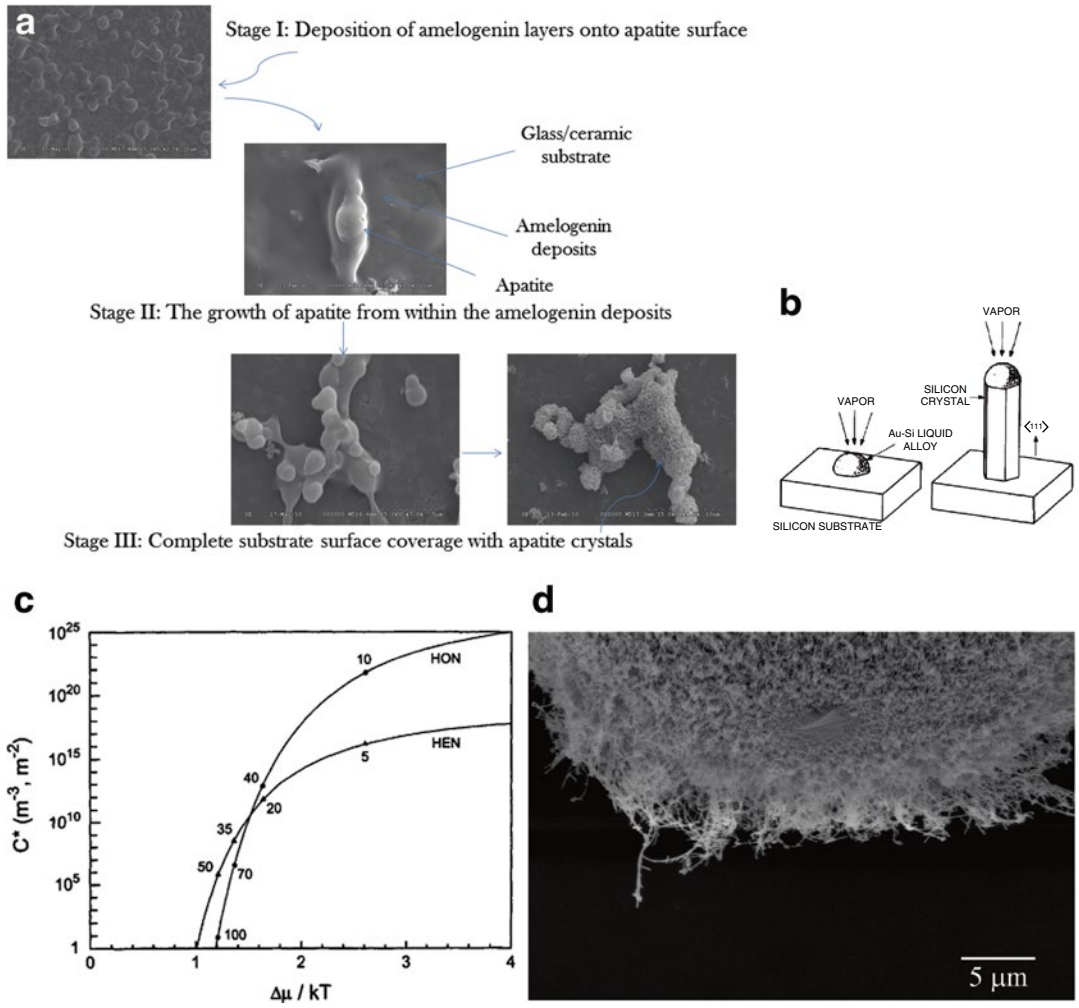

Fig. 13.3 (**a**) Evolution of the surface layers on crystal growth substrates in the course of a typical, 7-day long continuous titration experiment, during which amelogenin sols at the concentration of 400 μg/ml and a relatively high initial concentration of KH_2PO_4 are titrated with $CaCl_2$ and KH_2PO_4. (**b**) This process bears resemblance to the mechanism for the formation of silicon nanowires via the action of gold nanodroplets sputtered over the substrate surface and used as a means for ensuring sufficiently slow increase in the supersaturation ratio of silicon atoms introduced to them through vapor (Reproduced with permission from Wagner and Ellis (1964)). (**c**) As the supersaturation ratio for active species in the system is gradually increased and exceeds 1, the conditions for heterogeneous nucleation (HEN) are hit before those for homogeneous one (HON) (Reprinted with permission from Kashchiev (2000)). (**d**) Silicon nanowires outgrown from silica beads using the gold nanodroplet-assisted chemical vapor deposition process whose mechanism is similar to the growth of apatite from the surface of amelogenin-covered apatite

within amelogenin structure may be similarly important in ensuring the proper "gating" of the units of growth, as it occurs in ion channels on cell membranes (Zhaohua et al. 2008). In that sense, the 100–150 kDa lipoprotein ATPase complex that simultaneously releases the bound Ca^{2+} ions on one side and protons on the other may serve as a model for the possible role that amelogenin may play in the transport of ions onto the growing apatite faces that it is physisorbed to. This effect is particularly relevant since, as it could be seen from Eq. (13.1), an increase in the acidity of the medium is entailed by the formation of apatite. This implies that the conditions

for simultaneous controlled delivery of ions to the mineralization front and dissipation of the released H^+/H_3O^+ ions to the surrounding amelogenin gel need to be ensured for the conditions for the interaction between amelogenin and apatite to be set properly.

$$10Ca^{2+} + 6HPO_4^{2-} + 2H_2O = Ca_{10}(PO_4)_6(OH)_2 + 8H^+ \quad (13.1)$$

The idea that amelogenin hinders the crystal growth has found its support in the observations of disorganized apatite fibers in the amelogenin knockout mouse (Gibson et al. 2001). The fact that enamel formed in the absence of amelogenin is pathologically thin, however, could counteract this idea by indicating that amelogenin might be involved in the process of extension of the primary crystals by means of its ability to promote uniaxial crystal growth. Finally, the ability to hinder or foster crystallization oftentimes depends on the protein concentration (Gower 2008) and other structural modifications it may undergo, so that around a single protein wrapped around a single crystal could be expected to play the role of inhibitor of the growth of one and of promoter of the growth of other faces. This brings us over to the proteolytic aspect of amelogenesis, without the mention of which no truly consistent model thereof could be proposed.

13.4 The Role of Proteases or How Amelogenin Needs to Disappear in Order for Apatite to Appear

Those with a developed sense for aesthetics may agree that the interaction between amelogenin and apatite has a poetic beauty intrinsic to it. Namely, enamel is the only tissue in the human body whose formation is conditioned by the gradual disappearance of the agents that direct this process. One of the most intriguing features of amelogenesis comes from the fact that not only does its final product, the tooth enamel, present the hardest tissue in the vertebrate body, but its high mineral content coupled with an ultrafine architecture implies that in this process the extracellular matrix directs not only the crystal growth, but its own constructive degradation too. In that sense, the enamel protein matrix is unique in the realm of biomineralization as it fulfills the old truism of biology: "Intercellular matrix exists to be destroyed". Its role could also be described by the ancient Biblical verses: "Verily, verily, I say unto you, except a corn of wheat fall into the ground and die, it abideth alone: but if it die, it bringeth forth much fruit" (The Holy Bible 1609). One could even argue that this also makes amelogenesis somewhat a more intricate mineralization process compared to dentinogenesis during which the collagenous protein matrix essentially remains intact and kept in the same place. In view of this, understanding amelogenesis becomes directly conditioned by understanding the effects of the enzymatic hydrolysis of amelogenin on the crystal formation.

The major proteases of the enamel matrix include matrix metalloproteinase-20 (MMP-20, a.k.a. enamelysin), enamel matrix serine protease 1 (EMSP1, a.k.a. kallikrein-4), and cathepsin B. They are secreted into the extracellular space by ameloblasts with the role of catalyzing hydrolysis of specific peptide bonds in amelogenin molecules. An increasing amount of evidence suggests that the initial cleavage products carry out an array of assembly-related functions in the developing enamel matrix (Bartlett and Simmer 1999). The main support for this idea comes from the fact that enamel matrix proteases are expressed early during development. In fact, the initially secreted nascent proteins are present in the enamel matrix in a transient form and are relatively quickly processed to generate a wide spectrum of smaller peptides. The nascent amelogenin is thus broken down to several fragments that serve specific roles in the protein assembly of protein and the mineral growth.

For example, the expression of MMP-20, the enamel matrix protease hydrolyzing amelogenin

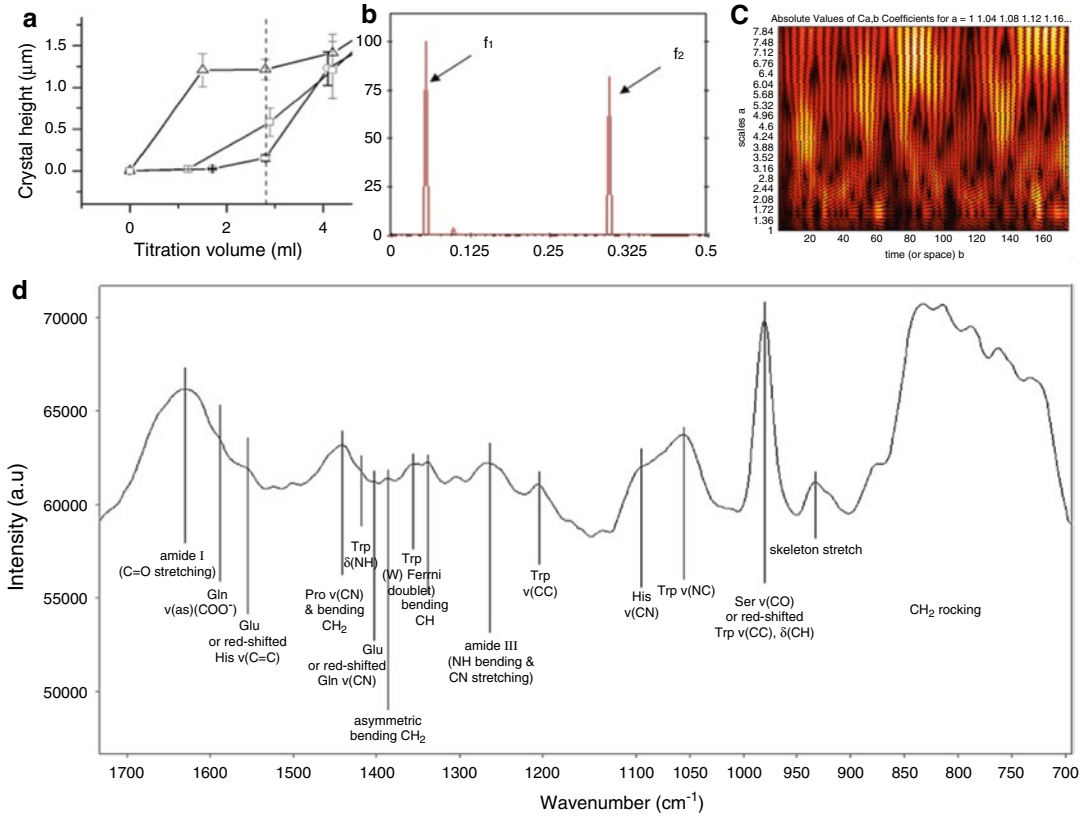

Fig. 13.4 (**a**) The average height of apatite crystals grown after different titration volumes compared between 0.4 mg/ml rH174 sample without MMP-20 (-o-), with MMP-20 in 10^5:1 weight ratio (-□-), and with MMP-20 in 10^3:1 weight ratio (-Δ-) with respect to rH174. (**b**) Raman spectrum of recombinant full-length human amelogenin dispersed in water at room temperature. (**c**) Multiple cross-spectral function and (**d**) wavelet scalogram of human amelogenin, showing the two characteristic radio frequencies for the protein to lie at $f_1 = 0.0547$ and $f_2 = 0.3438$ and the two "hottest spots" to be ^{70}Val–^{90}His and the C-terminal sequence from ^{145}Phe to ^{165}Thr, respectively, along with the primary sequence of the protein. For the relationship between the numerical values given here and the electron-ion interaction potential that describes the average energy states of all valence electrons in an amino acid, the variable used as a basis for the given calculations (See Ćosić and Pirogova 2007). The amino acid sequence of human amelogenin (h174) is the following: PLPPHPGHPGYINFSYEVLTPLKWYQ SIRPPYPSYGYEPMGGWLHHQIIPVLSQQHPP THTLQPHHHIPVVPAQQPVIPQQPMMPVPG QHSMTPIQHHQPNLPPPAQQPYQPQPVQPQPHQP MQPQP

in a highly controlled manner, peaks during the secretory stage and then gradually drops during the maturation, just as it is the case with amelogenin (Bartlett et al. 1998). The constancy of the ratio between enamel matrix components throughout relatively long periods of time (Simmer and Hu 2002) implies that the rate of generation and secretion of amelogenin corresponds to the rate of its cleavage. In view of this, enamel proteases might carry out not only the function of degrading amelogenin so as to provide free space for the sideway growth of enamel crystals, but also act as essential regulators of the activity of amelogenin and other enamel matrix proteins. The structure of amelogenin may thus be such that it contains several functional domains that become activated for different purposes and at different stages of amelogenesis (Snead 2003).

It has been shown that coupling the proteolysis using MMP-20 to apatite growth in the presence of amelogenin has the same effect on increasing the rate of crystal formation as quadrupling the concentration of amelogenin (Fig. 13.4a). These and similar findings have spo-

ken in favor of the immense importance of MMP-20 for the process of amelogenesis (Uskoković et al. 2011d). The essentiality of the role of enamel matrix proteases is supported by studies that have shown that the mutations not only in amelogenin genes, but in those that encode MMP-20 cause *amelogenesis imperfecta*, i.e., a pathological state typified by abnormal and significantly weakened enamel (Bartlett et al. 2006; Caterina et al. 2002). Inhibition of the activity of MMP-2, MMP-9 and MMP-20 by marimastat similarly led to an impairment of the mineralization of dental tissues in mice (Bourd-Boittin et al. 2005). KLK4 is another major protease in amelogenesis, known for its ability to rather aggressively degrade amelogenin towards the end of the maturation stage, similar to cathepsin B. That its role is equally crucial is known since mutation g.2142G>A on the gene coding for this protease causes an abnormal enzymatic activity, resulting in the enamel crystals of normal length but of insufficient thickness (Hart et al. 2004).

Hence, whereas the full-length amelogenin is only present at the surface, in the outer enamel layer, its cleavage products are exclusively found in the deeper, inner enamel layers where they also tend to organize into specific compartments. The C-terminal-containing cleavage products also tend to position at the enamel surface and are hardly found in the deeper layers, suggesting that the full-length molecules might be involved in the crystal growth only in the first stage during which the formation of elongated particles is initiated and is followed by the reorganization of the fibrous crystals into rods through finer peptide-mineral interaction mediated by the C-terminal-lacking peptides, small enough to protrude and line up in the inner enamel regions.

The relatively high content of small peptides resulting from the enzymatic hydrolysis of amelogenin unequivocally suggests their important role in conducting the crystal growth. A detailed analysis of the crystal growth effects of smaller polypeptides as the cleavage products of the full-length amelogenin could correspondingly present the logical next step in the investigation of the mechanism of amelogenesis. One of such molecules is tyrosine-rich amelogenin peptide (TRAP) obtained by cleaving a short sequence of amino acids (44) at the N-terminal of the nascent molecule (Ravindranath et al. 2007). Engineering of de novo peptides with compacted functionalities corresponding to their bigger biological counterparts presents another approach that is yet to be meticulously explored in the context of amelogenesis in particular and biomineralization in general. Phage peptide library screening may be an experimental method of choice to assess this, while, as far as theoretical methods are concerned, the evaluation of protein "hot spots" by means of the Continuous Wavelet Transform Resonant Recognition Model (CWT-RRM) presents one possibility too (De Trad et al. 2000). "Hot spot" sequences are usually found clustered in and around the active site of the folded protein and CWT-RRM analysis of human amelogenin resulted in the detection of two such sequences, one centrally located (^{70}Val–^{90}His) and one in the vicinity of the C-terminal (^{145}Phe–^{165}Thr). The main mammalian lineages, in fact, display highly conserved residues in the hydrophilic C-terminal region, while the central region of amelogenin molecules is more variable (Delgado et al. 2005), suggesting that C-terminal plays a major role in the protein-guided crystal growth. Usually proteins exhibit a single frequency peak during multiple cross-spectral RRM analysis, but in the case of amelogenin, two such peaks were detected. A single biological function of a protein is expected to correspond to a single frequency on this diagram and the doublet in this case suggests two different protein functions. The ambiguous and intrinsically antagonistic role of amelogenin, already hypothesized to be present in its presumed ability to act as a hinderer and a fosterer of crystal nucleation and growth, is thus being reaffirmed by means of one such analysis.

13.5 Attempts to Probe the Higher Orders of the Structure of Amelogenin

What we know today about the structure of amelogenin assemblies is far more versatile than what we know about its molecular structure. Namely, the typically observed morphology of amelogenin

aggregates in vitro is the one of nanospheres with the size at the order of tens of nanometers. Combined small-angle X-ray scattering (SAXS) and dynamic light scattering (DLS) experiments indicated that a certain ellipticity (with the aspect ratio in the range of 0.45–0.5) may be attributed to amelogenin assemblies (Aichmayer et al. 2010). Furthermore, limited proteolysis studies and experiments performed on polyelectrolyte multilayers have indicated that regions at both C- and N- termini are exposed on the surface of the nanospheres (Moradian-Oldak et al. 2002a; Gergely et al. 2007). Experiments in which C-terminal was cleaved prior to the interaction with apatite have demonstrated a reduced ability of amelogenin cleavage products to interact with apatite (Aoba et al. 1987; Moradian-Oldak et al. 2002b), suggesting that the hydrophilic C-terminal, naturally, should be the region of the protein in direct contact with apatite (Shaw et al. 2004). With both C- and N- terminals exposed on the nanosphere surface, it is expected that C- terminal would be involved in the attachment onto the mineral surface, while N- terminal and the hydrophobic core of the protein would be involved in protein-protein interactions.

The knowledge on secondary and tertiary structures of amelogenin molecules is, on the other hand, still very poor. Diffraction studies have been impeded by the pronounced hydrophobicity of the protein, which tends to clump the molecules together and prevent the monomers from adopting a crystalline arrangement in space. Tens of thousands of serendipitous crystallization attempts by numerous research groups are informally said to have failed. Only the amino acid sequence of amelogenin is currently known, although there is a prospect that both evolutionary structural alignment simulations (Sire et al. 2005) and ab initio modeling will provide an insight into other structural levels of this protein. Despite the fact that the sequence of amelogenin is 90 % evolutionarily conserved, its primary structure is rather unique in the animal kingdom, with only 24 % similarity to the closest structurally neighboring protein in the human body. Of course, although there are examples of exceptionally high structural similarity between proteins that share only 20 % of sequence similarity (e.g., hemoglobins), substitution of one or a few out of hundreds of residues in a protein sequence often results in drastic changes in its secondary and tertiary structures (Horst and Samudrala 2009). The main challenge for computational studies aimed to assess the higher orders of the structure of amelogenin, however, comes from a relatively high proportion of Pro residues: 49 out of 175 in the complete X chromosome sequence of human amelogenin (including the exon 4 otherwise missing in the full-length amelogenin secreted in the enamel matrix) and 42 out of 175 in the complete Y chromosome sequence. The large number of Pro residues along the primary structure of amelogenin presents a considerable limitation due to their structure-breaking role and deviations from the regular secondary structure elements that they induce. The Raman Amide I band of recombinant full-length human amelogenin detected at 1,620 cm^{-1} indicated intermolecular extended chains (Fig. 13.6), and is in agreement with the results of circular dichroism (CD) studies, which have suggested the existence of polyproline type II structure in porcine amelogenin (Lakshminarayanan et al. 2009; Delak et al. 2009).

A single ^{41}Pro→Thr mutation in recombinant full-length human amelogenin has been shown to result in significantly lower rates of apatite growth compared with the wild-type (Zhu et al. 2011). In view of the fact that the nearest proteolytic cleavage site lies between the residues ^{45}Trp and ^{46}Leu and that this mutation significantly reduces the enzymatic hydrolysis of amelogenin in the reaction with MMP-20, it has been suggested that proline residues might play a major role in aligning the cleavage-site residues along the active site of the enzyme (Tanimoto et al. 2008a) . In fact, the concentration of proline residues along the amelogenin sequence typically increases in the vicinity of the sites that are subject to proteolytic cleavage, suggesting that the hindered enzymatic interaction between amelogenin and MMP-20 may be the major cause of *amelogenesis imperfecta*.

High content of proline residues, however, does not necessarily predispose a protein for

adopting poly-L-proline helix of type II in aqueous solution, similar to the one adopted by native collagen or many globular proteins (10 % of individual amino acid residues in proteins exist in form of the polyproline conformation, and each protein on average contains one polyproline helix, although most of them are short, ranging from 4 to 6 residues in length) (Stapley and Creamer 1999). Whereas the sequence of collagen is composed of the repeating sequence of Gly-Pro-Tyr (with Pro residues preventing collagen from adopting α-helix and instead imposing a left-handed helix with ~3 residues per turn), proline residues in amelogenin are not positioned in such a periodic manner. Despite that, there are certain structural insights that can be derived from the high content of Pro residues. First of all, the side chains of residues in the polyproline helix protrude outward from the axis of the helix and are considerably separated by the extended nature of the helix, thus precluding hydrogen bonding interactions between adjacent side chains. As a result, both hydrophilic and hydrophobic side chains become exposed on the surface, providing favorable conditions for protein-protein interactions. The majority of side chains and backbone carbonyl and amide groups are thus also solvent-exposed, which is readily visible as kinks or bulges produced by a Pro residue in the middle of an α-helix or β–sheet, respectively (Eswar et al. 2003). Unlike secondary structures with intensive intramolecular hydrogen bonding, such as α-helix, the backbone carbonyl oxygen atoms are free to participate in hydrogen bonds across protein surfaces. Polyproline secondary structures also exhibit a significant conformational stability, which additionally contributes to their exploitation as binding sites. Proline-rich sequences are, in fact, common recognition sites for protein-protein interaction modules (Rath et al. 2005). An intrinsic predisposition of amelogenin for intermolecular interactions and for the formation of functional assemblies naturally follows.

Amelogenin sequence also has a relatively high content of glutamine: 26 out of 174 residues. The only exception among side chains that preclude the formation of intramolecular interactions between side chains of a polyproline protein is exactly glutamine, as it can participate in hydrogen bonding with the backbone carbonyl oxygen of the preceding residue. On the other hand, just as proline residues tend to participate in the formation of isolated extended strands that are conformationally distinct from polyproline helices, glutamines have also been implicated in the formation of aggregates through the extended strand formation. Polyglutamines are also some of the peptides that readily adopt the polyproline helical structure. Most proteins in human parotid and submandibular saliva, in fact, belong to the family of proline-rich proteins. On average, proline, glycine and glutamine account for 70–80 % of all the amino acids within these proteins that are, however, not unique to salivary glands in the oral cavity, but are found in the respiratory tract and pancreas (Bennick 1987). These proline-rich proteins are known for their ability to bind calcium and thus presumably assist in buffering the concentration of ionic Ca^{2+} in saliva. They have also been shown to adhere strongly to apatite, exhibiting a lubricating effect and contributing to the formation of dental pellicle. However, owing to a high content of the three amino acids, their sequence is, unlike the one of amelogenin, highly repetitive.

13.6 Combining Protein Assembly, Crystal Growth and Proteolysis in Experiments Attempting to Engineer the Artificial Enamel

From the previous sections of the discourse, it could be concluded that assembly of amelogenin and its proteolytic products into dynamically evolving geometries able to guide the crystal growth along the right directions presents the central challenge for the attempts to engineer enamel in the lab using amelogenin as the crystal growth agent. In view of interrelated (a) amelogenin assembly, (b) proteolytic hydrolysis and (c) the crystal growth, a triadic nature of amelogenesis as the basis for its biomimicry could be pro-

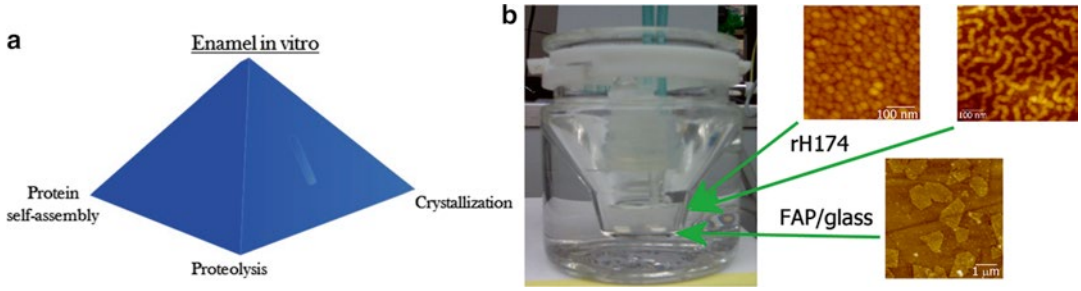

Fig. 13.5 (**a**) Biomimicry of amelogenesis as based on well understood and utilized three essential aspects of the process: protein self-assembly, proteolysis and crystallization. (**b**) The image of a borosilicate glass vessel for the continuous and computerized (Dosimat 755 and Tiamo 1.2, Brinkmann–Methrohm) titration of amelogenin (rH174) sols with different protein assembly geometries for the purpose of controlled growth of finely polished substrates containing apatite (FAP) crystals with (001) faces exposed on the surface and interspersed with a glass matrix

posed (Fig. 13.5a) and an experimental setting aimed at accomplishing this is shown in Fig. 13.5b. According to this model, the biologically relevant assembly of amelogenin is presumed to depend on its proteolytic hydrolysis, whereas the assembly of amelogenin nanospheres, naturally, affects the rate and selectivity of proteolysis by exposing specific active groups to the surface. Similarly, no uniaxial and accurately orchestrated growth of apatite fibers could be possible without the assembly of amelogenin into biologically relevant forms, while this assembly may occur only when coupled to the crystal growth through the amelogenin matrix, as proposed by Cölfen and Mann (2003). According to this model, the aggregation of primary particles of apatite in the form of filamentous crystals modifies the thixotropic gelatinous environment around them and produces conditions for the transformation of the protein nanospheres into soft filaments anchored on the surface of the growing crystals. For example, crystallites precipitated in the presence of monomeric rM179 and rM166 comprised acicular morphologies, whereas the pre-assembled full-length rM179 had no influence on the crystal morphology (Beniash et al. 2005), indicating that conditions for a co-assembly in which both phases would structurally change need to be established for a successful protein-crystal interaction to be promoted instead of attempting to use one phase as a static structural template for the transition of another. Finally, while proteolytic digestion is necessary to clear the space for the filling of the protein-occupied space by the newly grown crystals, the ongoing crystal growth may be involved in shifting the balance of active species in the system in favor of selective proteolysis.

The low levels of supersaturation, bordering the metastable state, appear to be crucial for providing the right conditions for amelogenin-guided crystal growth and the fabrication of enamel-like crystals (Fig. 13.6). Low rates of nucleation and crystal growth naturally favor the formation of elongated crystals. For example, when controlled degradation of urea is used to slowly increase alkalinity of the solution and provide conditions for precipitation, apatite crystals formed are either plate-shaped or needle-shaped (Jevtić and Uskoković 2007) . Single-crystal apatite fibers with 20–60 μm in length and 100–300 nm in diameter were thus obtained by precipitation using decomposition of urea (Aizawa et al. 2005). Although elongated apatite crystals have been obtained by methods involving rapid crystallization (Ashok et al. 2007), attempts to initiate nucleation and crystal growth at a higher rate than optimal by increasing the supersaturation ratio is expected to disrupt the continuity of amelogenin-guided crystal growth (Habelitz et al. 2005b).

Another essential requirement for the properly conducted amelogenesis is to increase the supersaturation ratio sufficiently slowly as well as with setting the precise ratio between Ca^{2+} and $H_xPO_4^{x-3}$ species. The concentrations of Ca^{2+} and

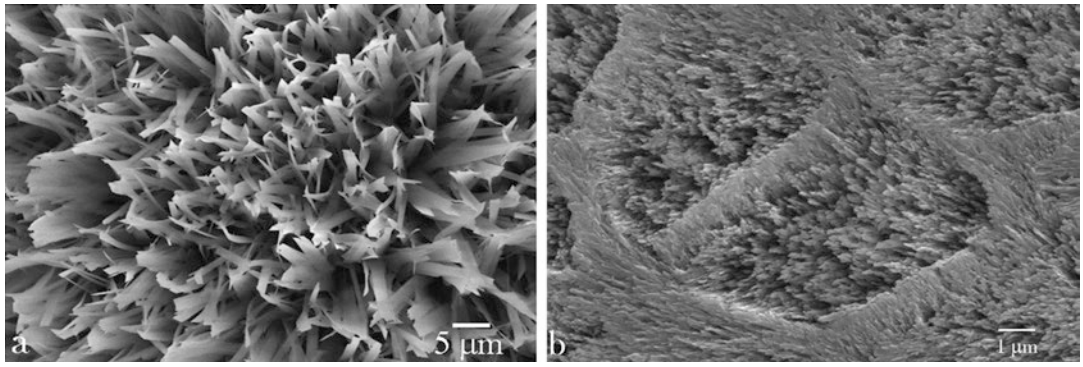

Fig. 13.6 (**a**) Microstructure of apatite fibers outgrown from fluoroapatite/glass substrates immersed in amelogenin sols at high initial phosphate concentration and pH 6.5, the conditions under which the interaction between amelogenin and apatite is expected to more intense owing to opposite surface charges, negative for apatite and positive for amelogenin. (**b**) Natural enamel displaying structural similarity to that synthesized in the lab (**a**), though composed of apatite fibers finer in diameter

$H_xPO_4^{x-3}$ ions in the fluid of developing enamel are 0.5 mM on average, and 2–5 mM, respectively, and the high initial concentrations of $H_xPO_4^{x-3}$ in amelogenin suspensions, together with the absence of Ca^{2+} prior to the onset of titration, which gradually raises its concentration in the system, proved best for the controlled surface growth of apatite fibers. Curiously enough, the same ratio between the concentration of calcium and phosphate ions (markedly different from the one within hydroxyapatite crystals, i.e., Ca/P = 1.667) is present in saliva, suggesting its favorableness for both the natural regeneration of enamel in the presence of proteins that would mimic the role play by amelogenin in the course of amelogenesis.

13.7 The Role of Other Protein Species, Fluoride, pH, Water and Dentin

By now we must have been convinced that amelogenin is an absolutely essential element for the proper replication of amelogenesis in vitro and engineering of artificial enamel. Not only have studies on transgenic mice shown that the missing C- or N- terminals in amalogenin induce severe defects in the resulting enamel (Paine et al. 2000, 2002; Fong et al. 2003), but a single point mutation (^{41}Pro→Thr) in the amelogenin gene causes severe dental enamel malformation known as *amelogenesis imperfecta* (Collier et al. 1997). However, amelogenin still constitutes 90, not 100 % of the composition of the enamel matrix. The prospect of attempts to engineer artificial enamel by means of harnessing only the right interaction between amelogenin and apatite, while discarding all other protein species of the enamel matrix, is dubious, to say the least. In that sense, despite the fact that reports on the role of enamel matrix proteins other than amelogenin in the enamel formation are comparatively scarce (Wang et al. 2005), evidence exists of the essentiality of macromolecular species present in minor amounts in the developing and maturating enamel matrix for the proper formation of the tissue. For example, mutations on the enamelin gene resulted in severe phenotypic *amelogenesis imperfecta* (Sawada et al. 2011; Lindemeyer and Gibson 2010; Masuya et al. 2005), demonstrating its essential role in the process of amelogenesis. Although its low concentration in the enamel matrix could easily trick us into thinking that we could do without it as well, this need not be necessarily so. For, there are many examples of macromolecular or amphiphilic additives that exhibit a cooperative effect on the assembly of the precipitated phase at low concentrations only (Mann et al. 2001). Polymeric or aliphatic additives introduced to repel colloidal entities, for one, oftentimes undergo aggregation at higher concen-

trations, leading to the loss of individuality or particles in the colloid and its irreversible destabilization (Uskoković 2013).

Ameloblastin is another protein of the enamel matrix expected to have a significant function, not only because of its localization at the secretory end of ameloblasts where the crystal growth is initiated, but because of both an augmented and inhibited expression of ameloblastin has been shown to result in *amelogenesis imperfecta* (Paine et al. 2003; Margolis et al. 2006). The roles of even less abundant components of the enamel matrix, such as KLK4, keratin K14, DLX3 or biglycan proteins, the mutant expressions of which are also known to produce the conditions of *amelogenesis imperfecta* (Stephanopoulos et al. 2005), have all but been investigated thoroughly and it is doubtful whether there would be any room for functionless ingredients in biosynthetic pathways.

Although adding fluoride to biomimetic experiments aimed toward replicating amelogenesis would also be a natural approach in view of its presence in natural enamel apatite, exceeding amounts thereof are known to result in increased porosity and weakening of the enamel structure (Lyaruu et al. 2014). It was also shown that increased levels of fluoride in developing enamel decrease the activity of MMP-20 (Zhang et al. 2006), resulting in the condition known as fluorosis. The role of fluoride ions in promoting elongation of apatite crystals has, however, been well documented. In a set of experiments, only the combination of amelogenin and fluoride led to formation of rod-like apatite crystals, while merely octacalcium phosphate precipitated in the absence of fluoride (Iijima and Moradian-Oldak 2005; Iijima et al. 2006). On the other hand, it was demonstrated that fluoride ions do not directly interact with amelogenins, but limit their effect on the process of amelogenesis to their incorporation into the apatite crystal lattice (Tanimoto et al. 2008b).

pH during amelogenesis varies within the range of more than a single unit, that is, from 6.0 to 7.2 (Sasaki et al. 1991), exhibiting variations from one end of ameloblasts to another. For this reason, pH is often considered to be one of the most important parameters to control during biomineralization events (Weaver et al. 2009) and phosphate, carbonate and protein species altogether work to buffer the system and prevent catastrophic drops or soars in acidity or alkalinity. Although the unavailability of techniques for measuring pH variations at the nanometer scale is to be blamed for the enigmatic status of this variable in the process of amelogenesis, its importance is beyond question. The need for precise orchestration of pH, even if not at the local scale, as it is most probably the case, certainly place an additional burden on biomimeticians of amelogenesis.

Early secretory enamel consists of 50–60 vol.% of water, 20–30 vol.% of protein, and about 15–20 vol.% of mineral. High concentrations of amelogenin (~200–300 mg/ml) in the developing enamel matrix imply that the latter resembles a gel more than an aqueous solution. Growing apatite in gelatinous media rather than in ordinary aqueous solutions thus presents a natural biomimetic choice (Wen et al. 2000; Petta et al. 2006). Crystallization of apatite from such dense media may favor the slow and controlled growth. Precipitation of fluoroapatite in gelatin per se, without the presence of amelogenin, thus resulted in spherical composites consisting of needle-shaped crystals and around 2 % of organic matter (Busch et al. 2001; Busch 2004). Density of the aqueous medium is larger compared to ordinary aqueous solutions not only in the enamel matrix, but in biological environments per se. Under such circumstances, water exhibits modified structure and properties. Cytoplasm typically contains about 400 g/dm^3 of macromolecules, which as such occupy 5–40 % of the total cell volume with an average separation between them of 1–2 nm. Within such nanoscopically confined conditions, water possesses an altered hydrogen bonding structure in comparison with the bulk water. Also, by playing various structural roles, water presents an essential component of a fully functional protein. Although it has been shown that structure and functionality of some enzymes can be preserved in non-polar media or even in vacuum (albeit the preservation of bound water even under such circumstances), it is suggested

that water "lubricates" the peptide chains and provides conditions for favorable molecular recognition effects. Consequently, the concepts of diffusion and solubility limits should be redefined with the transition to complex and dense media such as those from which enamel crystal grow.

The initial enamel crystals are nucleated along the dentin-enamel junction and a proper substrate is therefore of vital importance in ensuring the right final structure of the material. Epitaxial effects were many times proven as essential in self-assembly procedures (Uskoković 2008), and many biomineralization mechanisms (e.g., crystallization of thin flakes of nacre in the mollusk shells) depend on the interfacial structural matching between an organic substrate and an inorganic phase. Despite the fact that the hardness of enamel is a result of its nanoscale superstructural organization, the strength of enamel is also highly dependent on the supporting dentin. This interaction between the dentin substrate and superstructurally organized enamel crystals may be another factor of critical importance for replication of the assembly of fibrous apatite crystals in vitro. It is also known that signals originating from the dental papilla are required to activate the expression of amelogenin (Garant 2003), which points to an even wider scope of amelogenesis, in view of which the prospects of replicating the process by focusing only on a selected number of species and control parameters can be subjected to reasonable scrutiny.

13.8 Conclusion and Future Prospects

It may have become clear by now that the replication of amelogenesis in vitro stands for a daunting task that requires knowledge on the ability to orchestrate interactions between a multitude of polypeptides in precise correlation with setting the right conditions for diffusion of the ionic growth units and their precipitation in form of uniaxial crystals. In the end, it is logical to expect that the three major aforementioned aspects of amelogenesis – (1) the assembly of amelogenin and other enamel matrix proteins, (2) the proteolytic activity, and (3) crystallization – need to be in precise synergy with each other in order to produce the desired outcome. These endeavors are additionally made difficult because they are being designed to yield fundamental insights regarding amelogenesis, while at the same time to be harnessed for practical purposes. As much as it is natural, this entwinement of the practical and the fundamental aspects of the biomimetic settings aimed at replicating biomineralization in a beaker is also inherently illogical. For, how could we be expected to create a desired product without knowing the chemical mechanisms intrinsic to its formation and how could we be expected to understand the fundamental features of a process if we do not know how to replicate it? As of today, however, it is difficult to estimate which aspect of the process is more difficult to penetrate into: fundamental or practical. In any case, conceiving original experimental approaches to mimic amelogenesis presents the key, although two eyes need to be used to analyze the outcomes. The proteomic, life science eye would follow the protein-related aspects of the process, whereas the materials science eye would follow the crystal formation facets of it. Needless to add, these two eyes need to look in the same direction and in synergy from the top of the aforementioned pyramid (Fig. 13.5a) in order for the path of biomimetics of tooth enamel to be walked on successfully. In such a way, there is a chance that the future development of this field will transcend the broad speculations that dominate the contemporary literature reports on amelogenesis-related studies, though remain receptive to the effects of some of the most minor components of this fascinating biological process. For, if the science of the enamel growth teaches us something profound, it is that "small is beautiful" and that a tiny detail of this Universe, such as the enamel, hides many mysterious patterns, diligent plunging in the research of which may open the doors to understanding of much greater secrets of the physical reality in which we abide.

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Whole Tooth Regeneration as a Future Dental Treatment

14

Masamitsu Oshima and Takashi Tsuji

Abstract

Dental problems caused by dental caries, periodontal disease and tooth injury compromise the oral and general health issues. Current advances for the development of regenerative therapy have been influenced by our understanding of embryonic development, stem cell biology, and tissue engineering technology. Tooth regenerative therapy for tooth tissue repair and whole tooth replacement is currently expected a novel therapeutic concept with the full recovery of tooth physiological functions. Dental stem cells and cell-activating cytokines are thought to be candidate approach for tooth tissue regeneration because they have the potential to differentiate into tooth tissues *in vitro* and in vivo. Whole tooth replacement therapy is considered to be an attractive concept for next generation regenerative therapy as a form of bioengineered organ replacement. For realization of whole tooth regeneration, we have developed a novel three-dimensional cell manipulation method designated the "organ germ method". This method involves compartmentalisation of epithelial and mesenchymal cells at a high cell density to mimic multicellular assembly

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conditions and epithelial-mesenchymal interactions in organogenesis. The bioengineered tooth germ generates a structurally correct tooth *in vitro*, and erupted successfully with correct tooth structure when transplanted into the oral cavity. We have ectopically generated a bioengineered tooth unit composed of a mature tooth, periodontal ligament and alveolar bone, and that tooth unit was engrafted into an adult jawbone through bone integration. Bioengineered teeth were also able to perform physiological tooth functions such as mastication, periodontal ligament function and response to noxious stimuli. In this review, we describe recent findings and technologies underpinning whole tooth regenerative therapy.

Keywords
Whole tooth regeneration • Tooth replacement • Dental tissue engineering • Organ germ • Tooth germ • Stem cells

14.1 Introduction

Oral functions regarding mastication, swallowing and speech are an important aspect of good health and quality of life (Proffit et al. 2004). The tooth is an ectodermal organ whose development is regulated by reciprocal epithelial-mesenchymal interactions (Jussila et al. 2013; Tucker and Sharpe 2004; Ikeda and Tsuji 2008) and contains distinctive hard tissue structure composed of enamel, dentin and cementum (Avery 2002; Nanci 2012). Teeth also have soft connective tissues such as pulp and periodontal ligament (PDL) that include peripheral nerve fibres and blood vessels to maintain tooth homeostasis and physiological functions (Avery 2002; Nanci 2012). Tooth physiological functions are exerted efficiently by the characteristic three-dimensional multicellular structure that establishes functional synergy with the maxillofacial region (Avery 2002; Nanci 2012). Tooth loss due to dental caries, periodontal disease and traumatic injury causes fundamental problems for oral functions and associated general health issues (Proffit et al. 2004). To restore the occlusal function or aesthetic condition after tooth loss, several dental therapies that replace the tooth with artificial materials such as fixed dental bridges and removable dentures have been performed conventionally (Rosenstiel et al. 2001; Pokorny et al. 2008). Recently, osseointegrated dental implants that can restore function without affecting the healthy teeth have been adopted to treat tooth loss (Brenemark and Zarb 1985; Burns et al. 2003). Although these artificial therapies have been widely applied to rehabilitation of tooth loss, it is anticipated that further technological developments based on biological findings are needed to restore tooth physiological functions (Tucker and Sharpe 2004).

Recent regenerative therapies have been developed based on our understanding of embryonic development, stem cell biology and tissue engineering technology (Korbling and Estrov 2003; Brockes and Kumar 2005; Watt and Hogan 2000; Langer and Vacanti 1999; Atala 2005). One concept in regenerative therapies rely on the cell transplantation of purified tissue-derived stem cells or embryonic stem (ES) or induced pluripotent stem (iPS) cells. These stem cell transplantation, which targets structural and functional diseases such as malignant diseases, neurological disorders, myocardial infarction, and hepatic dysfunction, has been attempted to repair damaged tissues (Copelan 2006; Lindvall and Kokaia 2006; Segers and Lee 2008; Wang et al. 2003). In dentistry, basic research of stem/progenitor cells have provided new insights concerning tooth tissue-derived stem cells, including dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED) and stem cells from apical papilla (SCAP) that have been isolated from the dental pulp tissue (Huang et al. 2009; Gronthos et al. 2000; Miura et al. 2003; Sonoyama et al. 2008). These stem cells are thought to be a poten-

tial resource for stem cell-mediated tissue repair, including dentin or pulp regeneration, based on their high proliferation and multi-differentiation capacity (Mantesso and Sharpe 2009; Yen and Sharpe 2008). Also, periodontal ligament-derived stem cells (PDLSCs), which can differentiate into all periodontal cell types after transplantation, have also been identified, and have been attempted to develop cell sheet-engineering using PDLSCs for clinical use in periodontal tissue regeneration (Seo et al. 2004). Although these treatments contribute to partial tissue repair, many researchers anticipate the development of further therapeutic technologies using dental stem cells that can regenerate lost teeth (Mantesso and Sharpe 2009; Yen and Sharpe 2008).

Organ replacement regenerative therapy, unlike stem-cell transplantation, holds great promise for the replacement of dysfunctional organs via a regenerative strategy by reconstructing a fully functional bioengineered organ using three-dimensional cell manipulation *in vitro* (Atala 2005; Seo et al. 2004). It is expected that bioengineering technology will eventually enable the reconstruction of fully functional organs *in vitro* through the proper arrangement of several cell components. In the dental field, tooth regenerative therapy involves the replacement of a lost or damaged tooth with a bioengineered tooth reconstructed from stem cells and with the potential to generate a functional tooth unit including the whole tooth and periodontal tissue surrounding the alveolar bone (Purnell 2008; Volponi et al. 2010; Sharpe and Young 2005). It is anticipated that whole tooth replacement therapy will be established in the near future as a successful biological treatment that will provide essential functional recovery of lost teeth to satisfy aesthetic and physiological requirements (Volponi et al. 2010; Sharpe and Young 2005) (Fig. 14.1). Over the past three decades, many approaches for replacing lost teeth have been studied, including three-dimensional bioengineered teeth and tooth germ generation using biodegradable materials and cell aggregation methods (Sharpe and Young 2005; Duailibi et al. 2006). Recently, the first studies of fully functioning bioengineered tooth replacement with the correct tooth structure, masticatory performance, responsiveness to mechanical stress and perceptive potential following transplantation into a tooth-loss region were reported (Nakao et al. 2007; Ikeda et al. 2009; Oshima et al. 2011). In this chapter, we describe novel technologies for whole tooth replacement therapy that have the potential to provide functional recovery and

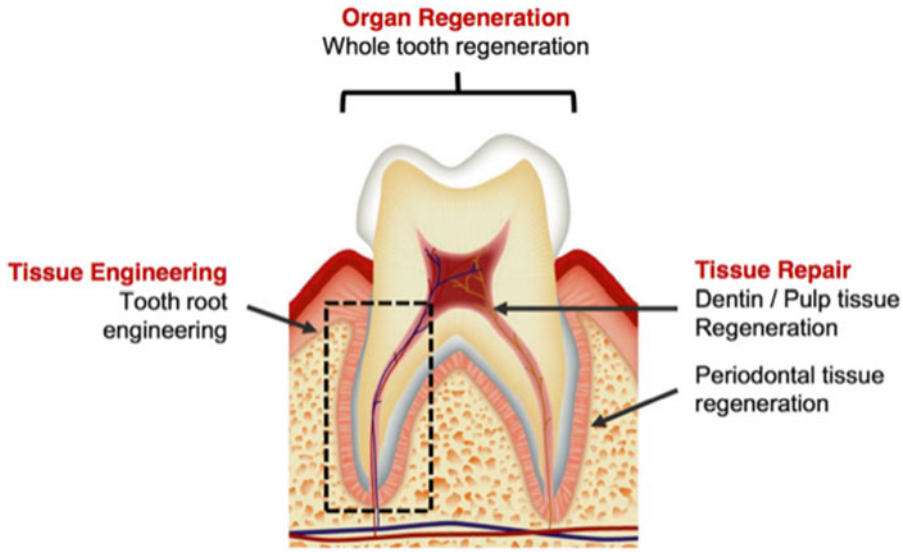

Fig. 14.1 Concepts of dental regenerative therapy. Recent approaches to developing technologies for tooth regenerative therapy have included tissue repair and whole-tooth replacement

entirely replace the current dental treatments based on artificial materials.

14.2 Developmental Process of Tooth Formation

Ectodermal organs, including the teeth, hair and salivary glands, arise from their respective organ germs through the reciprocal epithelial-mesenchymal interactions in the developing embryo (Thesleff 2003) (Fig. 14.2). The mechanism of tooth formation is also regulated by reciprocal epithelial and mesenchymal interactions, particularly those involved in stem cell, signalling molecule and transcription factor pathways (Jussila et al. 2013; Thesleff 2003). During early craniofacial development in mice, the dental lamina first thickens, followed by epithelial thickening at the sites of future teeth and subsequent epithelial budding to the neural crest-derived ecto-mesenchyme. Tooth-forming fields are specified at embryonic day (ED) 10–11 through the expression of homeobox genes e.g. *Msx1*, *Msx2*, *Lhx8* and *Barx1* and secretory molecules including fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (Ikeda and Tsuji 2008; Thesleff 2003; Bei 2009; Nakatomi et al. 2010). At ED 11.5, oral epithelium invaginates into the mesenchymal region, and then tooth bud is formed by the condensation of mesenchyme that is derived from neural crest cells. At ED 13.5–14.5, a transient epithelial signalling centre called the first enamel knot, which expresses several signalling molecules, including Shh, Wnts, BMPs and FGFs, is thought to regulate individual cell fates and to orchestrate epithelial morphogenesis through the epithelial-mesenchymal interactions (Ikeda and Tsuji 2008). The epithelial and mesenchymal cells in the tooth germ differentiate into the respective progenitor cells, including ameloblasts, odontoblasts and dental follicle cells at ED 16.5–18.5. These progenitor cells secrete a collagenous extracellular matrix that mineralises into the enamel and dentin tissue at the epithelium–mesenchyme interface (Fukumoto and Yamada 2005). Tooth

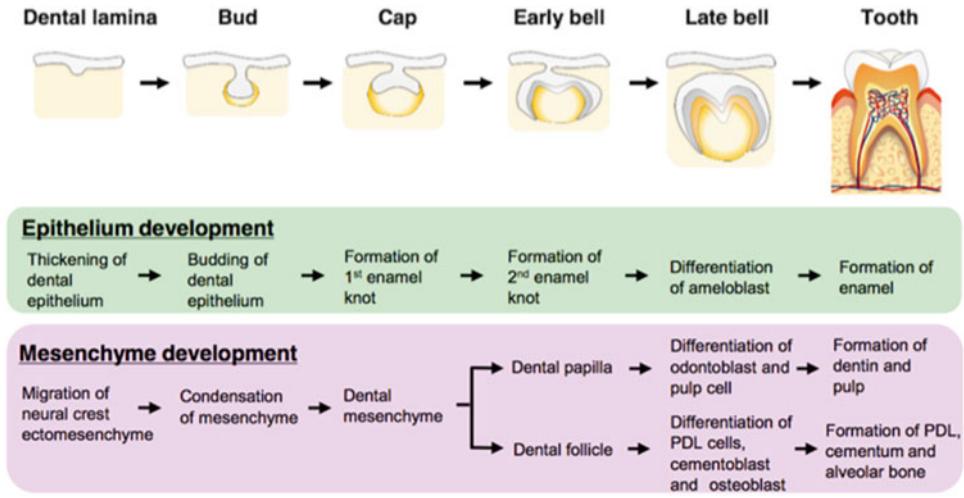

Fig. 14.2 Schematic representation of the developmental stages of a tooth. The tooth germ is formed from the dental lamina, which consists of an invaginated epithelium that is developed from the immature oral epithelium and condensed mesenchyme cells that is derived from neural crest cells (ED 11–12). The first enamel knot, which acts as a developmental signalling centre forms within the dental epithelium (ED 13–15). The secondary enamel knots are formed; these tissues play an important role in regulating the cusp position and number (ED 16). The epithelial and mesenchymal cells in the tooth germ differentiate into tooth tissue-forming cells including ameloblasts, odontoblasts and dental follicle cells (ED 18). Ameloblasts and odontoblasts accumulate enamel and dentin, respectively, at the boundary surface between the epithelium and mesenchyme. On the other hand, the dental follicle cells differentiate into periodontal tissues, including the periodontal ligament, cementum and alveolar bone

morphogenesis, including tooth size and shape, are thought to be regulated by signalling molecules such as the BMPs and FGFs emanating from the secondary enamel knots, which regulate the cusp pattern of the mature natural tooth in the early bell stage to form the tooth crown (Thesleff 2003). After tooth crown formation, tooth eruption into the oral cavity begins involving tooth-root elongation, and dental follicle cells form the periodontal tissues including cementum, PDL and alveolar bone onto root-dentin surface (Ikeda and Tsuji 2008; Foster et al. 2007; Yang et al. 2009).

14.3 Technological Development for Whole Tooth Regeneration by Using a Novel Three-Dimensional Cell Manipulation Method

Stem cell transplantation therapy is now considered an effective approach to restore partial organ functions at local damaged sites. However, current bioengineering technologies have not yet achieved their goal of reconstructing complex organs which is in stark contrast to the stem cell transplantation therapies used to repair the damaged tissues. The ultimate goal of regenerative therapy in the future is to develop organ replacement regenerative therapies that will restore lost or damaged tissues following disease, injury or aging with a fully functioning bioengineered complex organ (Atala 2005; Seo et al. 2004). To realize the reconstruction of bioengineered organs, one biological approach is based on recapitulating organogenesis by mimicking the reciprocal epithelial-mesenchymal interactions that occur in the organ developmental process, thereby developing fully functional bioengineered organ from a bioengineered organ germ by using immature stem cells via three-dimensional cell manipulation technology *in vitro* (Ikeda and Tsuji 2008; Sharpe and Young 2005). For whole tooth regeneration, an attractive concept has been proposed in which a bioengineered tooth germ would be transplanted into the tooth loss region and would develop into a functioning mature tooth (Ikeda et al. 2009) (Fig. 14.3, upper panel). Furthermore, it is expected

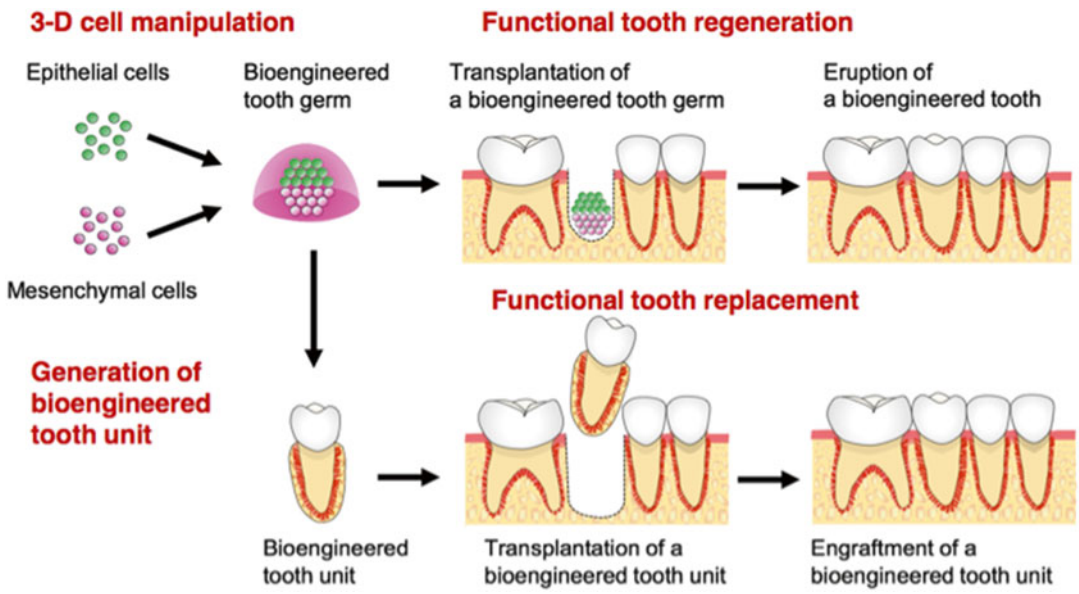

Fig. 14.3 Regenerative strategy of whole-tooth replacement. Fully functioning teeth can be regenerated in vivo by transplanting bioengineered tooth germs reconstituted from epithelial and mesenchymal cells via the organ germ method or by transplanting bioengineered tooth units with a periodontal ligament and alveolar bone that developed from bioengineered tooth germs

that it will be possible to transplant a bioengineered mature tooth unit, including tooth, PDL and alveolar bone, which will engraft through physiological bone integration of the recipient's jaw (Oshima et al. 2011) (Fig. 14.3, lower panel). Transplantation of a bioengineered tooth unit has also been proposed as a viable option to repair the large resorption defects in the alveolar bone after tooth loss (Oshima et al. 2011). To enable these whole tooth regenerative strategies, it will be important first to develop techniques for the manipulation of cells in three dimensions in order to reconstruct bioengineered tooth using completely dissociated epithelial and mesenchymal cells *in vitro*. To date, two conventional approaches and a novel cell manipulation method are currently being examined, as described below.

14.3.1 Biodegradable Scaffold Method

Scaffold technology represented by three-dimensional tissue engineering has contributed to the large-scale tissue regeneration for damaged tissues through seeding target cells on degradable materials such as natural molecules and synthetic polymers. This method has shown high utility in three-dimensional tissue engineering technology, and these preparations have been used in clinical applications including bone and cartilage regenerative therapies (Quarto et al. 2001; Cao et al. 1997; Caplan and Bruder 2001). Previous studies using collagen/gelatine sponges or polyglycolic acid/poly-L-lactate-co-glycolide copolymers (PLA/PLGA) have reported the partial generation of tooth tissue structure through seeding epithelial and mesenchymal cells isolated from porcine tooth germ (Honda et al. 2003, 2007; Young et al. 2002; Iwatsuki et al. 2006; Duailibi et al. 2004; Yelick and Vacanti 2006; Sumita et al. 2006). This scaffold-based method may be practical for controlling tooth shape and size; however, the fundamental problems regarding the regeneration of tooth itself have not been resolved. The presence of residual scaffold material after in vivo transplantation is considered to be the cause of the low frequency of tooth formation and the irregularity of the resulting tooth tissue structures *e.g.* the enamel-dentin complex and the cell arrangement of ameloblast/odontoblast lineages (Honda et al. 2003; Young et al. 2002). Fully generation of proper tooth structure using scaffolds requires the formation of complex junctions between the enamel, dentin and cementum that result from accurate spatiotemporal cell gradients of ameloblasts, odontoblasts and cementoblasts as well as natural tooth development (Volponi et al. 2010; Nakao et al. 2007).

14.3.2 Cell Aggregation Method

The cell aggregation method is known as a typical bioengineering protocol employed for the reconstitution of a bioengineered organ germ to reproduce the epithelial-mesenchymal interactions that occur during organogenesis (Purnell 2008; Volponi et al. 2010). Previous studies have reported that transplanting bioengineered cell aggregates derived from the ectodermal origin such as hair follicle and mammary gland, and demonstrated in the in vivo regeneration of each organ with the proper tissue structure and cellular arrangements (Zheng et al. 2005; Shackleton et al. 2006). In the dental field, many researchers have isolated the dental epithelial and mesenchymal tissues from embryonic tooth germs using stereomicroscopic guidance, and dissociating such tissues with surgical and enzymatic treatments to obtain single stem cells. It has been reported that bioengineering cell aggregates re-pelleted with dental epithelial and mesenchymal stem cells using the cellular centrifugation have the potential for tooth germ formation after in vivo transplantation (Hu et al. 2006; Yamamoto et al. 2003). Even when bioengineered cell aggregate were mixed with epithelial and mesenchymal stem cells isolated from tooth germ, the correct tooth structure could be generated by the self-reorganisation through the cell rearrangement of epithelial and mesenchymal cells (Song et al. 2006). Although this technique partially

replicated tooth organogenesis, further improvements in the frequency of bioengineered tooth development and correct tissue formation has been required.

14.3.3 Three-Dimensional Cell-Manipulation Method: The "Organ Germ Method"

To achieve precise replication of the developmental processes in organogenesis, an *in vitro* three-dimensional novel cell manipulation method designated as a bioengineered organ germ method has been recently established (Nakao et al. 2007). We investigated the possibility of developing a bioengineered tooth germ using completely dissociated single stem cells from epithelial and mesenchymal tissues of incisor or molar tooth germ at cap stage in ED 14.5 mice. The bioengineered cell aggregate reconstituting by epithelial or mesenchymal cells alone generated keratinized-epithelium or bone, respectively, on subrenal capsule transplantation. Bioengineered tooth germ that reconstituted the cell compartmentalization between epithelial and mesenchymal cells at a low cell density (0.5–1.0×10^8 cells/ml), or which did not form cell compartmentalization (*i.e.* mixed cell aggregate) at high-cell density (5.0×10^8 cells/ml), was also unable to generate a correct tooth structure (Nakao et al. 2007). The most significant breakthrough using our method is the achievement of three-dimensional cell compartmentalization of epithelial and mesenchymal cells at a high cell density in a collagen gel. This bioengineered tooth germ can achieve initial tooth development with the appropriate cell-to-cell compaction between epithelial and mesenchymal cells *in vitro* organ culture. Bioengineering tooth germ reproducing by this method successfully replicates the multicellular assembly, including ameloblasts, odontoblasts, pulp cells and dental follicle cells, based on the epithelial-mesenchymal interactions as well as natural tooth development, and could allow for large-scale organ reconstruction. The bioengineered tooth germ generates a structurally correct tooth after transplantation in an organ culture *in vitro* as well as following placement into a subrenal capsule in vivo (Nakao et al. 2007) (Fig. 14.4a). Tooth morphology is defined by not only the crown size and tooth length as macro-morphological feature but also the cusp numbers/position as micro-morphological feature. These morphological regulations are determined in the tooth-forming field by specific gene expression in immature oral epithelium and neural crest-derived mesenchyme in the embryonic jaw. It is considered that macro-patterning, including the number and size of teeth are spatiotemporally regulated by the patterned signalling molecules in accordance with an activator-inhibitor model. The tooth micro-patterning, including the position and number of cusps in the teeth, are also thought to be involved in the secondary enamel knots. Thus, the patterning of signalling molecules based on the reaction-diffusion mechanism, which is regulated by the reciprocal activation and inhibition of cell proliferation in epithelial and mesenchymal tissues, is important for the determination of tooth morphogenesis (Thesleff 2003). Bioengineered tooth germs were reconstructed using various cell-to-cell contact lengths between the epithelial and mesenchymal cell layers, and thereby the crown widths and cusp numbers of generated bioengineered teeth were dependent on the contact length of the bioengineered tooth germs (Ishida et al. 2011).

Moreover, an attractive technology for generating a bioengineered tooth unit composed of a mature tooth, PDL and alveolar bone through in vivo transplantation into the subrenal capsule has been developed (Fig. 14.4b, c). Multiple bioengineered tooth units surrounded by alveolar bone could also be generated through transplanting several tooth germs in a size-control device (Oshima et al. 2011). Each bioengineered tooth had the correct toot structure including the pulp cavities and the partitioned periodontal tissue structure (Fig. 14.4b). Hence, it would be possible to accomplish the multiple teeth

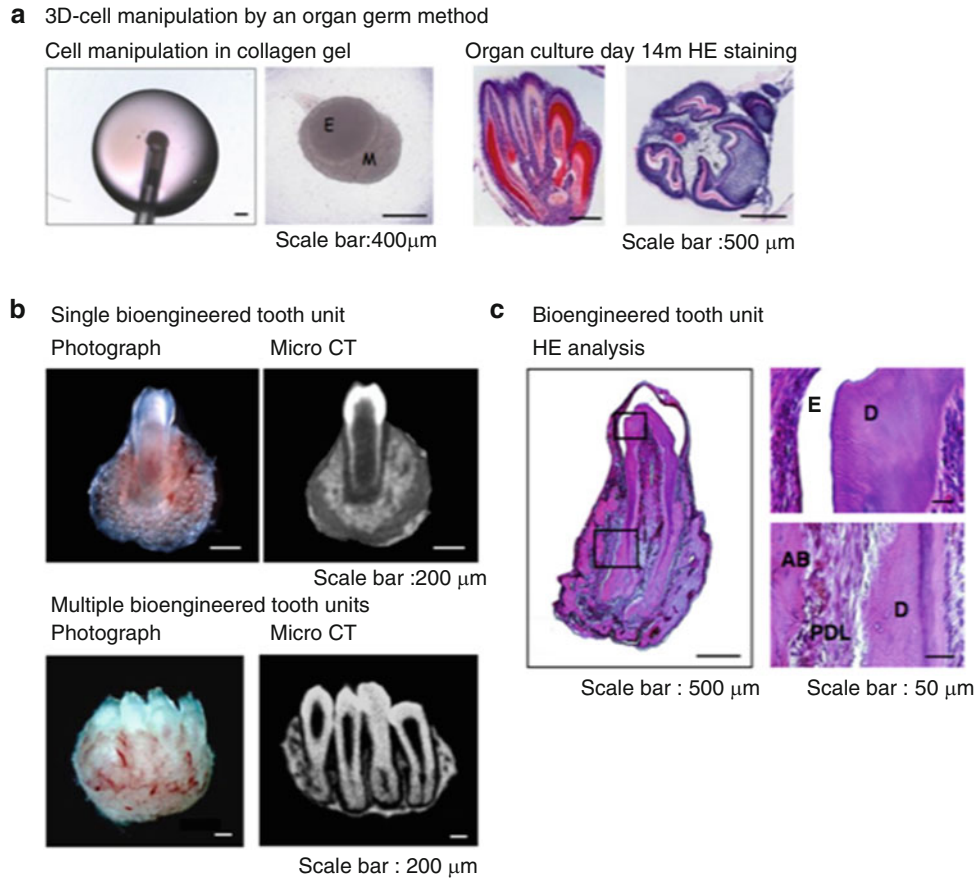

Fig. 14.4 The organ germ method: a three-dimensional cell-processing system. (**a**) Dissociated mesenchymal cells at a high density are injected into the centre of a collagen drop. Tooth germ-derived epithelial cells and mesenchymal cells are injected into the collagen drop at a high density (*left panel*). At 1 day of organ culture, the bioengineered tooth germ with the appropriate compartmentalization between the epithelial and mesenchymal cells and cell-to-cell compaction was formed (*centre-left panel*). Bioengineered molar tooth germs, which were reconstituted using dissociated cells isolated from incisor and molar tooth germ, developed in *in vitro* organ culture over 14 days (*right panels*). (**b**) Transplantation of this bioengineered tooth germ into a subrenal capsule for 30 days (*upper panels*). Multiple bioengineered tooth units surrounded by alveolar bone can also be generated by transplanting several tooth germs (*lower panels*). (**c**) A bioengineered tooth unit composed of a mature tooth, periodontal ligament and alveolar bone, was generated with the natural tooth structure such as enamel (*E*), dentin (*D*), the periodontal ligament (*PDL*) and alveolar bone

replacement in the case of edentulous jaw by using this regenerative transplantation method (Oshima et al. 2011). These technologies have the great promise to achieve functional tooth regeneration and also indicate a substantial advance in bioengineered organ replacement regenerative therapy.

14.4 Functional Whole-Tooth Replacement Using the Bioengineered Tooth

Regenerated organs should exhibit organ-intrinsic functions in cooperation with the surrounding environment, including vascular and

nervous system in the host. Previous studies *in Xenopus* showed that regenerated secondary organs can restore the physiological function of organs such as heartbeat and neural innervation (Sedohara et al. 2003; Tyler and Baker 2003). It is, thus, expected to demonstrate the organ replacement by using a bioengineered organ in mammalians. Oral functions such as mastication, pronunciation, and facial aesthetics have an important contribution to the quality of life because they facilitate both oral communication and general health. These functions are exerted by the teeth, masticatory muscles and temporomandibular joint under the control of the central nervous system (Proffit et al. 2004; Dawson 2006). For the realisation of tooth replacement regenerative therapy, a regenerated tooth developing from bioengineered germ or a transplanted bioengineered mature tooth unit must be capable of properly engrafting into the lost tooth region in an adult oral environment and acquiring full functionality, including sufficient masticatory performance, biological integration via periodontal attachment and afferent responsiveness to noxious stimulations in the maxillofacial region (Proffit et al. 2004; Dawson 2006).

14.4.1 Successful Transplantation of a Bioengineered Tooth Germ or a Bioengineered Mature Tooth Unit for Whole-Tooth Replacement

The critical issue regarding the successful tooth regenerative therapy via the transplantation of bioengineered tooth germ into the tooth loss region is whether the germ can erupt and occlude properly with the opposing tooth in an adult oral environment. Natural tooth eruption is autonomously regulated by biological mechanism that involves the tooth germ cell components and the surrounding alveolar/jawbone area (Wise et al. 2002; Wise and King 2008). In the tooth developmental process, dental follicle cells, which encircle around the developing tooth germ, generate the cementum, PDL and alveolar bone and subsequently affect the resorption of the alveolar bone overlying the tooth germ during tooth eruption (Dawson 2006; Wise and King 2008). So far, it has been reported that a transplanted natural tooth germ erupted in a murine toothless diastema region (Nakao et al. 2007; Ohazama et al. 2004). Recently, we have demonstrated that a bioengineered tooth germ can develop the correct tooth structure in an oral cavity and also successfully erupt 37 days after transplantation (Nakao et al. 2007; Ikeda et al. 2009). Bioengineered tooth erupts autonomously through the regulation of bone remodelling in the eruption pathway that faithfully reproduces the molecular mechanisms involved in natural tooth eruption. Subsequently, the erupting bioengineered tooth not only reached the occlusal plane but also maintained occlusal function with the opposing natural tooth at 49 days after transplantation (Ikeda et al. 2009) (Fig. 14.5a).

Transplantation of a bioengineered mature organ would lead to the immediate performance of the full functions in vivo and greatly affect on the survival outcomes in numerous diseases (Gridelli and Remuzzi 2000). In the case of a transplanted bioengineered mature tooth unit comprising mature tooth, PDL and alveolar bone, the most critical consideration is whether that unit can be engrafted into the tooth loss region through bone integration, which involves natural bone remodelling in the recipient. A bioengineered tooth unit transplanted at a position reaching the occlusal plane with the opposing upper first molar was successfully engrafted after 40 days and then maintained the periodontal ligament originating from the bioengineered tooth unit through successful bone integration and regeneration (Oshima et al. 2011) (Fig. 14.5b, c). The hardness of the enamel and dentin components in bioengineered teeth were equivalent to that of natural teeth upon analyse using the Knoop hardness test (Ikeda et al. 2009; Oshima et al. 2011). These approaches demonstrated that the potential to successfully recover masticatory performance and natural tooth tissue through state-of-the-art bioengineering technology.

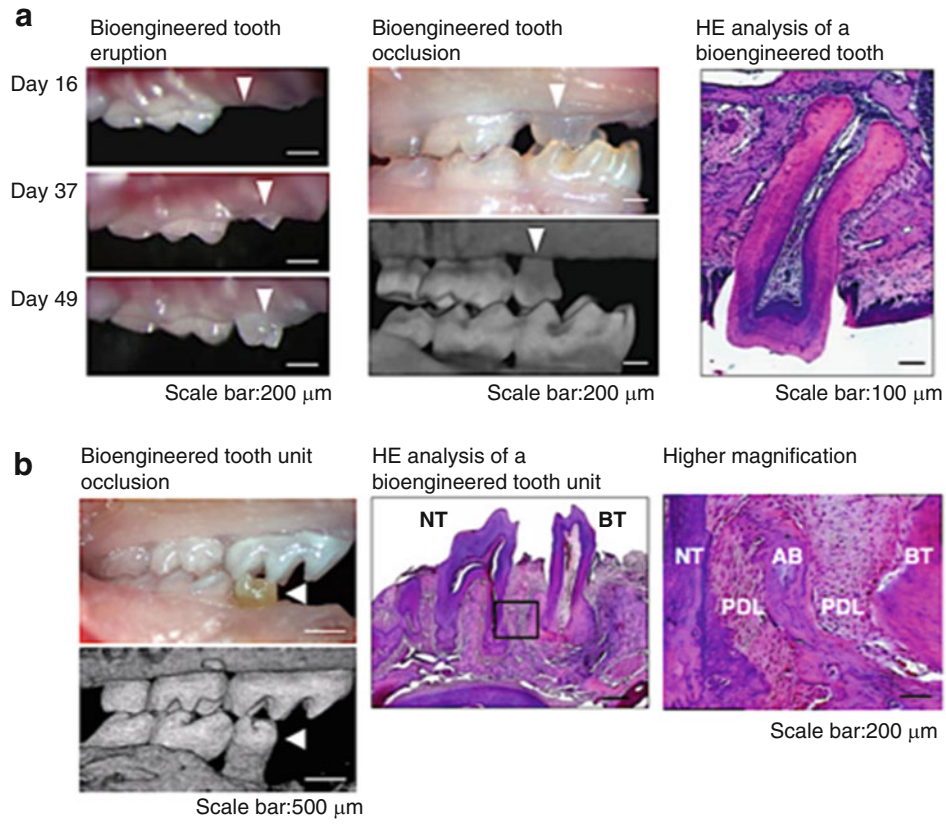

Fig. 14.5 Transplantation of a bioengineered tooth germ or a bioengineered tooth unit. (**a**) Bioengineered tooth germ erupted (*arrowheads*, *left panels*) and reached the occlusal plane with the opposing lower first molar at 49 days after transplantation (*arrowheads*, *centre panels*). The bioengineered tooth also formed a correct tooth structure comprising enamel, dentin, dental pulp, and alveolar bone (*right panels*). (**b**) The bioengineered tooth unit was engrafted through bone integration and occluded with the opposing upper first molar at 40 days after transplantation (*arrowheads*, *left panels*). The engrafted bioengineered tooth unit also had the correct tooth structure. *NT* natural tooth, *BT* bioengineered tooth, *AB* alveolar bone, *PDL* periodontal ligament (*centre* and *right panels*). (**c**) Three-dimensional superposition of micro-CT images of natural dentition (*gray*, *double dotted line*), a transplanted bioengineered tooth unit (*right panel*) and a no transplantation control (*left panel*) at day 0 in an extensive bone defect (*red*, *straight line*), and at 45 days after transplantation (*green*, *dotted line*) are represented. The superior edges of the recipient alveolar bone are indicated by each *line*

14.4.2 Biological Response of Bioengineered Tooth to Mechanical Stress

Full oral functions are essential for the cooperation with teeth and the maxillofacial region via the biological connection of periodontal ligaments. Tooth loss and periodontal disease cause fundamental problems for oral and general health issues (Proffit et al. 2004; Dawson 2006). The structural properties of periodontal tissue contribute to the physiological function of the tooth, including the absorption of occlusal loading, the maintenance of alveolar bone height and orthodontic tooth movement involving bone remodelling (Foster et al. 2007). Although autologous tooth transplantation can successfully restore physiological tooth functions, it has been known that the remaining healthy periodontal tissue around extracted tooth root is required for the accomplishment of highly survival rate and the prevention of ankyloses (Tsukiboshi 1993). In contrast, the absence of a PDL in a dental implant is associated with deficits in important tooth functions requiring the coordination of the teeth and the maxillofacial components through the connection of PDL (Avery 2002; Pokorny et al. 2008; Lindhe et al. 2008). Thus, biological regenerative therapy using functional periodontal tissue has been shown to be effective in restoring normal tooth functions as an alternative to artificial therapy. To analyse the movement of bioengineered tooth, we have experimentally developed an orthodontic tooth movement model. Experimental tooth movement consisted of a horizontal orthodontic force of about 10–15 g applied continuously to the bioengineered tooth in a buccal direction using a nickel-titanium wire with a diameter of 0.012 inch. (Ikeda et al. 2009; Oshima et al. 2011). We have demonstrated that bioengineered teeth successfully underwent the functional tooth movement equivalent to that of natural teeth through the proper localisation of osteoclasts and osteoblasts in response to mechanical stress (Ikeda et al. 2009; Oshima et al. 2011). These findings indicate that a bioengineered tooth can replicate critical dental functions and subsequently lead to the restoration and re-establishment of functional teeth within the maxillofacial region (Ikeda et al. 2009; Oshima et al. 2011).

14.4.3 Perceptive Potential for Noxious Stimulation in Bioengineered Tooth

The afferent nervous system is established by the growth of axons that navigate and establish connections with developing target organs during embryogenesis (Luukko et al. 2005). The teeth are the peripheral organs innervated by the sensory nerves and sympathetic or parasympathetic nerves in the maxillofacial region, and that afferent nervous system contribute to the regulation of tooth physiological functions and the perception of noxious stimulations (Luukko et al. 2005). It is thus thought that neuronal regeneration, which is associated with the re-entry of nerve fibres subsequent to the transplantation of a tooth germ or an autologous tooth, is required for the achievement of biological dental regenerative therapy (Kjaer et al. 1999). Current dental implants, which are directly connected to the alveolar bone as an osseointegration, cannot perceive noxious stimulations such as excessive occlusal loading and trauma because of the absence of neuronal innervation in the periodontal tissue (Brenemark and Zarb 1985; Burns et al. 2003). Therefore, it is expected that tooth regenerative therapy will achieve the functional recovery of the neuronal perceptive potential for noxious stimulation. We have demonstrated that sensory and sympathetic nerve fibres can innervate both the pulp tissue and the PDL region with the reconstruction of blood vessels in an engrafted bioengineered tooth (Ikeda et al. 2009; Oshima et al. 2011) (Fig. 14.6). Bioengineered teeth also represented the perceptive potential for nociceptive pain stimulation including pulp injury and orthodontic movement, and they can properly transduce these peripheral stimulation to the superficial layers of the medullary dorsal horn through c-Fos immunoreactive neurons in the central nervous system (Ikeda et al. 2009; Oshima et al. 2011). These findings indicate that bioengi-

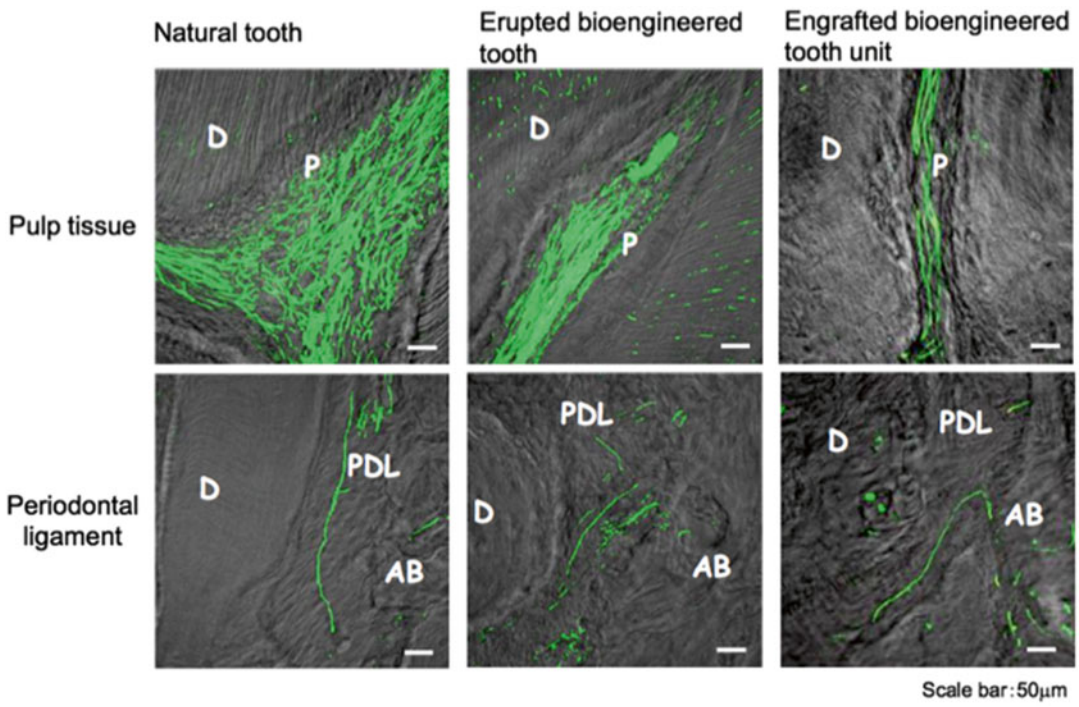

Fig. 14.6 Neuronal Innervation of a bioengineered tooth. The nerve innervation of the dental pulp and periodontal ligament in the natural and bioengineered teeth detected by using the immunostaining for neurofilament H. *D* dentin, *P* pulp, *PDL* periodontal ligament, *AB* alveolar bone

neered teeth can indeed restore the proprioceptive potential to recognize and respond to noxious stimulation within the maxillofacial region.

14.5 Conclusion and Future Consideration

Current regenerative technology has progressed remarkably, and many patients can be benefitted by the contributions of the tooth regenerative therapy for dental disorders. To address the desirable future clinical applications of whole-tooth replacement therapy, one of the major research hurdles remaining is the identification of appropriate cell sources (Ikeda and Tsuji 2008). Of course, the cell source may be optimised by using the patient's own cells for regenerative therapy to avoid immunological rejection. In the dental field, recent studies of stem-cell biology have led to the identification of candidate cell sources based on tooth organogenesis for tooth tissue regeneration and tooth replacement therapy (Purnell 2008; Volponi et al. 2010). These dental stem cells, such as DPSCs, SHED, SCAP, PDLSCs and dental-follicle stem cells, can differentiate into several dental-cell lineages and also contribute to the turnover and supply of various progenitor cells (Gronthos et al. 2000; Miura et al. 2003; Sonoyama et al. 2008; Seo et al. 2004). Although these linages would be valuable cell sources for stem-cell transplantation therapy aimed toward dental tissue regeneration (Egusa et al. 2012, 2013), the tooth inductive potential cells, which can replicate an epithelial-mesenchymal interaction for whole-tooth regeneration, has not yet been identified. Pluripotent stem cells including ES cells and iPS cells are also candidate cell sources that are capable of differentiating into endodermal, ectodermal and mesodermal cell types (Yan et al. 2010). Recently, iPS cells have been established from several oral tissues such as pulp, PDL, gingiva and oral mucosa, and they have represented the ability to

differentiate into dental epithelial and mesenchymal cells (Egusa et al. 2010; Arakaki et al. 2012; Otsu et al. 2012). Meanwhile, the identification of master genes for reprogramming non-dental cells to differentiate into dental epithelial and mesenchymal cells is considered to an important direction for future tooth regenerative therapy. Notably, the self-organisation of various tissues such as the optic cup and adenohypophysis using uniform pluripotent stem cells in three-dimensional culture has been reported (Eiraku et al. 2011; Suga et al. 2011; Sasai 2013). A three-dimensional *in vitro* organogenesis system using the proper induced stem cells would contribute to the development of regenerative strategies for whole teeth and other organs.

The different tooth types such as incisors, canines, premolars and molars have unique morphological features that are programmed at predetermined sites in the oral cavity during tooth development. Several studies have proposed molecular mechanisms for tooth morphology regulation (Ishida et al. 2011; Cai et al. 2007). Tooth size, crown and root shape are important aspects upon generating a bioengineered tooth with proper functional occlusion and aesthetics. Further studies are required to develop a bioengineering technology that can regulate tooth morphology, including tissue engineering using scaffolds and the identification of morphogenesis-related genes/cytokines to achieve the appropriate morphogenesis. Tooth regenerative therapy is now regarded as a crucial model for future organ replacement regenerative therapies for severe diseases and will contribute substantially to the knowledge and technology of whole-organ regeneration (Ikeda and Tsuji 2008; Volponi et al. 2010).

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