Chapter 12
Nanocrystalline Apatite-Based Biomaterials and Stem Cells in Orthopaedics

Feza Korkusuz, Muharrem Timuçin, and Petek Korkusuz

Abstract Nanocrystalline apatite-based biomaterials and stem cells are emerging research fields in orthopaedic surgery and traumatology that have the potential of improving quality of life of the elderly and enhance health-related socio-economic challenges. Nanocrystalline apatite-based biomaterials and especially calcium phosphate nano-biomaterials exploit new physical, chemical and biological properties that have the possibility to increase surface area and improve tissue integration. Stem cells of adult origin decrease inflammation, increase vascularity and are able to replace degenerated tissue cells during the process of regeneration. The bone is the only human tissue that regenerates. Musculoskeletal disorders including osteoporotic fractures and osteoarthritis decrease quality of life in the elderly and cause severe burden on economics. Nanocrystalline calcium phosphate bioceramics have the ability to prevent or treat osteoporotic fractures when combined with stem cells. These biomaterials may also be used for drug delivery purposes to treat bone infections when combined with stem cell as they can assist in treating osteoarthritis. Current research challenges are trying to overcome the toxicity and carcinogenesis with these cells and nanomaterials. Long-term stability of these cells and materials is another challenge for these materials. This chapter deals with nanocrystalline calcium phosphate bioceramics and mesenchymal stem cells.

F. Korkusuz, M.D. (✉)
Department of Sports Medicine, Hacettepe University Faculty of Medicine, Sihhiye, Ankara 06100, Turkey
e-mail: feza.korkusuz@hacettepe.edu.tr

M. Timuçin, Ph.D.
Department of Metallurgical and Materials Engineering, Middle East Technical University, Çankaya, Ankara 06800, Turkey
e-mail: timucin@metu.edu.tr

P. Korkusuz, M.D., Ph.D
Department of Histology and Embryology, Hacettepe University Faculty of Medicine, Sihhiye, Ankara 06100, Turkey
e-mail: petek@hacettepe.edu.tr

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12.1 Introduction

Medical materials also named as "biomaterials" are used alone or in combination with cells and signalling molecules to engineer and regenerate missing or injured tissues and organs [1]. Metals such as stainless steel, cobalt chromium and titanium alloys and various ceramics and polymers were successfully used to replace musculoskeletal tissues for decades. In fact, in early civilisations of Mesopotamia and Egypt, medical materials called "implants" were used to restore missing teeth and defects of bones. Material is the matter from which a thing is or can be made, and a biomaterial is defined as a biological or synthetic substance which can be introduced into body tissue as part of an implanted medical device or used to replace an organ and/or bodily function. Nanomaterial is a material having particles or constituents of nanoscale dimensions or one that is produced by nanotechnology. Bioceramics are non-metallic synthetic grafts used to regenerate and/or replace bones and joints [2–4]. Annually, 3–5 % of the world's population will have some form of medical implants inserted into their body. Fifty percent of these implants are used to replace degenerated joints and restore fractured bones. Although metals are the most commonly used implants, their longevity is limited and they need to be revised due to loosening. Studies to improve metallic implants mechanical and surface properties are advancing their clinical outcome. Particle disease [5] however is a leading cause of total joint revision procedures with no approved drug therapy to prevent or inhibit osteolysis. To prevent this disease, a new quest has emerged to investigate the possibility of using non-metallic implants including ceramics and polymers. Nanocrystalline apatite-based biomaterials were developed for biomedical applications [6–8]. We have produced and characterised various apatite-based biomaterials of micro- and nano-size to be used in bone regeneration. We replaced trace amount of calcium with trace elements in some of our formulations to better stimulate bone formation. This chapter covers our last 20 years of experience on apatite-based bioceramics and their interaction with cells and tissues.

12.2 Production of Nanocrystalline Apatite-Based Biomaterials

In the last 30 years, a class of oxidic inorganic compounds containing calcium and phosphorus in their structure has evolved into a variety of synthetic bone substitutes owing to the fact that the inorganic portion of the natural bone resembles closely the hydrated calcium phosphate compound known as hydroxyapatite. This compound

is commonly represented by the formula $Ca_{10}(HPO_4)_6(OH)_2$ and is referred to as HAp in the literature. Chemically, the bone apatite is distinguished from synthetic HAp by a deficiency of calcium in its constitution and incorporation of a variety of ions such as Na^+, K^+, Mg^{2+}, Zn^{2+}, Sr^{2+}, Si^{4+}, F^- and CO_3^{2-} in trace quantities, i.e. <1 %. These entities play important roles on the behaviour and the performance of the skeletal bone.

The synthetic mineral HAp was found to possess a multitude of useful biological properties which renders it as an indispensible material for replacement and repair of the natural bone tissue in the human body. It has excellent biocompatibility and bioactivity [9]. However, the resorption rate in the body is rather low. A second calcium phosphate compound, known as tricalcium phosphate, designated as TCP, also displayed desirable biological properties with faster resorption. Therefore, for adjustable resorption and remodelling, HAp and TCP can be combined in a biphasic composite with different proportions. The ratio of Ca to P in HAp is 1.67; this is very close to that in the natural bone. The formula for TCP is $3CaO{\cdot}P_2O_5$, hence its Ca to P ratio is lower.

Various techniques have been developed for synthesising calcium phosphate compounds mentioned above. The methods for producing HAp or TCP, and their combinations, results in fine ceramic powders with controlled chemical purity. The powders are then used to manufacture customised grafting materials for specific clinical applications. The grafts may be in the form of powder, granules or bulk ceramics sintered following the compaction of the powder in a suitable die. In bulk form, the sintered ceramic may be manufactured with desired pore architecture so that it mimics the natural bone in the body. Recently, HAp and TCP combined with biocompatible polymers are used for the production of various types of scaffolds. Another potential application is the coating of the surfaces of metallic or ceramic implants with thin layers of HAp or TCP in order to produce a biocompatible interface between the implant and the soft tissue.

The chemical route for obtaining HAp or TCP powder is based on an acid-base reaction which results in the precipitation of a solid precursor from aqueous solution. For this purpose the solution of a calcium salt of known molarity is combined by stirring with a solution containing phosphate ions of known strength in a reactor under controlled temperature and pH conditions [10–13]. By observing correct stoichiometry a solid precursor of the desired calcium phosphate compound is formed. This slurry is aged and then the precursor is separated from the liquor by centrifugal filtration and repeated washings. The cake so obtained is calcined at temperatures above 700 °C in order to arrive at the calcium phosphate powder of interest. The formation of HAp or TCP throughout the chemical process can be described by the following ionic reactions:

$$10Ca^{2+} + 6PO_4{}^{3-} + 2OH^- \rightarrow Ca_{10}(PO_4)_6(OH)_2 \tag{12.1}$$

$$3Ca^{2+} + 2PO_4{}^{3-} \rightarrow 3CaO \cdot P_2O_5 \tag{12.2}$$

A novel approach in chemical synthesis of nanocrystalline HAp powder has been reported [14] in which the application of microwave irradiation during the process resulted in spherulitic HAp particles which exhibited long-term flow ability even after 3 years of storage in non-hermetically sealed containers. Still another novelty was the use of sol-gel technique for the synthesis of ultrafine HAp powders [15–17]. In general, the amorphous precursors of the sol-gel route were found to crystallise to HAp at lower temperatures, but the products suffered from carbonate inclusions.

In a solid-liquid reaction approach [18, 19], the calcium phosphate compound was synthesised by reacting a dilute phosphoric acid solution with an aqueous slurry of $Ca(OH)_2$, again under controlled temperature and pH. The resulting mass is then dried by heating, and then the precursor was calcined in a muffle furnace to obtain the CaP powder. The advantage of this process was that all participating ions were preserved in the solid mass, minimising the compositional variations, but the powder particle size would be coarser.

Although HAp possesses desirable biocompatibility, its medical applications were reported to be limited due to poor mechanical properties and inefficacy in its osseo-behaviour. For example, low tensile strength and low fracture toughness have been the major causes which inhibited the use of HAp ceramics in load bearing applications. One way of enhancing the strength and osseointegration is to use siliconised HAp. Silicon (Si) was incorporated in the HAp structure by partial replacement of the phosphorus as described in the following formula [20, 21]:

$$Ca_{10}(PO_4)_{6-x}(SiO_4)_x(OH)_{2-x} \tag{12.3}$$

In a review on the synthesis of silicon substituted HAp and α-TCP [22], it was reported that silicon tends to inhibit grain growth in CaP materials resulting in finer microstructure, which constitute the basis for improved strength. In addition, it has been found that silicon played a significant role on the bone and the cartilage systems. The increased bioactivity and apposition were attributed to a number of different factors such as transformation of the material surface to a biologically equivalent apatite, increased solubility of the material and a more electronegative surface, all encouraging enhanced biomimetic precipitation on the surface.

12.2.1 Combining with Trace Elements

The calcium phosphate powders and ceramics used in our bone substitution studies included pure HAp, HAp+TCP composites, Si- and/or Sr-doped HAp and HAp+wollastonite composites. As defined recently [23], we used the acid-base reaction technique for manufacturing powders of HAp containing Si and trace elements for biomedical purposes. The HAp powder modified with Si was produced by partial substitution of trace elements for P in the molecular formula of HAp.

The Si addition was made to the $Ca(OH)_2$ slurry in the form of an organometallic solution of Si known as TEOS. The formula of HAp modified by silicon was $Ca_{10}(PO4)_5{\cdot}5(SiO_4)_{0.5}(OH)_{1.5}$. Trace elements were added to the Ca site of the HAp in an amount of 250, 500 and 1,000 ppm. An equivalent amount of Ca was reduced in the $Ca(OH)_2$ suspension. The HAp powder which contained trace elements and Si as co-dopants had 250, 500 and 1,000 ppm of trace element and 0.5 atom of Si in the molecular formula.

Wollastonite is a calcium silicate compound formulated as $CaSiO_3$. Wollastonite has osteoconductive properties by its own [24]. Wollastonite was added to HAp in order to produce HAp-wollastonite composites in this study. Synthetic wollastonite was manufactured by the reaction of an intimate mixture of $CaCO_3$ and SiO_2 powders in accordance with the following reaction:

$$CaCO_3 + SiO_2 \rightarrow CaSiO_3 \tag{12.4}$$

This reaction was carried out at 1,250 °C for a total duration of 72 h. The formation of wollastonite was verified by X-ray diffraction (XRD) analysis.

A frit was introduced into HAp-wollastonite powder mixtures to facilitate sintering of the compacts. Frit had a chemical composition on weight percent basis as 5.5 % Na_2O, 12.1 % CaO, 11.8 % Al_2O_3 and 60.6 % SiO_2.

12.2.2 Characterisation

12.2.2.1 X-Ray Diffraction Analysis (XRD)

X-ray diffraction analysis is used for material characterisation and to determine the crystal structure of nanocrystalline apatite-based biomaterials. Crystallographic descriptions of trace elements incorporated into the HAp composites are carried out according to the Joint Committee on Powder Diffraction and Standards (JCPDS, HA, 09-0432).

12.2.2.2 Porosity Test

Pore formation in ceramics causes attachment and proliferation of osteoblasts. Pore formation in the composites is tested according to the standard test method for water absorption, bulk density, apparent porosity and apparent specific gravity of fired whiteware products (ASTM C 373-88). For obtaining porosity firstly, weight of ceramics (W_{dry}) is measured before placing them into water. After 24 h of immersion in water, weight of suspended samples (W_{susp}) was measured. The composites are placed onto paper towel for wiping out of the water from the surface and saturated weights (W_{sat}) are measured. In the present study, xylene was used as immersion liquid instead of water.

The results of the test are expressed by using the formula given below:

$$d_{\text{bulk}} = W_{\text{dry}} \times 0.861 / \left(W_{\text{sat}} - W_{\text{susp}}\right) \tag{12.5}$$

where d_{bulk} is the bulk density of the ceramic and 0.861 is the density of xylene.

The following formulae were used for obtaining sintered density in terms of percentage of the theoretical density (%TD) and the percentage porosity (%P):

$$d_{\text{bulk}}/d_{\text{th}} \times 100 = \%\text{TD} \tag{12.6}$$

$$100 - \%\text{TD} = \%\text{P} \tag{12.7}$$

The theoretical density of hydroxyapatite was taken as 3.156 g/cm^3.

12.2.2.3 Scanning Electron Microscopy (SEM)

The scanning electron microscope equipped with an X-ray microanalysis system was used for imaging higher magnifications of the material. SEM examinations are performed on broken surfaces after coating materials with a gold layer by the sputter coating equipment.

12.3 Bone Structure and Function

The bone itself is a composite consisting of HAp nanorods embedded into a collagen matrix. It is a protein matrix strengthened basically with calcium and phosphate. Mostly type I collagen and cells form its organic component where minerals establish its inorganic phase. The bone is a metabolically active vascular tissue that has the capability to regenerate.

Bones are categorised as long, short, flat and irregular according to their shapes. Long bones are separated into parts according to their functions. The outer shell that is named as the *cortex* (Fig. 12.1) is thicker and the middle part that contains the bone marrow that is named as the *medulla* (Fig. 12.2) is narrower at the center of the long bone. This is the narrow shaft of bone called the *diaphysis*. The areas next to the diaphysis and towards the endings of the bone are named as *metaphysis*. The cortex becomes narrower and the medulla is wider in the metaphysis. Blood circulation and metabolic activity is higher in the metaphysis related to its structure, whereas the diaphysis is stronger but metabolically less active. The metaphysis ends at the *physis* which is of cartilage named also as the *growth plate* in children and growing adolescent. Longitudinal growth of bones occurs from the physis, whereas transverse growth occurs from the *apophysis*. The physis and apophysis calcify at the end of the growth period. The area from the physis to the end of the bone

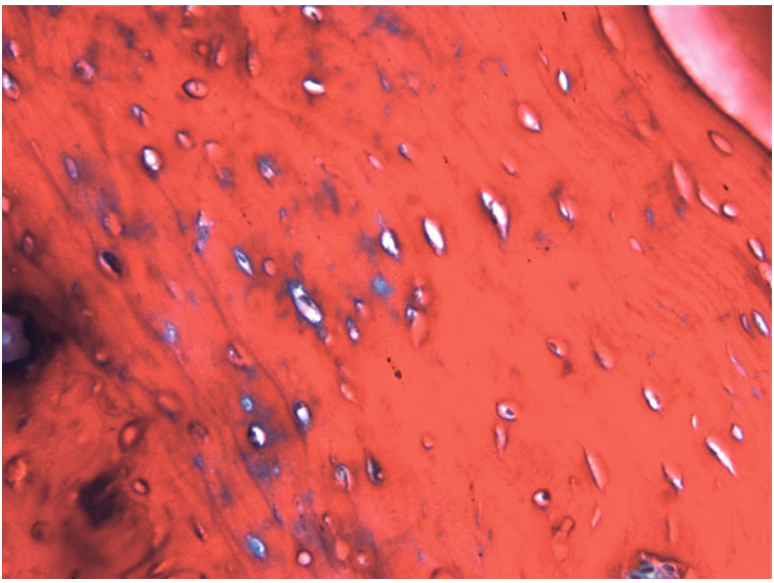

Fig. 12.1 Cortical bone

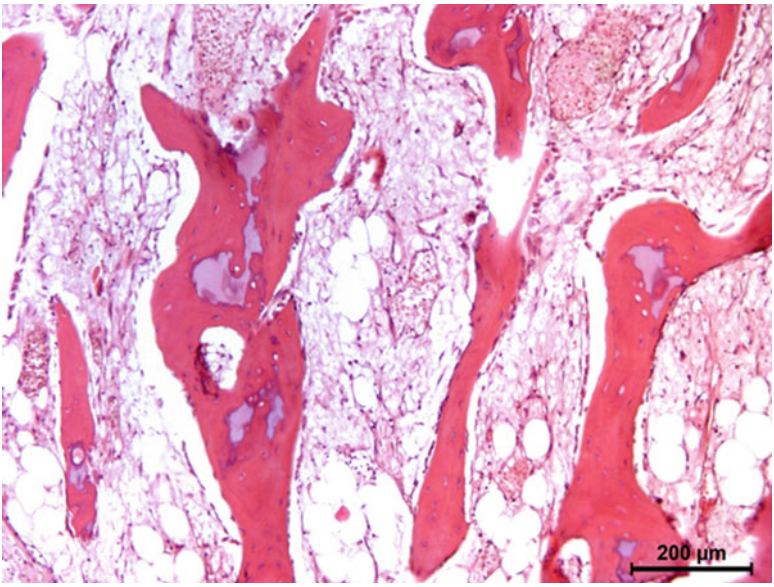

Fig. 12.2 Cancellous bone

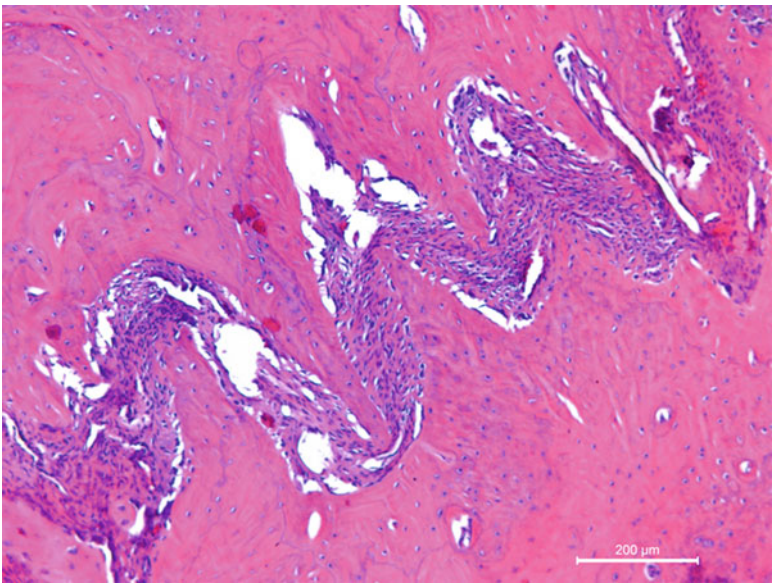

Fig. 12.3 Flat bones of the cranium connect each other with connective tissue

is named as the *epiphysis*. Almost each long bone ends with *articular cartilage* constituting a joint that allows movement. Samples to short bones are the ones at the wrist and ankle joints. These bones are tightly packed with ligaments allowing essential but limited motion. Bones of the cranium (Fig. 12.3) are typical flat bones and bones of the spine are examples of irregular bones.

Bones protect internal organs from outer powers. They allow movement as all muscles attach to them. Bones produce new blood cells in their bone marrow and the bone marrow is one of the most well-known sources of mesenchymal stem cells. The bone also serves as the depot of minerals and trace elements. For example. calcium is stored in bones and when needed it is released from there by certain hormones.

Mechanical properties of bone are important for protection and movement functions. The minerals that constitute the inorganic part of the bone attain strength that can indirectly be measured by dual energy X-ray absorptiometry (DXA) [25]. An alternative technology named *vibration analysis* is assessed to quantify bone strength recently [26]. The organic part of the bone is mainly of collagen type 1. This component gives elasticity to the bone. Due to its unique composition of organic and inorganic components, the bone is as strong as steel but lighter. As the bone is a living tissue, cells regularly exchange its components. This turnover of organic and inorganic parts of the bone is characterised as homeostasis. As one part of the bone is broken down (catabolised), it is regenerated (anabolised) immediately in children and adults. With ageing and due to the genetic code of individuals, the breaking down of the bone by *osteoclasts* turns to be faster than new bone production by *osteoblasts*, leading to a condition of brittle bone.

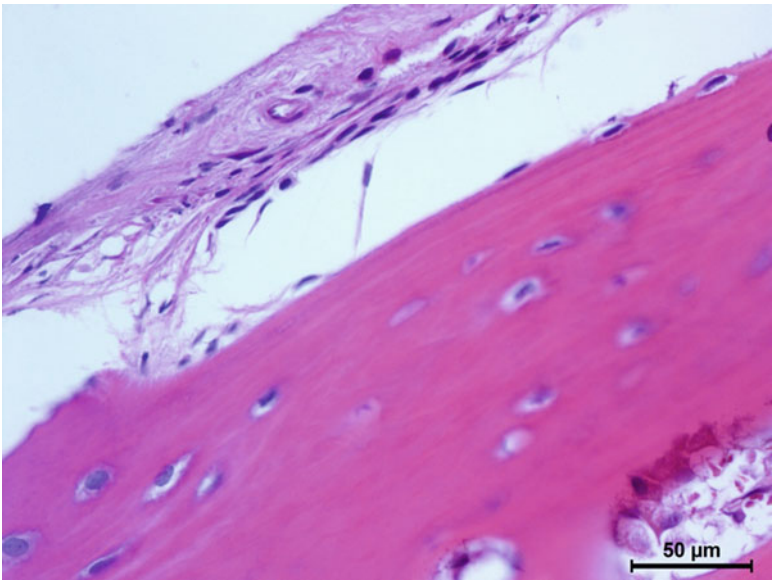

Fig. 12.4 The periosteum

Osteoporosis is defined as loss of bone mineral density (BMD) and deterioration of bone microarchitecture leading to low quality of the bone that may end up with fractures [27]. From the mechanical point of view, whenever the organic component is removed the bone becomes brittle. When the inorganic component is removed the bone becomes highly elastic but it cannot bear weight. The balance of the bone is maintained by its cells through various internal and external stimulators. Bone morphogenic proteins (BMP) which are mostly members of the transforming growth factor (TGF) beta family are the most important signalling molecules that act on bone cell proliferation and differentiation. BMPs are also important in bone growth and regeneration. Systemic growth factors, neural mediators [28] and physical, electrical and magnetic stimulators are proposed to mediate bone formation and regeneration through BMPs [29].

When the unity of the bone is interrupted by a fracture, the initial stage of response will be inflammation. The skin of the bone that is called the *periosteum* (Fig. 12.4) prevents the leakage of blood into other tissues. The so-called haematoma entrapped in the periosteum will short after play a major effect in regeneration. The necrotic tissue at the fracture ends is resorbed by osteoclasts when internal or external fixation is attained. This is followed by the repair of blood cells and appearance of osteoblast. Osteoblasts repair the injured bone, whereas the haematoma is calcified by these cells. After initial healing is achieved, osteoblasts will reshape the bone working together with osteoclasts. This final stage of repair is named as regeneration as the original tissue is reconstructed. Osteocytes are the cells of bone tissue that are responsible of communication. In case of injury, these cells

invite the osteoclasts and osteoblasts to initiate repair. Cortical bone, cancellous bone and the periosteum have cells and mediators that can initiate and continue with the process of regeneration. Still the most important factors that take role in bone regeneration are adequate and appropriate blood circulation and attainment of stability.

12.4 Stem Cells and Cell Response to Nanocrystalline Apatite-Based Biomaterials

Mesenchymal stem cells (MSCs) have the potential to convert to osteoblasts when essential [30, 31]. These cells are frequently used together with natural or synthetic matrices to regenerate the bone [31]. Apatite-based biomaterials are currently used in non-weight-bearing bone sites as osteoconductive materials. In a recent study, we were able to produce 300 and 500 nm nano-HAp powder clusters (Figs. 12.5 and 12.6) by heating them up to 300 °C and 1,000 °C, respectively. When these nano-HAp powder clusters were combined with MSCs in culture, they attached (Fig. 12.7) and proliferated well (Fig. 12.8) on the surface of the clusters.

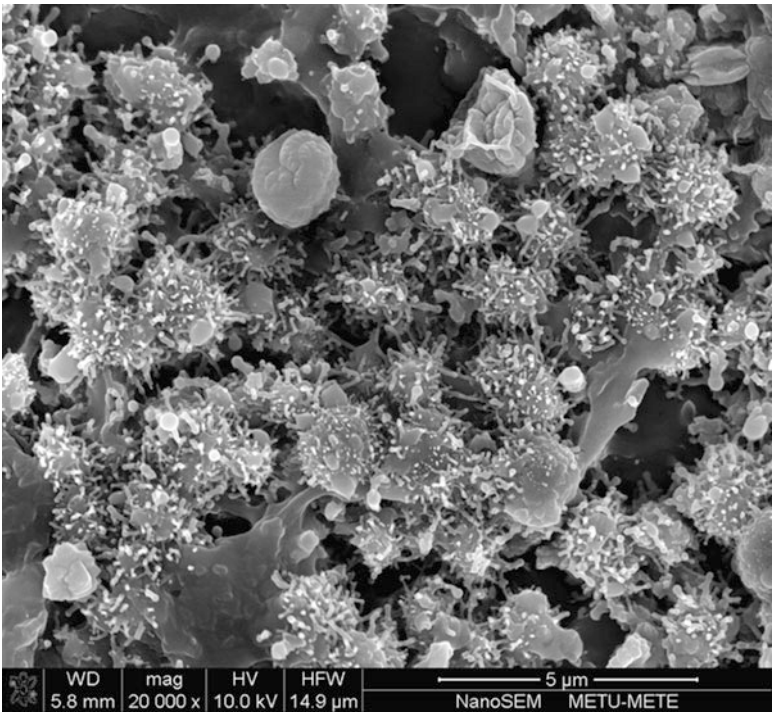

Fig. 12.5 Scanning electron micrographs on 300 and 500 nm nano-HAp powder clusters

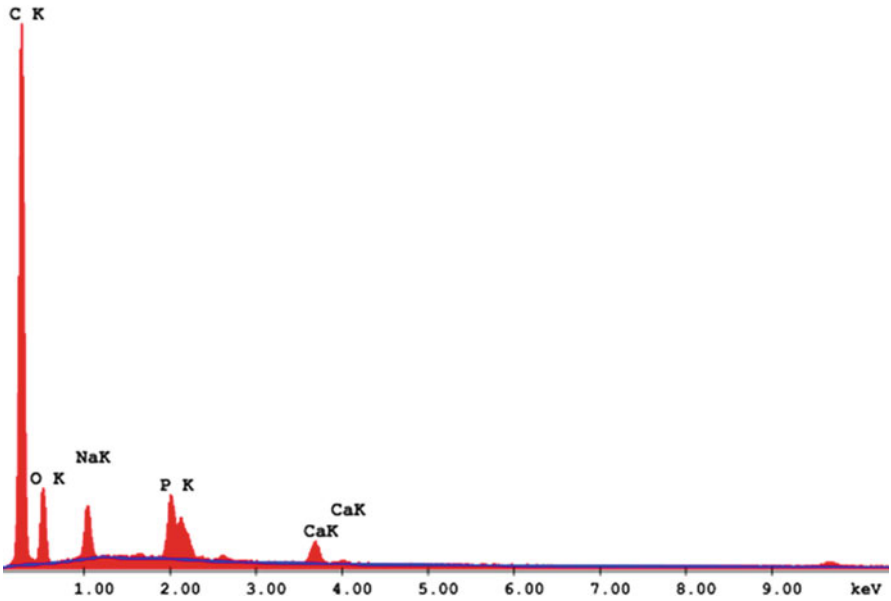

Fig. 12.6 XRD of nano-HAp powder clusters

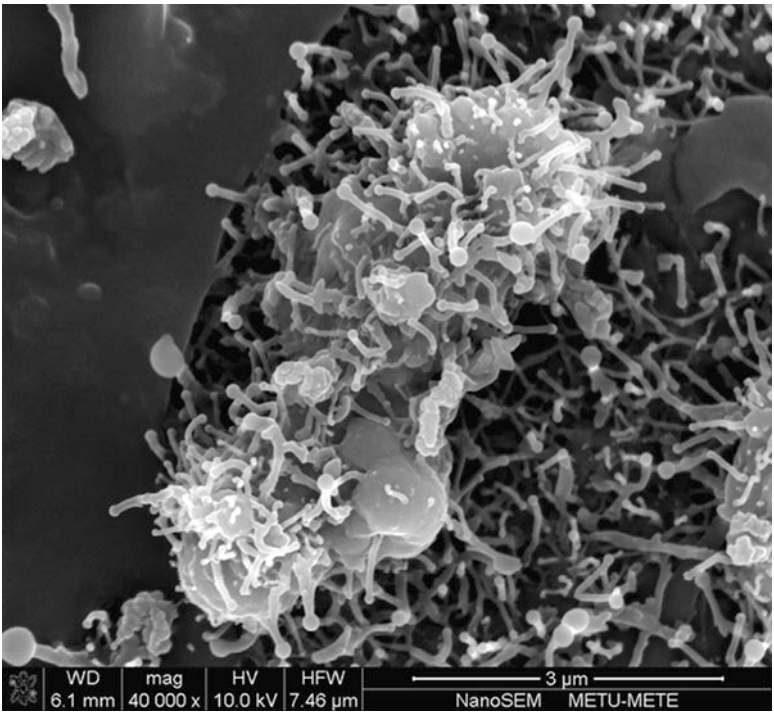

Fig. 12.7 MCSs attached on nano-HAp powder clusters

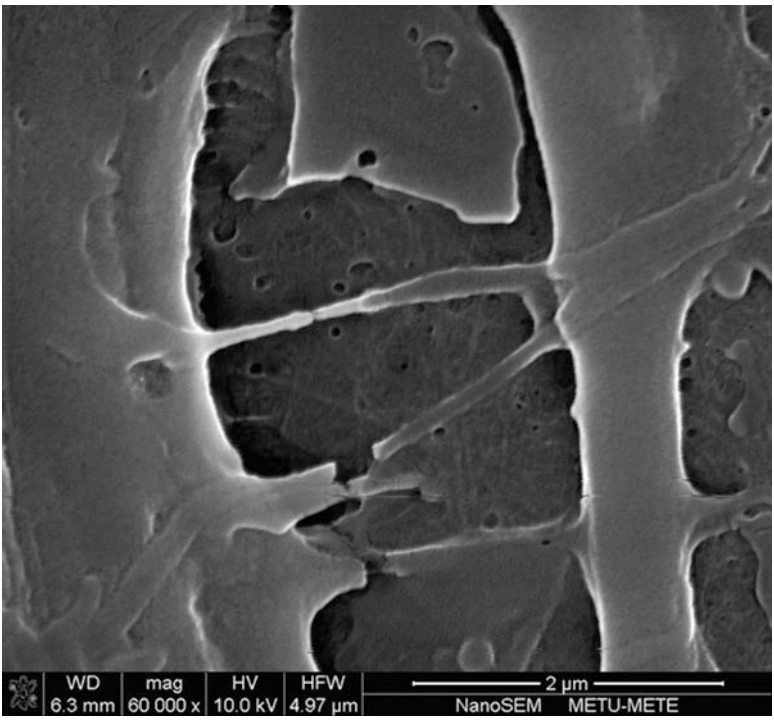

Fig. 12.8 Proliferation and extracellular matrix formation of MSCs on nano-HAp powder clusters

Attachment and proliferation of MSCs on nano-HAp was assessed using a real-time cell electronic system (Fig. 12.9).

Transmission electron microscopy (Fig. 12.10) and scanning electron microscopy (Fig. 12.11) revealed that the nano-HAp was biocompatible. Cells grew and exhibited their extracellular matrix on the nano-HAp powder clusters (Fig. 12.12). Over a period of time, all the nano-HAp powder clusters were covered with the extracellular matrix of MSCs (Fig. 12.13).

12.5 Tissue Response to Nanocrystalline Apatite-Based Biomaterials

Synthesis of nanocrystalline apatite-based biomaterials is recently well studied [32–34]. As nano-HAp is a relatively new material, few in vivo studies were carried out [35]. Our recent clinical experience with trace element-containing HAp [23] revealed that the basic material that we used to produce our nano-HAp powders was Acceptable.

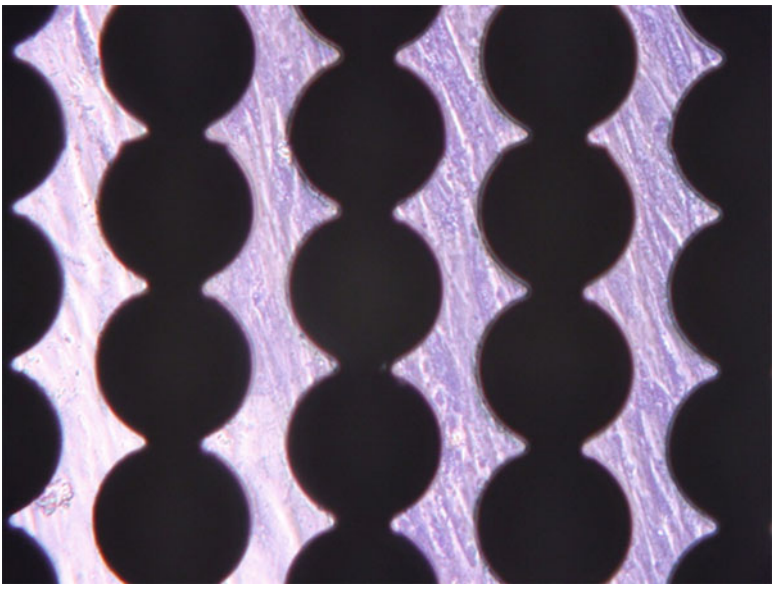

Fig. 12.9 MSCs growing on nano-HAP ceramic powder clusters in a real-time cell electronic system

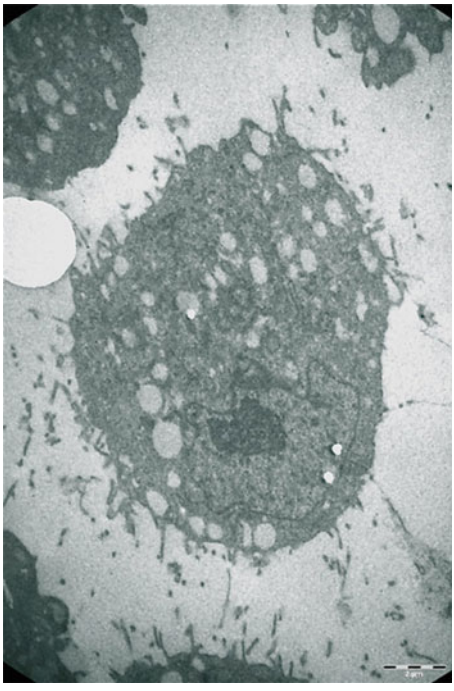

Fig. 12.10 Transmission electron microscopy of MSCs implanted on nano-HAp powder clusters revealed their biocompatibility

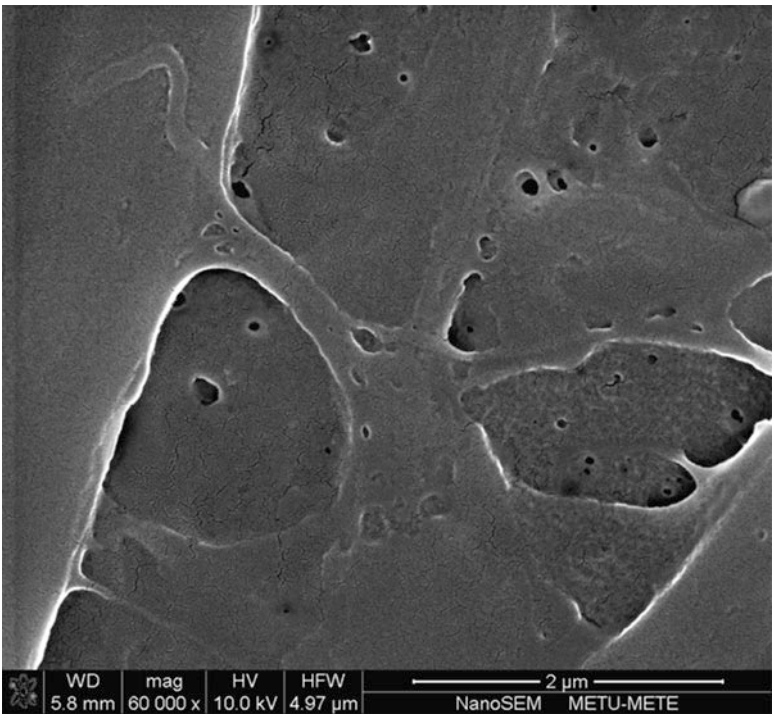

Fig. 12.11 Scanning electron microscopy of MSCs implanted on nano-HAp powder clusters revealed that they exhibited growth and extracellular matrix production

12.6 Growth Factor and Drug Delivery

Growth factors, cells and scaffolds are essential components of regenerative medicine. As stated previously, BMPs are used to regenerate the bone alone or in combination with cells and scaffolds [36]. BMP-2-loaded nanoparticles combined with fibrin presented potent effects of MSCs during bone regeneration [36]. Such composites were also efficient in regenerating mandibular defects [37]. Nano-HAp combined with poly(lactide-co-glycolide) promoted MSC adhesion and osteogenic differentiation recently [38]. What we discovered from our studies were in line with these recent findings. Nano-HAp can be combined with polymers to slow down the release of growth factors and antibiotics. Nano-HAp can also be used to attach MSC that will be delivered to the bone.

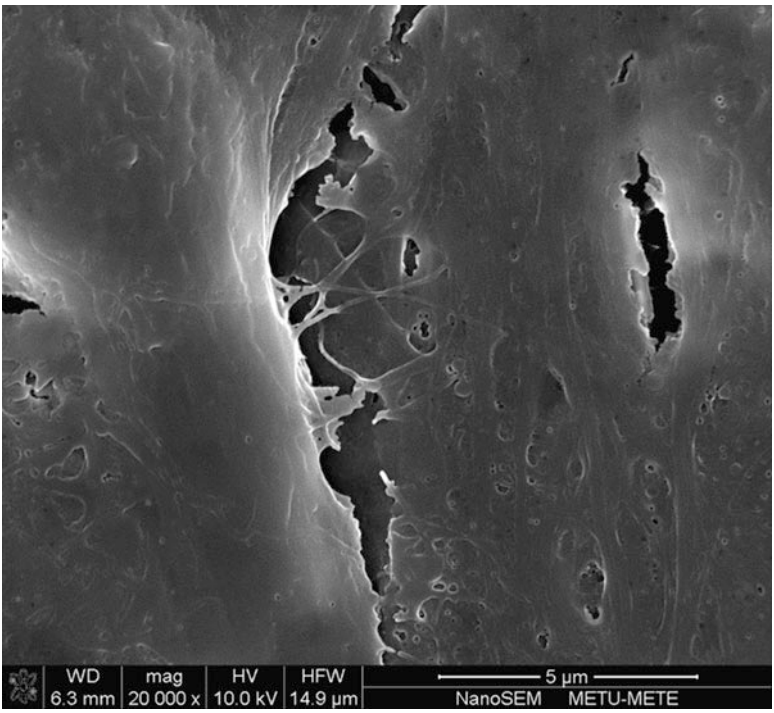

Fig. 12.12 MSCs immediately covered the nano-HAp ceramics

12.7 Implant Surface Coatings

Integration of implants into the bone largely depends on surface properties. Nano-HAp coating had potential benefits to enhance implant osseointegration in rats [39]. A recent review assessed the effectiveness of coating orthopaedic implants with nano-HAp [40–42]. Trace elements such as Mg and Sr can be dropped on to the nano-HAp coating of implants [39]. We concur with the idea that expanding the surface area of the bone-implant interface will aid better osseointegration.

In conclusion, our findings with nano-HAp powders revealed that this biomaterial is biocompatible and allows MSC attachment and proliferation. Attached cells produce the extracellular matrix, and they may aid implant integration and/or release of BMP or antibiotics.

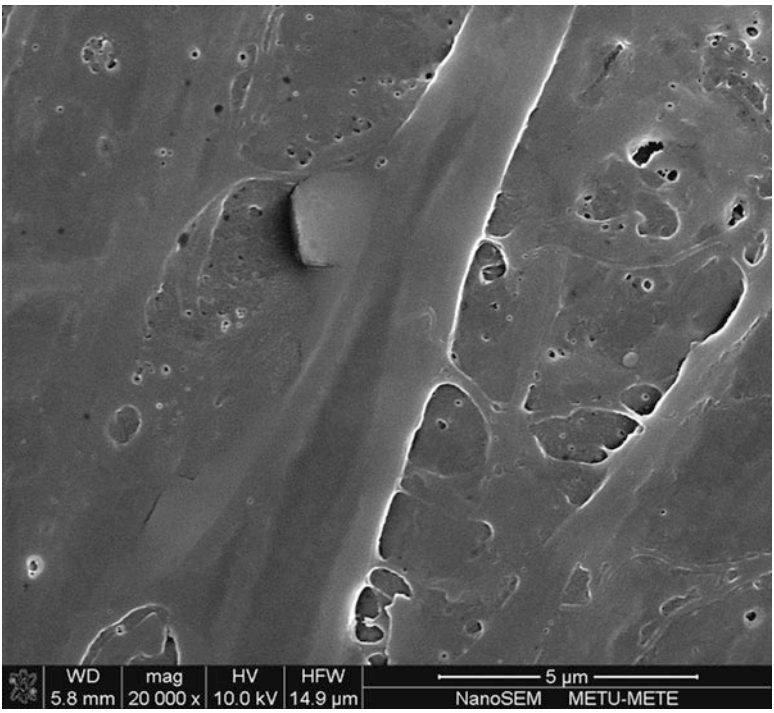

Fig. 12.13 In about 1–2 weeks, all nano-HAp surfaces were covered with the extracellular matrix of the MSCs

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