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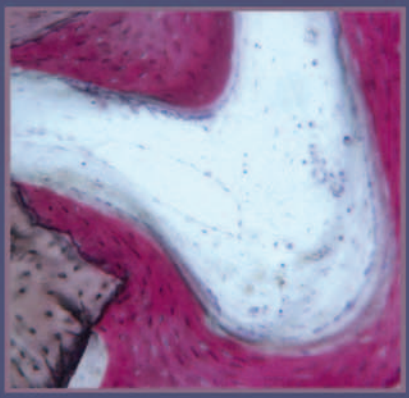

Bone Response to Dental Implant Materials

Edited by Adriano Piattelli

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Bone Response to Dental Implant Materials

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Bone Response to Dental Implant Materials

Edited by

Adriano Piattelli

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ISBN: 978-0-08-100287-2 (print)
ISBN: 978-0-08-100288-9 (online)

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

For information on all Woodhead Publishing publications visit our website at https://www.elsevier.com/

Publisher: Matthew Deans
Acquisition Editor: Laura Overend
Editorial Project Manager: Lucy Beg
Production Project Manager: Poulouse Joseph
Designer: Mark Rogers

Typeset by TNQ Books and Journals

Contents

List of contributors

H. Alexander Orthogen LLC, Springfield, NJ, United States

S. Anil Prince Sattam Bin Abdulaziz University, AlKharj, Saudi Arabia

L. Bagán-Debon University of Valencia, Valencia, Spain

J.V. Bagán-Sebastián University of Valencia, Valencia, Spain

J.L. Calvo-Guirado Universidad Católica San Antonio (UCAM), Murcia, Spain

E.P. Chalisserry Pukyong National University, Busan, Korea

A. De Sanctis University "G. d'Annunzio" of Chieti-Pescara, Pescara, Italy

J. Diaz-Marcos Scientific and Technological Centers of the University of Barcelona, Barcelona, Spain

J. Duyck KU Leuven, Leuven, Belgium; U.Z. St. Raphaël, Leuven, Belgium

A. Fontana University "G. d'Annunzio", Chieti, Italy

G. Frisardi Epochè – Orofacial Pain Centre, Rome, Italy; University of Sassari, Sassari, Italy

F. Frisardi Epochè – Orofacial Pain Centre, Rome, Italy

S.A. Gattone University "G. d'Annunzio" of Chieti-Pescara, Pescara, Italy

S. Gehrke Universidad Católica San Antonio (UCAM), Murcia, Spain

A. Giuliani Università Politecnica delle Marche, Ancona, Italy

F. Iaculli University "G. d'Annunzio", Chieti, Italy

G. Iezzi University "G. d'Annunzio", Chieti, Italy

O.T. Jensen University of Utah School of Dentistry, Salt Lake City, UT, United States

S.-K. Kim Pukyong National University, Busan, Korea

J.E. Maté Sánchez de Val Universidad Católica San Antonio (UCAM), Murcia, Spain

C. Murray European University College, Dubai, UAE

M. Peñarrocha-Diago University of Valencia, Valencia, Spain

V. Perrotti University "G. d'Annunzio", Chieti, Italy

A. Piattelli University "G. d'Annunzio", Chieti, Italy

R.L. Reuben Heriot-Watt University, Edinburgh, United Kingdom

J. Ricci New York University College of Dentistry, New York, NY, United States

M.S. Shim Incheon National University, Incheon, Republic of Korea

D. Soto-Peñazola University of Valencia, Valencia, Spain

E.M. Staderini Western Switzerland Universities of Applied Sciences, Geneva, Switzerland

P.P. Valentini University Tor Vergata, Rome, Italy

K. Vandamme KU Leuven, Leuven, Belgium; U.Z. St. Raphaël, Leuven, Belgium

J. Venkatesan Incheon National University, Incheon, Republic of Korea

I.-S. Yeo Seoul National University, Seoul, Korea

B. Zavan University of Padova, Padova, Italy

Introduction to bone response to dental implant materials

1

V. Perrotti, F. Iaculli, A. Fontana, A. Piattelli, G. Iezzi
University "G. d'Annunzio", Chieti, Italy

1.1 Introduction

1.1.1 Bone structure in the aspect of functionality

Bone tissue, originating from mesenchymal tissue, is a type of specialized connective tissue that functions as a support. It is involved in many processes, which are essential for the human body. Bone is uniquely designed for its role of providing mechanical stability to the skeleton, which is needed for load bearing, locomotion, and protection of internal organs; it presents characteristics such as strength, hardness, and resistance to pressure, traction, and torsion. Furthermore, the homeostasis of calcium level in blood is maintained because the mineral calcium, which is stored in the bone, is mobilized from the storage reserve to enter the blood. The diversity of the bone functionality can be attributed to its complex structure. Indeed, most of the unique properties of the bone are related to its specific constitution.

Bone is composed of cells and an intercellular matrix rich in organic compounds, mainly type I collagen fibers embedded in a ground substance consisting of proteoglycans, glycoproteins, as well as inorganic minerals. The collagen fibers form bundles or fibrils, which resist the pulling forces, whereas the minerals provide stiffness, which resists bending and compression. Bone minerals are mainly in the form of crystals of calcium phosphate—calcium hydroxyapatite (HA) and when associated with collagen fibers give the specific hardness to the bone.

Although the bone is populated by a variety of different cells, its functional integrity is guaranteed by four principal cell types: the osteoclasts (OCLs), bone-destroying cells; the osteoblasts (OBLs), bone-forming cells; the osteocytes (OCTs), bone-maintaining cells; and the endothelial cells (ECs), bone-related angiogenic cells. All of them have defined tasks and are thus essential for the maintenance of a healthy bone tissue.

OCLs are large, multinucleated cells formed by the self-fusion of macrophages (Fig. 1.1). They are located on the bone surface in shallow pits called resorption pits or Howship's lacunae. The main function of OCLs is resorption of the bone tissue. The OCLs are able to resorb the strong matrix by secreting acid and collagenase. Resorption plays a crucial role in the maintenance, repair, and remodeling of bones. OCLs are formed by the fusion of mononuclear precursors derived from the pluripotential hematopoietic stem cells and share more committed hematopoietic progenitors with cells of the mononuclear phagocyte system [1].

Bone Response to Dental Implant Materials. http://dx.doi.org/10.1016/B978-0-08-100287-2.00001-X
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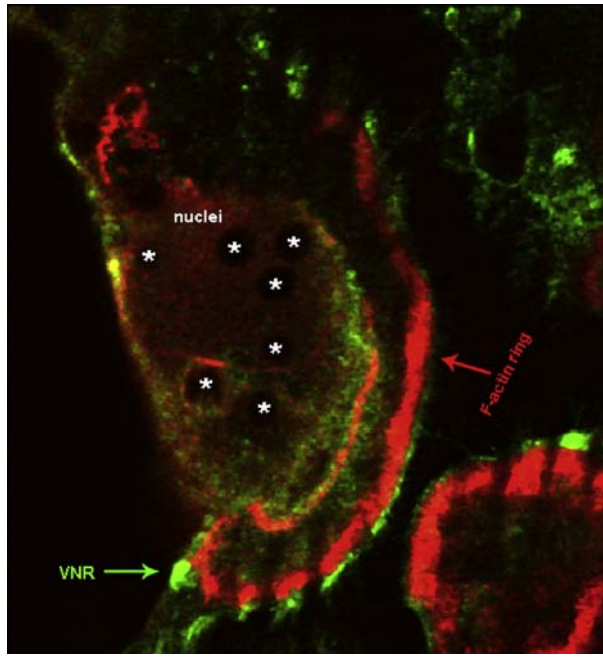

Figure 1.1 High-power *XY* view of a multinucleated OCL (asterisks) in peripheral blood mononuclear cell cultures of the bovine bone. Red indicates positive staining for F-actin—enriched patches and rings with phalloidin—tetramethylrhodamine B isothiocyanate (TRITC) indicating activated OCLs (*red arrows*). Green indicates positive staining for the monoclonal antibody 23C6 to detect human integrin alpha V beta 3 complex of the vitronectin receptor, scale bar $^{1}/_{4}$ 10 mm.

OBLs are mononucleate cells of mesenchymal origin that are responsible for the bone formation; they are located mostly on the surface of the bone, as a single layer of mononuclear cells (Fig. 1.2). Their function is to produce the organic components of the bone matrix. When active, they show high alkaline phosphatase activity. OBLs eventually become trapped in the matrix they produce and become OCTs.

OCTs are star-shaped cells that occupy the lacunae in the bone matrix and are the most common cell types in the bone (Figs. 1.3 and 1.4). They show thin cytoplasmic processes called filopodia that form a network of small canals called canaliculi. This network is essential for the exchange of nutrients and waste. OCTs are very long-living cells, with a half-life of 25 years, and are not capable of division. These cells have a mechanosensory activity, they have reduced synthetic activity, but are also able to break down the bone matrix through a mechanism called osteocytic osteolysis that releases calcium ions for calcium homeostasis and has an important role in phosphate metabolism. Besides these functions in molecular synthesis and modification, OCTs are able to transmit signal over long distances through canaliculi. There is growing evidence that OCTs are regulatory cells that control the function of OBLs and OCLs.

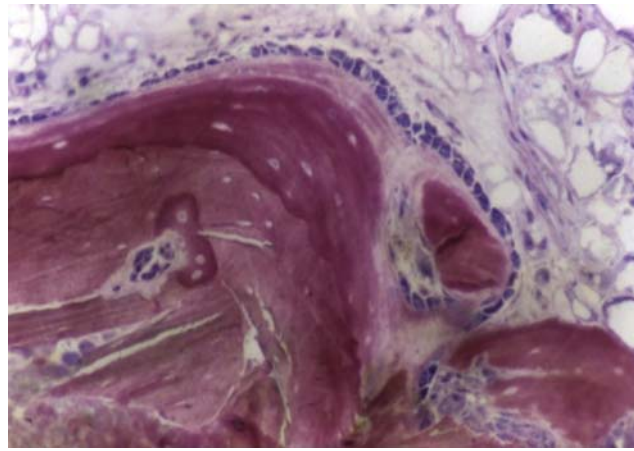

Figure 1.2 A rim of OBLs producing osteoid matrix. Toluidine blue and acid fuchsin staining; original magnification 200×.

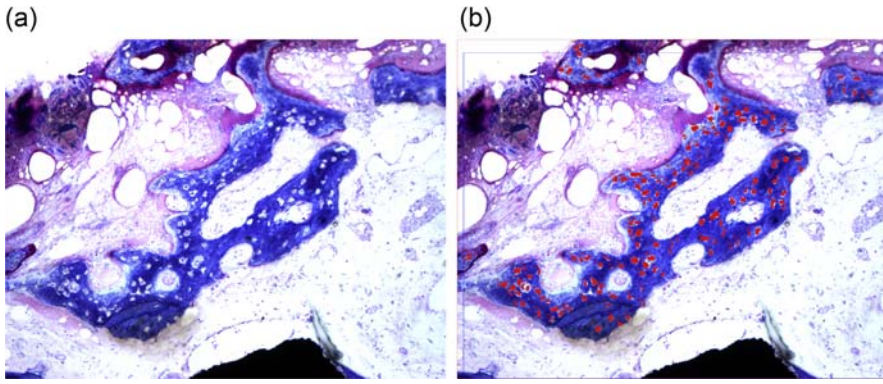

Figure 1.3 (a) Histological image showing OCTs in the peri-implant bone tissue of samples retrieved from humans after a loading period of 4 weeks−7 months. (b) Images showing how the count of the number of OCTs was undertaken. OCTs lacunae were highlighted in red. Toluidine blue and acid fuchsin staining; original magnification 100×.

ECs are very flat, they form pavement-like patterns on the inside of the vessels and are known to function in a variety of important physiological processes. Essentially, ECs secrete a number of mediators (factors), which may elicit biological responses by various signal-transduction mechanisms. Such mediators are implicated in regulating the permeability of the endothelium and can promote chemotactic responses, such as inflammation and blood clotting.

It is well established that bone formation is an angiogenesis-dependent process [2], and ECs have long been known for their role in the formation of blood vessels that supply oxygen and nutrients to the developing bone tissue. However, it has been

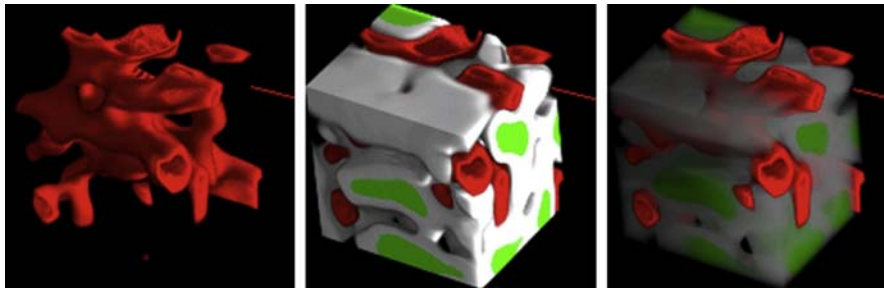

Figure 1.4 Regenerative potential of collagenated biomaterial grafts. Representative subvolume of a collagenated biomaterial as retrieved from in vivo test after 12 months and studied by synchrotron radiation−based, phase-contrast microtomography. Legend: *red phase*, regenerated vessels; *white phase*, newly formed bone and bone under remodeling; *green phase*, fully mineralized bone and residual scaffold.
Courtesy Dr. Alessandra Giuliani, Università Politecnica delle Marche, Ancona, Italy.

suggested, more recently, that ECs may play a more direct role in bone development and formation through their interactions with osteoprogenitor cells [3] and, under certain conditions, their production of specific bone-inductive factors [4].

At the macroscopic level, the bone is arranged in two architectural forms: dense compact bone (cortical, around 80% of the total skeleton) and cancellous (trabecular, around 20% of the total skeleton) bone (Fig. 1.5). Cortical bone is dense and made of multiple stacked layers with less than 10% porosity.

It is organized in cylindrical shaped elements called osteons, composed of concentric lamellae (Fig. 1.6).

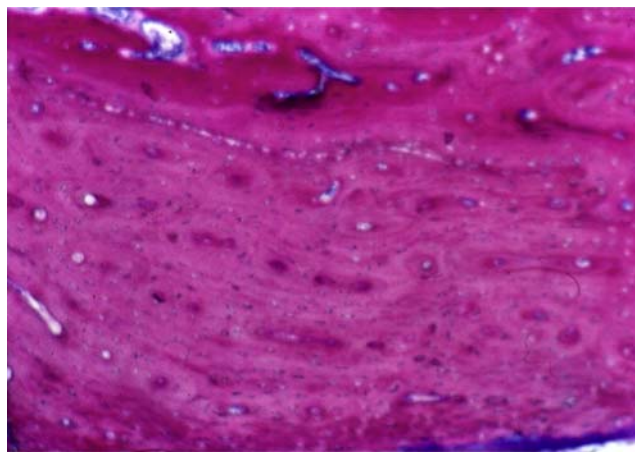

Figure 1.5 Histological image of the dense cortical bone tissue. Toluidine blue and acid fuchsin staining; original magnification 200×.

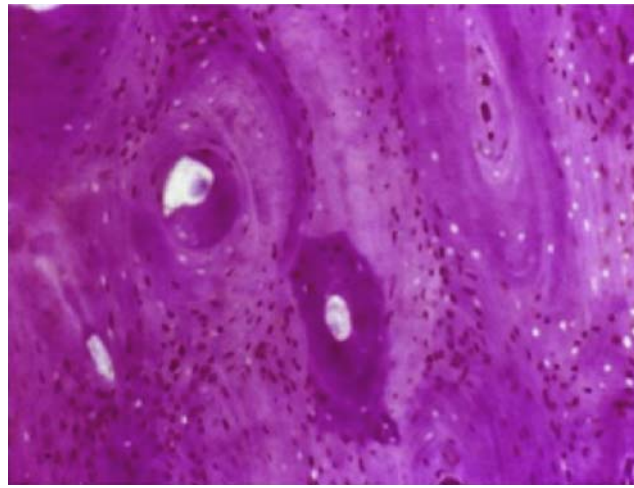

Figure 1.6 Histological image of osteons consisting of concentric layers, or lamellae, of compact bone tissue that surround a central canal, the Haversian canal. Toluidine blue and acid fuchsin staining; original magnification 100×.

The space between osteons is occupied by interstitial lamellae, which are remnants of osteons partially resorbed during bone remodeling. Osteons are cylindrical structures that are usually several millimeters long and around 0.2 mm in diameter.

The center of an osteon is made of a central canal, called the Haversian canal, that contains the bone's nerve and blood supply. On the surface of the osteon, the boundary is formed by the cement line (Fig. 1.7).

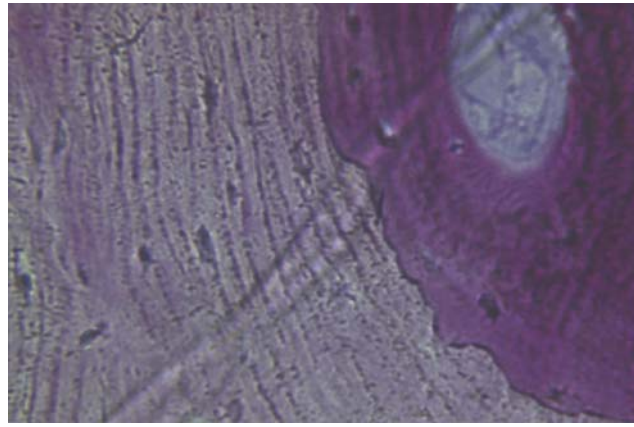

Figure 1.7 Histological image of a secondary osteon, showing the cement line formed as a result of bone remodeling process. Toluidine blue and acid fuchsin staining; original magnification 200×.

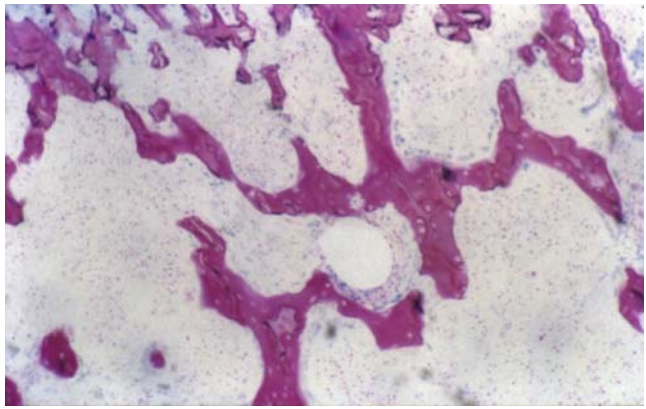

Figure 1.8 Histological image of the trabecular bone with wide marrow spaces. Toluidine blue and acid fuchsin staining; original magnification 100×.

Cortical bone is usually found on the surface of bones. In contrast, cancellous bone is organized in a porous sponge-like pattern (50−90% porosity) and it consists of a honeycomb of branching bars, plates, and rods of various sizes called trabeculae and oriented according to the direction of the physiological load (Fig. 1.8).

It is much softer, weaker, and more flexible than the cortical bone and therefore has a higher surface area to mass ratio, which makes it suitable for metabolic activity such as the exchange of calcium ions. It is found in most areas of the bone that is not under high mechanical stress. Cancellous bone makes up the bulk of the interior of most bones. The difference in tissue arrangement between the two types of bone provides increased resistance to torsion and bending; the resistance to torsion and bending by cortical bone is around 20 times superior compared to that by cancellous bone.

At the microscopic level, cortical and cancellous bone may consist of woven or lamellar bone. Woven bone is organized in a small number of randomly oriented collagen fibers and contains a high proportion of OCTs (four times the number of OCTs per unit of volume compared to lamellar bone; Fig. 1.9).

Lamellar bone is highly organized in concentric sheets filled with many collagen fibers parallel to other fibers in the same layer and contains a low proportion of OCTs. After a fracture, woven bone quickly forms and is gradually replaced by slow-growing lamellar bone through a process known as “bony substitution.”

Bone is a dynamic, highly vascularized tissue with the unique capacity to heal and remodel without leaving a scar. The dynamics of bone formation involves three different processes:

- Growth
- Modeling
- Remodeling

During childhood and the early years of adulthood, while the epiphyses are still open, the skeleton grows in length (growth), the bones expand in diameter and achieve their external shape (modeling). During bone modeling, OBLs and OCLs work

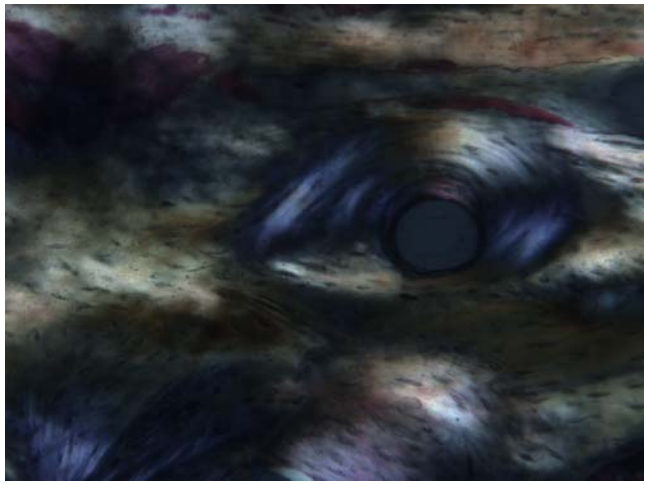

Figure 1.9 A light micrograph under the polarized light of human bone, where an osteon, typical of lamellar bone, is evident. Toluidine blue and acid fuchsin staining; original magnification 100×.

independently of each other and on different bone surfaces. The net balance is positive and it results in bone expansion, with the bone formation exceeding bone resorption. Bones reach their final external form and high bone density during this period. Both the growth and the modeling processes are controlled by hormones and mechanical forces. Following growth, bone volume remains static, with resorption and formation being in balance. Around the age 20–25 years, peak bone mass is achieved as a result of these processes. However, in later life resorption exceeds formation, leading to a slow decline in the bone mass. There is thus an unavoidable loss of the bone mass with age and a disruption of the trabecular network, which makes fortuitous osteoclastic perforations possible. Loss of the bone mass with age is unavoidable and is caused by the third process—bone remodeling. The latter process occurs once growth and modeling of the skeleton have been completed. It is likely that the major reason for remodeling is to enable the bones to respond and adapt to mechanical stresses, for example, as a result of physical exercise and during mechanical loading (e.g. orthodontic tooth movement or implant loading). Moreover, bone remodeling is designed to maintain a physiologically and mechanically competent skeleton and to repair areas of microdamage. Wolff's law states that bones develop a structure most suited to resist the forces acting upon them, adapting both the internal architecture and the external conformation to the change in external loading conditions. This change follows precise mathematical laws. When a change in loading pattern occurs, stress and strain fields in the bone are modified accordingly. Bone tissue detects the local change in strain and then adapts accordingly. The internal architecture is adapted in terms of change in density and disposition of trabeculae and osteons, the external conformation in terms of shape and dimensions. When strain is intensified, the new bone is formed. The process is complex and requires interaction between different cell phenotypes that are regulated by a variety of biochemical and mechanical factors.

Many oral conditions could lead to bone loss, such as infection, trauma, resorption after tooth extraction or surgical bone resection, and aging. It is imperative to restore the bone loss, which is the first step in any further prosthetic restoration. Various surgical solutions have been developed that allow the recovery of the lost bone. These techniques are combined with the use of biocompatible materials acting as scaffolds in supporting the bone regeneration.

1.1.2 Bone remodeling

The current concept of bone remodeling is based on the hypothesis that OCL precursors become activated and differentiate into OCLs and this begins the process of bone resorption. This step is followed by a bone formation phase. The number of sites entering the bone formation phase, called the activation frequency, together with the individual rates of the two processes, determines the rate of tissue turnover [5]. The signal that initiates bone remodeling has not been identified yet. Recently, it has been shown that mechanical stress can be sensed by OCTs and that these cells secrete paracrine factors such as insulin-like growth factor I (IGF-I) in response to mechanical forces [6]. Although IGF may act as a coupling factor in the bone remodeling cycle, the signal that initiates the cycle remains elusive. The sequence of events in the normal remodeling cycle is always the same, osteoclastic bone resorption, a reversal phase, followed by osteoblastic bone formation to repair the defect.

The termination of bone resorption and the initiation of bone formation in the resorption lacunae occur through a coupling mechanism [7]. The coupling process ensures that an equivalent amount of bone is laid down following the previous resorption phase. The detailed nature of the activation and coupling mechanism is still unknown, although the roles of some growth factors and proteinases such as transforming growth factor-β1 (TGF-β), IGF-I, IGF-II, and plasminogen activators have been indicated [8]. Whether the activation of OBLs begins simultaneously with OCLs' recruitment or at some later stage during the lacunar development is still not clear. Bone remodeling is regulated by systemic hormones and by local factors, which affect cells of both the OCL and OBL lineage and exert their effects on the replication of undifferentiated cells, the recruitment of cells, and the differentiated function of cells [9]. The end product of remodeling is the maintenance of a mineralized bone matrix and the major organic component of this matrix is collagen I (COL-1). The local factors are synthesized by skeletal cells and include growth factors, cytokines, and prostaglandins. Growth factors have effects on cells of the same class (autocrine factors) or on cells of another class within the tissue (paracrine factors).

Growth factors are also present in the circulation and may act as systemic regulators of skeletal metabolism, but the locally produced factors have more direct and important functions in cell growth. Growth factors may play a critical role in the coupling of bone formation to bone resorption and possibly in the pathophysiology of bone disorders.

Bone resorption is stimulated or inhibited by signals from other parts of the body, depending on the demand for calcium. Calcium-sensing membrane receptors in the parathyroid gland monitor calcium levels in the extracellular fluid. Low levels of

calcium stimulate the release of parathyroid hormone (PTH) from chief cells of the parathyroid gland. In addition to its effects on the kidney and intestine, PTH also - increases the number and activity of OCLs to release calcium from the bone and thus stimulates bone resorption. High levels of calcium in the blood, on the other hand, leads to decreased PTH release from the parathyroid gland, decreasing the number and activity of OCLs, resulting in less bone resorption.

OBLs stimulate osteoclastic differentiation of OCL precursors through Wingless-related integration site 5a (Wnt5a) signaling. The matricellular signaling effected by TGF-β1 and IGF-1 is integrated with the Sema4D-Plexin B1-mediated OCL−OBL interaction. Sema4D, whose secretion by OCLs is stimulated by increased OCL differentiation factor receptor activator of nuclear factor kappa-B ligand (RANKL), inhibits OBLs' differentiation. OBLs are induced to migrate to the resorption sites and differentiate through the secretion of Wnt10b by OCLs at the end of the resorption phase. OBLs, in turn, inhibit osteoclastogenesis (and therefore bone resorption) via osteoprotegerin (OPG) and RANKL secretion.

OCTs regulate bone formation through the release of Wnt antagonists, Sclerostin and Dickkopf-related protein 1, which in turn are inhibited by mechanosignals and PTH. Wnt signaling in OCTs controls the production of OPG, a decoy receptor for the key RANKL. In the bone resorption cavity, calcium, TGF-β1, and IGF-1 are released in response to osteoclastic activity. A number of paracrine signals are stimulated in OCTs following changes in skeletal loading, including prostaglandin I2 and prostaglandin E2, nitric oxide, and IGF. Recent studies have raised the intriguing possibility that the OCT apoptosis may be part of the mechanism whereby OCLs are targeted to sites of bone resorption as it is elevated in the bone that is being remodeled. Estrogen suppression, a known stimulant of bone resorption, increases OCT apoptosis, and changes in bone loading are also associated with OCT apoptosis. The phenotype of the OCTs appears deficient in some receptors found on the OBL. However, the OCT is well adapted for its role in bone homeostasis and maintains intracellular signaling to respond to the unique demands of its location.

It is well established that the bone formation is an angiogenesis-dependent process [2], and ECs have long been known for their role in the formation of blood vessels that supply oxygen and nutrients to the developing bone tissue. However, it has been suggested, more recently, that ECs may play a more direct role in the bone development and formation, through their interactions with osteoprogenitor cells [3] and, under certain conditions, their production of specific bone-inductive factors [4]. ECs secrete a number of mediators (factors), which may elicit biological responses by various signal-transduction mechanisms. Such mediators are implicated in regulating the permeability of the endothelium and can promote chemotactic responses in a variety of important physiological processes, such as bone formation, remodeling, and healing. Indeed, it is the capillary that supplies oxygen and nutrients and removes calcium and waste products of resorption. One of the most important nutrients transported via the vasculature to the basic multicellular unit is oxygen. In the absence of oxygen, OBLs cannot produce collagen effectively and their proliferation is reduced. Cellular responses to changes in oxygen tension are directed through the activity of the hypoxia-inducible factor (HIF), which is capable of activating the gene transcription

in response to low oxygen levels. OBL-specific knockdown of HIF1α or HIF2α has demonstrated important roles for HIF in controlling bone formation and vascularity. Furthermore, low oxygen environments encourage OCL HIF1α stabilization leading to increased OCL number.

1.1.3 The modern concept of biocompatibility

For over 50 years, biocompatibility consisted of implantable medical devices that should remain in contact with the tissues of the human body for a long time, without showing any adverse effect on those tissues from a chemical and biological point of view.

The first generation of implantable devices was designed and developed during the 1940s, and over the next few decades it became obvious that the best biological performance would be achieved with materials that showed the least chemical reactivity.

The selection criteria for implantable biomaterials included a list of events that had to be avoided, such as the local or systemic release of some products of corrosion or degradation, additives or contaminants of the main biomaterial, and their subsequent biological reaction. So, materials were selected if they were nontoxic, nonimmunogenic, nonthrombogenic, noncarcinogenic, and nonirritant.

Three important factors initiated a reevaluation of the biocompatibility concept:

1. The response to specific materials could vary from one application site to another, showing that the biocompatibility was dependent both on the material characteristics and on its application;
2. The material should specifically react with the surrounding tissues in a positive way, avoiding any adverse effect;
3. The material should degrade over time in the body rather than remain indefinitely.

Accordingly, biocompatibility was redefined in 1987 as follows: "Biocompatibility refers to the ability of a material to perform with an appropriate best response in a specific situation" [10]. Because this definition appeared to be too general, because specific mechanisms, such as individual involved processes or innovation of new biomaterials, were not provided, a modern approach defined biocompatibility as "a complex that depends on the characteristics of a material and on the biological host system."

Once grafted, the biomaterial should interact with cells of the host tissue, producing an appropriate response, which would lead to the desired clinical outcome through a combination of positive effects on critical cells and the avoidance of negative impact on others. The critical cells could be embryonic stem cells, ECs, or OBLs. The time scale may be minutes, hours, days, or years and the clinical outcome could be tissue replacement, functional support, tissue regeneration, etc. The biomaterial influences the events within the biological environment by either mechanical or molecular signaling processes, or more commonly by both. The biomaterial encounters macromolecules in the environment and becomes coated by an adsorbed layer typically composed of proteins, which may be coupled with biomaterial-derived ions or molecules. All subsequent interactions will take place between macromolecule-coated biomaterial and

surrounding tissues. Although because a material may affect different biological systems in different ways, there is not a material with unique biocompatibility characteristics.

Bone substitute materials should have osteoconductive properties, become integrated in bone and replace it, allow ingrowth of blood vessels, and be easy to use as well as cost-effective. However, the modern concept of biocompatibility implicates that biomaterials, besides osteoconductivity, should also show osteoinductive and even osteogenic properties. Osteoconductive materials are composed of a matrix that acts as a scaffold for the bone deposition. Osteoinductive materials contain molecules that stimulate differentiation of progenitor cells into OBLs. Some biomaterials even contain osteogenic cells, OBLs, or OBL precursors, which are capable of forming bone if placed in the proper environment.

Autologous bone (AB) is the only material characterized by osteogenic properties with the best results in bone regeneration, although its limited availability and the need for an additional surgical procedure to harvest the bone are nowadays considered disadvantages in its use.

It is extremely important to evaluate the interaction of a biomaterial with the host in the attempt to establish its biocompatibility and investigate their interactions. A good biomaterial should stimulate some cells of the receiving site, such as OBLs, OCLs, cells of innate, and adaptive immunity and platelets. Therefore, the host cells can be divided into three groups [11]:

1. Target cells
2. Defensive cells
3. Interfering cells

The target cells are the cells at which the therapy is aimed. They could be OBLs in bone contacting device, stems cells in a tissue engineering bioreactor, or cancer cells in a polymer-chemotherapeutic agent [12,13]. The defensive cells are cells of innate and adaptive immunity and platelets. Their existence is based on the need to repel and remove adverse external agents. The interfering cells are those that are in their natural habitat and essentially get in the way and interfere with the response, for example, fibroblasts in the soft connective tissue [14] or OCLs in the bone [15]. The activity of these cells can lead to hyperplasia or tissue resorption, or other undesirable events.

The involvement of defensive cells in the entire process is inevitable and the critical question is whether their responses are controlled or uncontrolled. In the latter case, the cells of the immune system react to the presence of the biomaterial, resulting in the release of a variety of proinflammatory mediators. The combined cellular and humoral answer during the inflammatory process can lead to an accelerating and aggressive reaction that destructs both biomaterial and host tissue [16]. In other cases, the presence of the irritant biomaterial may lead to giant cell formation and granulation tissue generation [17]. Interfering cells form part of the normal anatomical structure into which the biomaterial may be grafted and their influence can have an important effect on the clinical outcomes. The biomaterial components are usually nonspecific and may induce uncontrolled response of both defensive and interfering cells, which may lead to excessive tissue growth, tissue loss, and the loss of function because of the perturbation of normal homeostasis [18].

Moreover, the biomaterial components can be uptaken by the surrounding cells through a variety of mechanisms such as phagocytosis, pinocytosis, endocytosis, or the direct transit through the plasma membrane. Once inside the cell, the component can directly affect some cellular metabolic pathways or it can be degraded in endosomes and lysosomes or be altered by the cell enzymes. The products of these processes also influence the cell metabolism. The generation of reactive oxygen species could be induced and, together with alterations in organelle function, can result in cell damage or interfere with apoptotic and necrotic pathways [19].

1.2 Biomaterials

Several different biomaterials have been used in bone regeneration procedures and all of them seem to be able to favor the formation of a significant amount of vital bone.

A biomaterial should act as a scaffold for the formation of bone, possesses pore volume, pore interconnectivity, and pores size adequate to allow the invasion of osteogenic cells and blood vessels, and have mechanical features similar to the tissues to be regenerated. Biomaterials should, moreover, present a biologic stability, help in the volume maintenance, and allow for bone remodeling. Macro- and microporosity and the interconnecting porous structure of the grafted biomaterial play a relevant role in supporting the penetration, proliferation, and differentiation of OBLs and the ingrowth of newly formed blood vessels into the biomaterial particles.

Some researchers believe that a biomaterial should be completely resorbed and replaced by newly formed bone.

1.2.1 Autologous bone

AB is the golden standard of the grafting material due to the presence of vital OBL and growth factors. It has osteogenic, osteoinductive, and osteoconductive capabilities. Histology shows that it is a highly osteoconductive material, and most of the particles are partially and/or completely surrounded by newly formed bone, in tight contact with the particles. A complete absence of inflammatory cells, multinucleated giant cells, or foreign body reaction cells should be noted. However, its main disadvantage is related to its quantity obtained from intraoral sources, and often an additional surgical procedure is required, with a higher morbidity. Furthermore, AB can present a rapid and unpredictable resorption [20].

1.2.2 Porous phycogenic hydroxyapatite

Porous phycogenic hydroxyapatite (PHA) derived from calcifying maritime algae (*Coralline officialis*) is a biologic HA, prepared by the hydrothermal conversion of the calcium carbonate in the presence of ammonium phosphate at about 700°C. This process helps in preserving the porous structure of the biomaterial. Its composition is pure inorganic calcium phosphate. The pores have a mean diameter of 5−10 μm

with a periodical septation with a mean length of 50−100 μm and interconnected by means of small perforations with a mean diameter of 1−3 μm. Every pore is lined by fluorhydroxyapatite crystallites, with a size of 25−35 nm [11]. The particles are interconnected by microperforations, having a mean diameter of 1 μm. Its elevated microporosity should be helpful in the ingrowth of osteogenic cells and blood vessels.

PHA is a biocompatible, osteoconductive, and resorbable biomaterial. Most of the biomaterial particles appeared to be surrounded by newly formed bone, and in a few fields some particles seemed to be partially resorbed and substituted by newly formed bone. No inflammatory cell infiltrate or foreign body reaction cells were present; the bone was always in tight contact with the biomaterial particles with no intervening gaps (Figs. 1.10 and 1.11). Bone was present inside many biomaterial particles [21,22].

1.2.3 Collagenized porcine biomaterial

Collagenized porcine biomaterial is composed of carbonated nanocrystalline HA, containing organic material.

Most of the grafted biomaterial particles were surrounded by the newly formed bone with large OCT lacunae, always in tight contact with the particles, and no gaps were observed at the bone−biomaterial interface. No inflammatory cells and multinucleated giant cells were present. Some of the grafted particles were bridged and cemented by the newly formed bone. Many bone trabeculae were undergoing remodeling. Porcine bone is a highly osteoconductive biomaterial. It undergoes resorption, with the presence of active resorption signs (Fig. 1.12). OBLs and newly formed bone were commonly found on the surface of the biomaterial particles [23−25].

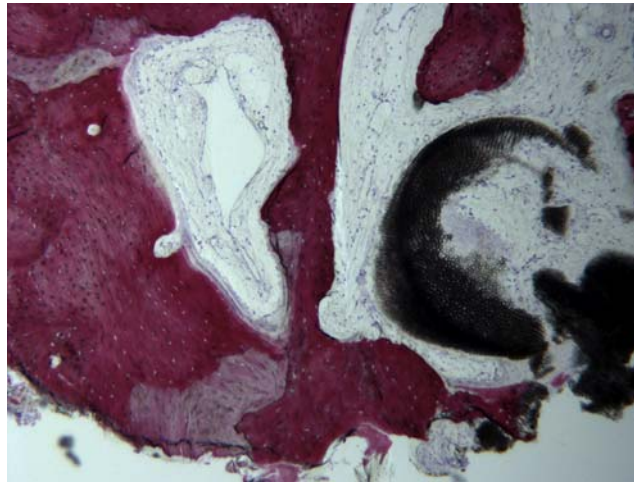

Figure 1.10 Histological image of a sample retrieved 6 months after a sinus lift in a human subject. Newly formed bone with remodeling areas and residual porous PHA material can be observed. Toluidine blue and acid fuchsin staining; original magnification 40×.

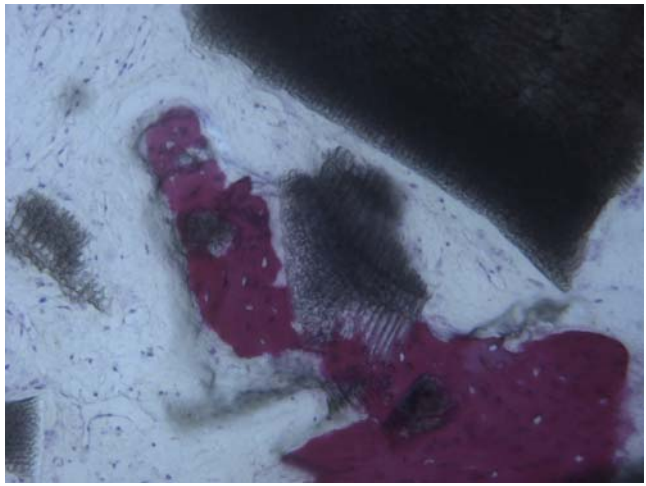

Figure 1.11 High-power histological image of porous PHA particles partially surrounded by the newly formed bone. Toluidine blue and acid fuchsin staining; original magnification 100×.

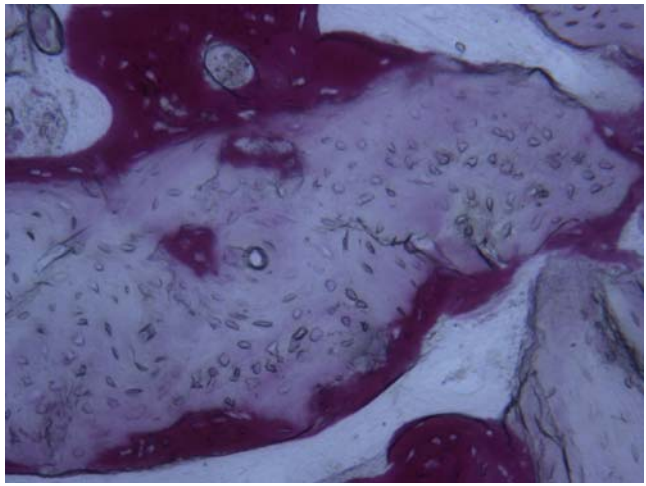

Figure 1.12 Histological image of a collagenized porcine material used to regenerate a postextraction socket in humans. The sample was retrieved after a 3-month healing time. The biomaterial particle is surrounded by the newly formed bone, which can be seen also in the inner part of the granule. The material's margin appears indented. Toluidine blue and acid fuchsin staining; original magnification 100×.

1.2.4 Anorganic bovine bone

Anorganic bovine bone (ABB) is a deproteinized bovine bone with a 75−80% degree of porosity and a size of the crystals of about 10 nm. It presents large pores and a high connectivity. ABB is one of the most used biomaterials and it has shown good

osteoconductive properties. No inflammatory cell infiltrate, foreign body response, and other adverse effects are present. A high quantity of new bone formation has been reported with the use of ABB. Usually, ABB particles seem to be almost completely surrounded by the newly formed bone. No gaps or connective, fibrous tissue were observed at the bone−biomaterial interface. Some particles seemed to be bridged by the newly formed bone. Due to its low resorption rate, ABB may significantly contribute in the prevention of volume tissue loss in grafted sites opposing, for example, the sinus pressure due to repneumatization. The ABB particles and the newly formed bone produce a dense hard tissue supportive, also over the long term, of loaded implants (Fig. 1.13). This biomaterial has, thus, a long-term, three-dimensional stability [26,27].

1.2.5 Biphasic calcium phosphate

Biphasic calcium phosphate (BCP) is composed of a combination of HA and tricalcium phosphate (TCP) and used in bone regeneration procedures. It has different ratios of HA/TCP, giving rise to balanced phases of activity, a more stable phase of HA, and a more soluble phase of TCP. The resorption rate of the material is dependent on the HA/TCP ratio (a higher TCP means a higher solubility); this material gradually dissolves in the body, determining the new bone formation by the release of calcium and phosphate ions. BCPs are highly biocompatible, and they do not provoke a foreign body or a toxic response. Most of the grafted BCP particles were partially surrounded by the newly formed bone with no gaps (Fig. 1.14). Some particles were bridged by the newly formed bone. Resorption was observed at the surface of some particles [28,29,30].

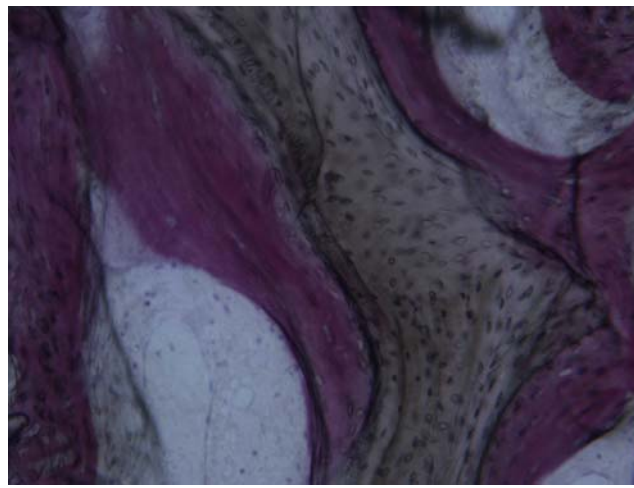

Figure 1.13 Histological image of an ABB particle integrated into the bone tissue and bridging the newly formed bone trabeculae. Toluidine blue and acid fuchsin staining; original magnification 100×.

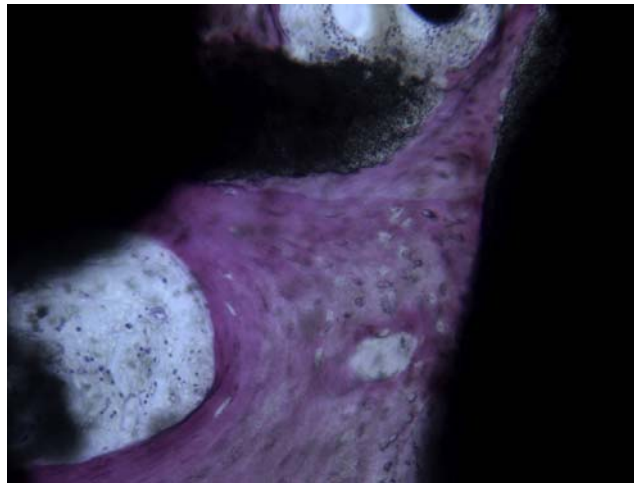

Figure 1.14 Histological image of BCP material retrieved 6 months after a sinus augmentation procedure in humans. In some portions, both next to newly formed bone and to a marrow space, signs of dissolution of the biomaterial particles can be observed. Toluidine blue and acid fuchsin staining; original magnification 100×.

1.2.6 Calcium carbonate

Different types of corals (aragonite or calcite forms of calcium carbonate) have been successfully used as a bone grafting biomaterial. They have a porous structure (150−500 μm), similar to the cancellous bone. These biomaterials can combine good mechanical properties with an open porosity. Their interconnected porous architecture, high compressive breaking stress, good biocompatibility, and resorbability, suggest their use as scaffolds for bone tissue engineering. Newly formed bone, with wide marrow spaces and wide OCT lacunae, was usually found around many biomaterial particles and some were bridged by the newly formed bone (Fig. 1.15). No inflammatory cell infiltrate, foreign body reaction, and fibrous connective tissue at the interface were observed [31,32].

1.3 Challenges and further trends

1.3.1 Graphene

Graphene is a flat monolayer of carbon atoms in a two-dimensional (2D) honeycomb lattice with high aspect ratio layer geometry and a very high specific surface area. It has attracted attention in recent years due to its exceptional thermal, mechanical, and electrical properties. Indeed, although graphene was originally developed for nanoelectronics applications, research interests in this material are continuously expanding to other fields, such as biomedicine and regenerative engineering.

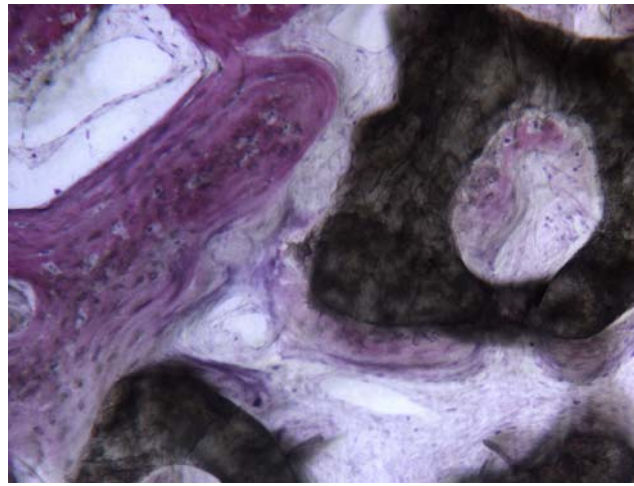

Figure 1.15 Histological image of calcium carbonate, where newly formed bone and osteoid matrix next to the particle can be observed. Toluidine blue and acid fuchsin staining; original magnification 100×.

Chemical bonding and structure were described during the 1930s. Philip Russell Wallace was the first who calculated in 1949 the electronic band structure, although the scientists did not pay special attention to this material until 2004. Andre Geim and Konstantin Novoselov, professor and doctoral student at the University of Manchester, respectively, isolated the first samples of graphene from graphite by mechanical exfoliation process [33]. Graphene can be obtained following both the bottom-up and the top-down approaches. Examples of the synthesis from simpler molecular entities (i.e. bottom-up) are chemical vapor deposition [34], anodic bonding [35], growth on silicon carbide [36], molecular beam epitaxy [37], unzipping of carbon nanotubes [38], self-assembly and polymerization of elected surfactants [39], and chemical syntesis [40]. Liquid-phase exfoliation [41] and mechanical exfoliation [33] of graphite are examples of cost-effective top-down strategies for the preparation of graphene.

Graphene, due to its 2D nature, owns unique electronic properties and is the thinnest ever tested material with an extremely high effective surface area ($\sim$2600 m^2/g). Graphene is a carbon allotrope in which each atom is connected with the neighboring carbon through a strong C—C covalent bond and shares with one of the two neighboring carbons one electron to form a π bond. Defect-free samples of graphene have a Young's modulus of 1.0 TPa and a superior intrinsic strength of $\sim$130 GPa. Graphene is one of the strongest materials known with a breaking strength over 100 times greater than a hypothetical steel film of the same thickness. With the functionalization of graphene, there is a reduction of mechanical robustness due to the introduction of defects. Thus, the mechanical properties of functionalized graphene are still much better than those of various traditional materials. Due to the out-of-plane quadratic dispersion of phonon, graphene possesses superior thermal transport properties and the intrinsic thermal conductivity (K) of this material is found to be as high as 5000 W m/K; thus, the graphene is a heat conductor one order better than copper.

Thanks to its 2D structure, each carbon atom can undergo chemical attack from both sides with respect to its plane resulting in the most reactive and lightest among all carbon allotropes and allowing to tune graphene properties at will. The possibility to favor its hydrophilicity (i.e. graphene oxide, GO) or tune its affinity to different materials via functionalization of the high exposed surface is responsible for the potential use of graphene in biological and medical applications. Particularly attractive appears to be the adaptability of graphene to flat or irregular surfaces due to its capacity to bend and its elasticity, and, therefore, its ability to be incorporated into any tissue or on any surface and scaffold (such as chitosan or HA), whether it is solid, in the form of hydrogels, or three dimensional. Moreover, worth noting is its transparency that differentiates graphene from black carbon analogs such as graphite, carbon nanotubes, and amorphous carbon. All these properties as well as the low cost of production process, playback on a large scale, and the availability of graphene makes it a really valuable and an exceptional additive for biomaterials.

1.3.2 Biomedical applications

Several studies have been conducted to study possible applications of graphene in the field of biomedicine. Studies clearly indicate that graphene and its related substrates are excellent nanoplatforms for promoting the adhesion, proliferation, and differentiation of various cells, such as human mesenchymal stem cells [42] and demonstrated that graphene synthesized by chemical vapor deposition was biocompatible with human OBL as well as mesenchymal stem cells, stimulating cell differentiation and growth [43]. Other interesting studies in the same line have been performed by other researchers [44–46], who demonstrated that graphene-based sheets were not harmful for human mesenchymal stem cells and concluded that those sheets accelerated their specific differentiation to bone cells (OBL) hypothesizing a great potential for this material in the field of bone regeneration. Also [47,48], in their studies in mice defects in which four types of scaffolds were used (titanium, titanium with bone morphogenetic protein 2 (BMP-2), titanium with GO, and titanium with BMP-2 and GO), the researchers concluded that Ti covered with GO allows a major amount of BMP-2 dose adhesion and its major liberation without modifying its structure and bioactivity. This enables a better human mesenchymal stem cell differentiation into OBLs and, therefore, a better bone neoformation in comparison with the other three scaffolds (Ti, Ti and GO, and Ti and BMP-2).

The exceptional physical properties of graphene certainly have a huge potential when combined with sophisticated derivatives and composites to provide functional, biologically active surfaces. The capability of biofunctionalization of graphene and its derivative, GO, has brought these nanomaterials under the spotlight and has drawn intense attention for a plethora of applications in biotechnology including bioassays, drug delivery, biosensors, photothermal anticancer therapy, and electrical stimulation of cells [49]. Several researchers have also evaluated the potential of graphene in neural and bone regeneration; however, few studies evaluated the ability of graphene to be biocompatible, osteoinductive, and osteoconductive when added to biomaterials.

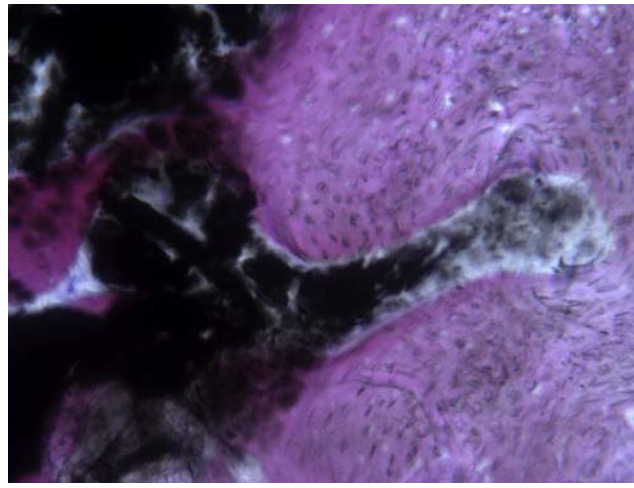

Figure 1.16 Histological image of a sample of PB coated with 50 μg/mL graphene oxide retrieved from sheep. Newly formed bone in contact with remnants of the graphene oxide coating can be observed. Toluidine blue and acid fuchsin staining; original magnification 100×.

Nowadays, the majority of biomaterials able to act as bone substitutes are limited to be osteoconductive and then only in part failed from the point of view of the processes of bone regeneration [50]. Studies report that graphene-HA are promising composites for scaffolds' fabrication in bone tissue engineering due to their ability to support proliferation and differentiation of human fetal osteoblastic (hFOB) cells [51]. Scientists developed a new effective procedure for the production of GO-coated porcine bone (PB) granules and analyzed their in vitro and in vivo potential. The results showed no toxic effects of GO-coated PB samples on primary human gingival fibroblasts and no inflammatory response around the grafted particles when implanted in vivo. Newly formed bone was detected around GO-coated PB particles (Fig. 1.16), although a small loss of GO was observed (Fig. 1.17), suggesting to use less GO concentrated samples.

In order for graphene to be used in biological and medical fields, biocompatibility as well as toxicological and ecological tests has been performed. It has been demonstrated that the in vivo effect of graphene-based materials (GBM) depends on their physical−chemical properties, concentration, time of exposure, and administration route, and also on the characteristics of the animals used. Most studies report no occurrence of adult animal death; however, there are some reports of GBM accumulation and histological findings associated with inflammation, and, more rarely, fibrosis.

In conclusion, before clinical applications, a systematic comparative study, for example, a deep meta-analysis, is highly desired to address the relative safety concerns (subtracting false-negative and false-positive effects) of graphene and derivatives. Although cell viability in vitro is not affected, graphene nanocytoxicity in a clinical setting using humans remains unknown, and further studies are needed to better evaluate the potential applications of this wonderful material.

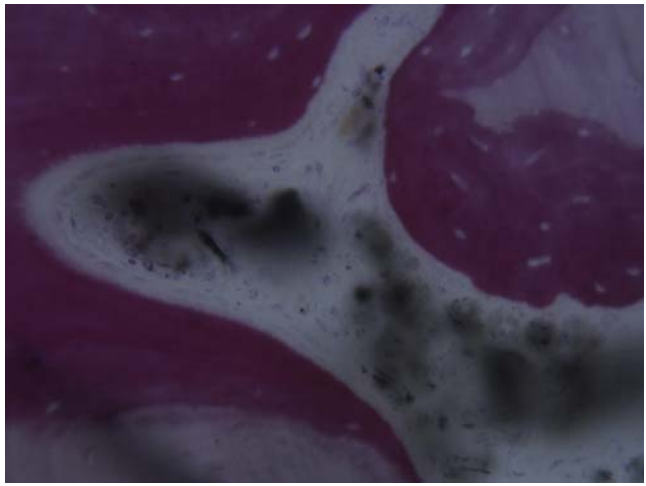

Figure 1.17 Histological image of a sample retrieved from sheep where PB particles coated with 50 μg/mL graphene oxide were used to fill bone defects. It is evident how the coating, although detached from the particles, is biocompatible as osteoblasts are depositing osteoid matrix next to the remnants of the coating. Toluidine blue and acid fuchsin staining; original magnification 200×.

Acknowledgment

This work has been supported by PRIN20102ZLNJ5 financed by Ministry of Education, University and Research (MIUR) Italy.

References

[1] Fujikawa Y, Quinn JM, Sabokbar A, McGee JO, Athanasou NA. The human osteoclast precursor circulates in the monocyte fraction. Endocrinology 1996;137(9):4058−60.
[2] Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 1999;5:623−8.
[3] Villars F, Guillotin B, Amedee T, Dutoya S, Bordenave L, Bareille R, et al. Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication. Am J Physiol Cell Physiol 2002;282:775−85.
[4] Bouletreau PJ, Warren SM, Spector JA, Peled ZM, Gerrets RP, Greenwald JA, et al. Hypoxia and VEGF up-regulate BMP-2 mRNA and protein expression in microvascular endothelial cells: implications for fracture healing. Plast Reconstr Surg 2002;109: 2384−97.
[5] Siddiqui JA, Partridge NC. Physiological bone remodeling: systemic regulation and growth factor involvement. Physiology (Bethesda) 2016;31(3):233−45.
[6] Mikuni-Takagaki Y. Mechanical responses and signal transduction pathways in stretched osteocytes. J Bone Min Metab 1999;17(1):57−60 (review).

[7] Florencio-Silva R, Sasso GR, Sasso-Cerri E, Simões MJ, Cerri PS. Biology of bone tissue: structure, function, and factors that influence bone cells. Biomed Res Int 2015;2015:421746.
[8] Sims NA, Martin TJ. Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. Bonekey Rep 2014;3:481.
[9] Capulli M, Paone R, Rucci N. Osteoblast and osteocyte: games without frontiers. Arch Biochem Biophys 2014;561:3−12.
[10] Williams DF. Consensus and definitions in biomaterials. Adv Biomaterials 1988;8:11−6.
[11] Williams DF. There is no such thing as a biocompatible material. Biomaterials 2014; 35(38):10009−14.
[12] Park JH, Olivares-Navarrete R, Wasilewski CE, Boyan BD, Tannenbaum R, Schwartz Z. Use of polyelectrolyte thin films to modulate osteoblast response to microstructured titanium surfaces. Biomaterials 2012;33(21):5267−77.
[13] Ghaedi M, Mendez JJ, Bove PF, Sivarapatna A, Raredon MS, Niklason LE. Alveolar epithelial differentiation of human induced pluripotent stem cells in a rotating bioreactor. Biomaterials 2014;35(2):699−710.
[14] Eckes B, Zigrino P, Kessler D, Holtkötter O, Shephard P, Mauch C, et al. Fibroblast-matrix interactions in wound healing and fibrosis. Matrix Biol 2000;19(4):325−32 (review).
[15] Costa DO, Prowse PD, Chrones T, Sims SM, Hamilton DW, Rizkalla AS, et al. The differential regulation of osteoblast and osteoclast activity by surface topography of hydroxyapatite coatings. Biomaterials 2013;34(30):7215−26.
[16] Franz S, Rammelt S, Scharnweber D, Simon JC. Immune responses to implants−a review of the implications for the design of immunomodulatory biomaterials. Biomaterials 2011; 32(28):6692−709.
[17] Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008;20(2):86−100.
[18] Schoen FJ. Mechanisms of function and disease of natural and replacement heart valves. Annu Rev Pathol 2012;7:161−83.
[19] Singh N, Manshian B, Jenkins GJ, Griffiths SM, Williams PM, Maffeis TG, et al. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 2009;30(23−24):3891−914.
[20] Soardi CM, Spinato S, Zaffe D, Wang HL. Atrophic maxillary floor augmentation by mineralized human bone allografts in sinuses of different size: an histologic and histomorphometric analysis. Clin Oral Implants Res 2011;22:560−6.
[21] Ewers R. Maxilla sinus grafting with marine algae derived bone forming material: a clinical report of long-term results. Oral Maxillofac Surg 2005;63:1712−23.
[22] Thorwarth M, Wehrhan F, Srour S, Schultze-Mosgau S, Felszeghy E, Bader RD, et al. Evaluation of substitutes for bone: comparison of microradiographic and histological assessments. Br J Oral Maxillofac Surg 2007;45:41−7.
[23] Barone A, Ricci M, Covani U, Nannmark U, Azarmehr I, Calvo-Guirado JL. Maxillary sinus augmentation using prehydrated corticocancellous porcine bone: hystomorphometric evaluation after 6 months. Clin Implant Dent Relat Res 2012;14:373−9.
[24] Calvo-Guirado JL, Ramirez Fernandez MP, Negri B, Delgado Riuz RA, Sanchez De-Val JEM, Gomez-Moreno G. Experimental model of bone response to collagenized xenografts of porcine origin (Osteobiol® MP3): a radiological and histomorphometric study. Clin Implant Dent Relat Res 2013;15:143−51.
[25] Ramirez Fernandez MP, Calvo-Guirado JL, Arcesio-Delgado Ruiz R, Maté Sanchez De-Val JE, Ortega VV, Olmes LM. Bone response to hydroxyapatites with open porosity of animal origin (porcine [OsteoBiol mp3] and bovine [Endobon]): a radiological and histomorphometric study. Clin Oral Implants Res 2011;22:767−73.

[26] Sartori S, Silvestri M, Forni F, Icaro Cornaglia A, Tepei P, Cattaneo V. Ten-year follow-up in a maxillary sinus augmentation using anorganic bovine bone (Bio-Oss). A case report with histomorphometric evaluation. Clin Oral Implants Res 2003;14:369−72.
[27] Polyzois I, Renvert S, Bosshardt DD, Lang NP, Claffey N. Effect of Bio-Oss® on osseointegration of dental implants surrounded by circumferential bone defects of different dimensions: an experimental study in the dog. Clin Oral Implants Res 2007;18:304−10.
[28] Artzi Z, Weinreb M, Carmeli G, Lev-Dor R, Dard M, Nemcovsky CE. Histomorphometric assessment of bone formation in sinus augmentation utilizing a combination of autogenous and hydroxyapatite/biphasic tricalcium phosphate graft materials: at 6 and 9 months in humans. Clin Oral Implants Res 2008;19:686−92.
[29] Covani U, Orlando B, Giacomelli L, Cornelini R, Barone A. Implant survival after sinus elevation with Straumann® BoneCeramic in clinical practice: ad-interim results of a prospective study at a 15-month follow-up. Clin Oral Implants Res 2011;22:481−4.
[30] Giuliani A, Manescu A, Larsson E, Tromba G, Luongo G, Piattelli A, et al. In vivo regenerative properties of coralline-derived (biocoral) scaffold grafts in human maxillary defects: demonstrative and comparative study with Beta-tricalcium phosphate and biphasic calcium phosphate by synchrotron radiation x-ray microtomography. Clin Implant Dent Relat Res 2014;16:736−50.
[31] Wikesjo UM, Lim WH, Razi SS, Sigurdsson TJ, Tatakis DN, Hardwick WR. Periodontal repair in dogs: a bioabsorbable calcium carbonate coral implant enhances space provision for alveolar bone regeneration in conjunction with guided tissue regeneration. J Periodontol 2003;74:957−64.
[32] Demers C, Hamdy CR, Corsi K, Chellat F, Tabriziam M, Yahia L. Natural coral exoskeleton as a bone graft substitute: a review. Biomed Mater Eng 2002;12:15−35.
[33] Geim AK, Novoselov KS. The rise of graphene. Nat Mater 2007;6:183−1291.
[34] Zhang Y, Zhang L, Zhou C. Review of chemical vapor deposition of graphene and related applications. Acc Chem Res 2013;46:2329−39.
[35] Balan A, Kamar R, Boukhicha M, Beyssac O, Bouillard JC, Taverna D, et al. Anodic bonded graphene. J Phys D Appl Phys 2010;43:374013.
[36] Rollings E, Gweon GH, Zhou SY, Mun BS, McChesney JL, Hussain BS, et al. Synthesis and characterization of atomically-thin graphite films on a silicon carbide substrate. J Phys Chem Solids 2006;67:2172−7.
[37] Lippert G, Dabrowski J, Yamamoto Y, Herziger F, Maultzsch J, Baringhaus J, et al. Molecular beam epitaxy of graphene on mica. Phys Status Solidi B 2012;249:2507−10.
[38] Kosynkin DV, Higginbotham AL, Sinitskii A, Lomeda JR, Dimiev A, Price K, et al. Longitudinal unzipping of carbon nanotubes to form graphene nanoribbons. Nature 2009; 458:872−6.
[39] Zhang W, Cui J, Tao C, Wu Y, Li Z, Ma L, et al. A strategy for producing pure single-layer graphene sheets based on a confined self-assembly approach. Angew Chem 2009;48:5864−8.
[40] Yang X, Dou X, Rouhanipour A, Zhi L, Räder HJ, Müller K. Two dimensional graphene nanoribbons. J Am Chem Soc 2008;130:4216−7.
[41] Coleman JN. Liquid exfoliation of defect-free graphene. Acc Chem Res 2013;46:14−22.
[42] Kalbacova M, Broz A, Kong J, Kalbac M. Graphene substrates promote adherence of human osteoblasts and mesenchymal stromal cells. Carbon 2010;48:4323−9.
[43] Talukdar Y, Rashkow JT, Lalwani G, Kanakia S, Sitharaman B. The effects of graphene nanostructures on mesenchymal stem cells. Biomaterials 2014;35:4863−77.
[44] Fan HL, Wang LL, Zhao KK, Li N, Shi Z, Ge Z, et al. Fabrication, mechanical properties, and biocompatibility of graphene-reinforced chitosan composites. Biomacromolecules 2010;11:2345−51.

[45] Nayak TR, Andersen H, Makam VS, Khaw C, Bae S, Xu X, et al. Graphene for controlled and accelerated osteogenic differentiation of human mesenchymal stem cells. ACS Nano 2011;5:4670−8.
[46] Shen H, Zhang L, Liu M, Zhang Z. Biomedical applications of graphene. Theranostics 2012;2:283−94.
[47] La WG, Park S, Yoon HH, Jeong GJ, Lee TJ, Bhang SH, et al. Delivery of a therapeutic protein for bone regeneration from a substrate coated with graphene oxide. Small 2013;9: 4051−60.
[48] La WG, Jin M, Park S, Yoon HH, Jeong GJ, Bhang SH, et al. Delivery of bone morphogenetic protein-2 and substance P using graphene oxide for bone regeneration. Int J Nanomedicine 2014;9(Suppl. 1):107−16.
[49] Chen GY, Pang DW, Hwang SM, Tuan HY, Hu YC. A graphene-based platform for induced pluripotent stem cells culture and differentiation. Biomaterials 2012;33:418−27.
[50] Oyefusi A, Olanipekun O, Neelgund GM, Peterson D, Stone JM, Williams E, et al. Hydroxyapatite grafted carbon nanotubes and graphene nanosheets: promising bone implant materials. Spectrochim Acta A Mol Biomol Spectrosc 2014;132:410−6.
[51] Ettorre V, De Marco P, Zara S, Perrotti V, Scarano A, Di Crescenzo A, et al. In vitro and in vivo characterization of graphene oxide coated porcine bone granules. Carbon 2016; 103:291−8.