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Bioinspired and Biomimetic Functional Hybrids as Tools for Regeneration of Orthopedic Interfaces

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1. Introduction

Multidisciplinary approaches in tissue engineering and nanotechnology have resulted in the availability of new materials whose design and fabrication is inspired by the natural constituents of living organisms. This "bioinspired" approach of designing and manufacturing materials is referred to as biomimetism because materials made by this method exhibit the same structural and functional properties that are seen in naturally occurring biological materials. Thus in general terms, biomimetism or biomimetics as it is also called [Vincent 2003], can be defined as a two way path leading from biology to engineering and back, but in more scientific terms, biomimetic production of materials follows the principles and/or processes of biology to obtain end products that are useful for biological and non biological applications. Some examples of such successfully developed products are: (1) commercially important organic compounds that are made by *in vitro* replication of a specific *in vivo* metabolic pathway e.g. coloring agents - lycopene, β-carotene, and astaxanthin [Dixon 2005, Chemler & Koffas 2008] and anti-cancer drugs - paclitaxel, shikonin, geraniol [Chemler & Koffas 2008; Kolewe et al., 2008] (2) super-hydrophobic and superadhesive glues that are manufactured by imitating processes similar to those seen in the lotus leaf, bee hive, gecko foot and rose petals [Feng & Jiang, 2006; Feng et al., 2003; Nystrom et al., 2006, Nystrom et al., 2010, Lee et al., 2007; Kamino, 2008, Liu et al., 2010] (3) design of anti-fogging and antireflection coatings that are inspired by materials seen in mosquito eyes [Liu et al., 2010]. Along with these successful attempts there are also examples, where the application of biomimetic designing has so far not yielded the desired product. Engineering of Type I collagen is one such case where the natural target molecule exhibits divergent properties in different tissues ranging from the high Young's modulus bearing substance in the matrix of the bone, to being the highly deformable elastomer in tendons, and exhibiting ideal optical properties in the multi-layered corneal tissue but it has not been possible to incorporate all these properties into a biomimetically produced collagen molecule [Weiner & Wagner, 1998, Meek & Fullwood, 2001].

Most strategies in biomimetic material design and manufacturing involve the generation of hierarchical assemblies of multiple components; therefore the bioactive and self healing products that are thus obtained get referred to as "functional hybrids". Although these

products are useful in diverse fields, such as the auto industry, computer logics and the wine industry, their main application is seen in the biomedical field – in particular manufacturing of biomedical devices for orthopedic applications. The design and manufacturing of orthopedic bimimetic implants is meant to replace parts of the hard and weight-bearing bones [Rigo et al., 2004; Chakraborty et al., 2009; Kapoor et al., 2010; Nair et al., 2009; Geary et al., 2008] and replacing soft orthopedic tissue [Balasundaram & Webster, 2007]. Further improvements in the implant's properties, which could include the incorporation of bioactive molecules such as long chain proteins or short peptide sequences [Chakraborty et al., 2009; Kapoor et al., 2010; Balasundaram & Webster, 2007; Keselowsky et al., 2005; Le et al., 2006; Stevens et al., 2008; Sato &Webster, 2004], are necessary to make them more acceptable by the cells at the site and thus ensure their long term success. This is an ongoing process and several new developments have taken in this field in recent years.
In this chapter we have focused on the anatomical organization of the orthopedic interfaces which are mimicked for tissue engineering applications and we have addressed some challenges that need to be met in the design and manufacturing of materials that can be used more effectively to replace damaged, malformed or diseased orthopedic tissue.

2.0 Normal bone anatomy and functions:

In order to efficiently biomimic the structure and function of bone components it is necessary to know the chemical biology and structural organization of the bone and its parts. Since the bone tissue contains uniquely large amounts of inorganic constituents along with the organic material, hence the details of how these two parts are biologically synthesized and combined are very important to be understood before efficient substitutes for them can be designed and manufactured.

2.1 Gross anatomy of the human bones

The simplest classification of the human bone subtypes is shown in Figure 1. It is interesting to note that a significant amount of tissue engineering work on making orthopedic devices has concentrated on the components of the long bones i.e. the 4 limbs of the appendicular skeleton. The internal organization and structure of the axial bones is much more complex than the tubular and cuboidal bones of the appendicular skeleton although the basic constituents of both types of bones are similar. Perhaps it is for this reason that the availability of biomimetically produced tissue engineered orthopedic products is significantly more for the appendicular bone parts than for the axial bone components with the possible exception of products related to teeth and intervertebral discs of the spine.
Each long bone can be divided into three regions, namely the epiphysis, the metaphysis and the diaphysis (Figure 2). The epiphyses make the two rounded ends of the long bone which is supported underneath by the metaphysic layer. In adult bones the two layers encapsulate the growth plate or physis itself which is the main generator of osteogenic cells during the developmental phase. Both the metaphysis and epiphysis are composed of a soft trabecular (spongy) bone that is surrounded by a relatively thin shell of hard cortical bone. The diaphysis which is the hollow cylindrical shaft of the bone is made up of the dense cortical hard bone that surrounds the softer bone marrow area. The main length of the diaphysial hard bone area is lined by two thin connective tissue layers - the periosteum on the outside and endosteum on the inside. Most of the hard region of the bone which makes up the bone

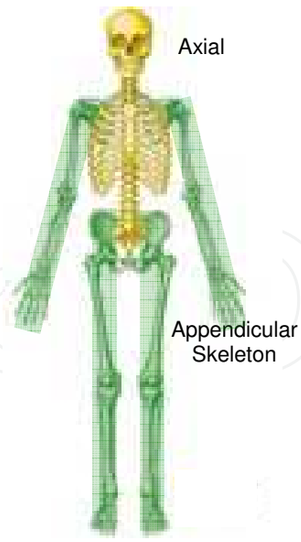


Fig. 1. Classification of bones in the human skeletal system into axial skeleton (yellow color) and appendicular skeleton (green shaded areas). All the long (tubular) bones. e.g. femur, humerus, and short (cuboidal) bones e.g. carpals, tarsals etc. are in the appendicular skeleton whereas the flat and irregular bones e.g. the vertebrae, the maxillofacial bones, bones of the skull etc. constitute the axial skeleton.

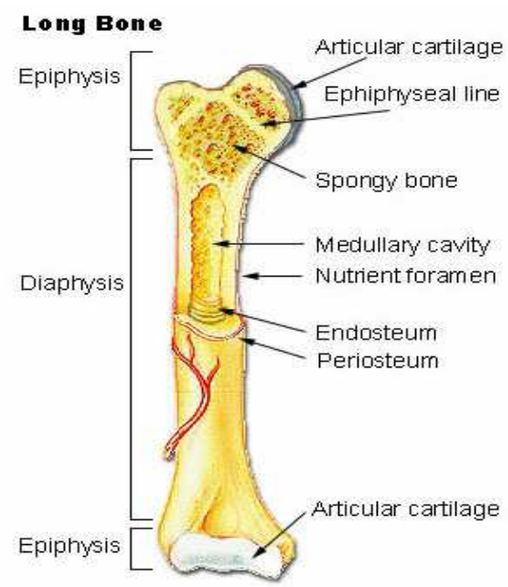


Fig. 2. The gross structure of a long appendicular bone. The epi-physeal regions at the two ends of the bone comprise the softer part of the bone whereas diaphysis constitutes the hard part. The hard of the bone lies in the cortical region and it surrounds a softer part in the medullary region. The medullary region is lined by a membrane supported endosteal layer which acts like a stem cell generating niche. It also acts as the site to produce osteoclast cells which are main cells that take part in bone resorbtion. The periosteum lies as the outer covering the entire bone and is the site of osteoblasts and osteocytes to the cortical bone mass.

mass and is responsible for its rigidity and load bearing capacity lies between these layers; it undergoes the maximum mineralization and therefore it contains the maximum inorganic content in the entire skeletal system. Due to their proximity with the bonemass the periosteum and the endosteum make up the two distinct orthopedic interfaces in long bones. They respectively play key roles in the formation and degeneration of the bone tissue. The cellular and biochemical organization of these two orthopedic interfaces along with the large mass of mineralised bone tissue that lies between them are the main targets of biomimetic designing and manufacturing products for the human skeletal system.

Structurally the periosteum is a vascularised membranous layer that covers the entire outer surface of all bones and functionally it acts as the regenerative orthopedic interface for the entire diaphysial region of the bone,. Externally it combines with the fibers and ligaments of the skeletal muscles and internally it provides attachment to the flattened osteoprogenitor cells which divide by mitosis and differentiate into osteoblasts and then osteocytes. The existence of the periosteum is essential for the regeneration of the bone after trauma injury. The endosteum, which makes the degenerative interface of the bonemass, lines the inner side of the mineralized cortical bone and has two surfaces - one which faces the outer mineralized side of the bone mass and another which faces the inner non mineralized sinusoidal bone marrow. The inner surface of endosteum makes several endosteal niches which harbor multipotent stem cells that generate hematopoietic, muscular, adipose and mesenchymal cell precursors in the marrow region. The outer surface of endosteum acts as the site for producing differentiated osteoclast cells that migrate into the mineralized bone matrix, between the periosteum and endosteum, and participate in its breakdown. Osteoclasts also remove the dead osteocytes that lie embedded in the matrix. The endosteum thus plays a key role in the bone remodeling by actively assisting the bone resorption process through osteoclasts.

2.2 Histological and biochemical organization

In general the bone tissue exhibits a unique histological organization, it exhibits the general properties of vertebrate connective tissues, but its matrix is uniquely dense, semi-rigid, porous and highly calcified because it is made up of an organic matrix and an inorganic mineral component. In a typical appendicular bone the matrix is composed of approximately 30-35% organic and 65-70% inorganic components. The organic component is called the osteoid which is composed of type I collagen and ground substances like glycoproteins, proteoglycans, peptides, carbohydrates and lipids. Mineralization of the osteoid, which can occur by several methods (see Section 3) constitutes the inorganic components of the bone and these constituents include calcium phosphate- hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and calcium carbonate along with similar salts of magnesium, fluoride and sodium in lesser quantity [Clarke 2008; Kalfas 2001].

The cellular component of bone tissue comprises three main cell types: osteoblasts, osteocytes and the osteoclasts. As mentioned above osteoblasts line the periosteal layer and they are cuboidal to flat in shape. They secrete the unmineralized organic matrix which later mineralizes and leads to increase in organic component of bone matrix. Osteoblasts, as they migrate into the matrix or line the canaliculi the thin cylindrical spaces or canals seen in the bone mass, differentiate into osteocytes, which possess long thin cytoplasmic processes called the filopodia. The osteocyte lined canaliculi help in the passage of nutrients and oxygen between the blood vessels and matrix localized osteocytes. Osteocytes also break down the bone matrix by osteocytic osteolysis to release calcium for calcium homeostasis.

They also maintain extracellular phosphorus concentration. The third main category of cells in the bone mass are the osteoclasts. These are bone resorbing cells which are multinucleated and carry out the process of bone resorption. They are generated from the shallow depressions on the inner side of the endosteum called howship lacunae. A schematic representation of the cellular and inorganic organization of the bone mass is seen in Figure 3 below.

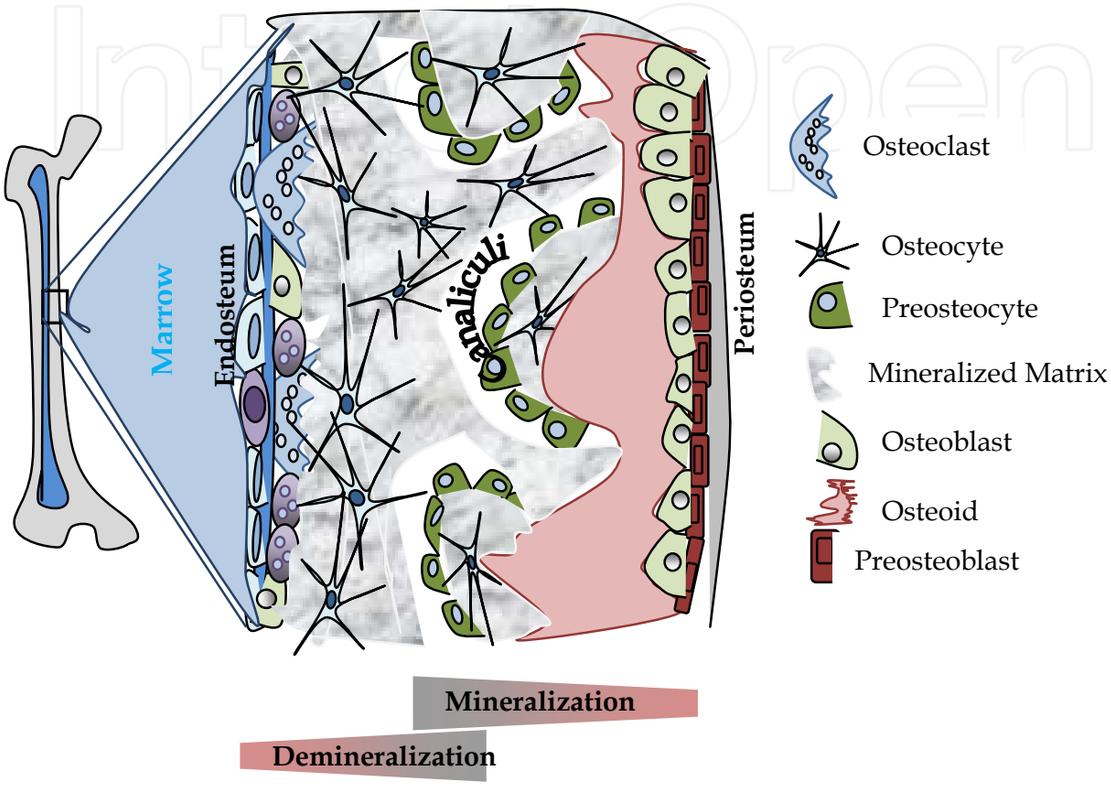


Fig. 3. A figurative description of the cellular organization in two orthopedic interfaces the periosteum and the endosteum that surround the bone matrix in the hard cortical bone.

3. Biomimicry of bone components

The capacity of bone tissue components, both cellular and inorganic, to self-regenerate, particularly after trauma related injuries, has attracted the interest of many scientists [Alves et al., 2010]. During this regeneration process, we observe the recreation of mineral rich tissues of different constitutions and hence this process is also referred to as biomineralization [Palmer, 2008]. Studying the process of biomineralization helps us in understanding the mechanisms by which living organisms deposit mineralized crystals within matrix [Sarikaya, 1999]. Among the approximately 40 different constituents found in the naturally formed biominerals, carbonates, phosphates and silicates of calcium are the most common [Stephen, 1988]. These salts have a significant role to play in determining the physiochemical properties and thermal stability in hard bone tissue [Sarikaya, 1999; Cai & Tang, 2009].

In general terms, biomineralization process can be either biologically induced or biologically controlled. In biologically induced mineralization (BIM) the shape and organization of the

crystals is not directly under cellular control and it is determined entirely by inorganic processes. As a result of this the shape and organization of the inorganic compounds made by BIM is of a low order. In contrast to this biologically controlled mineralization (BCM) is cell dependent and it shows a well balanced organization of the mineralizing salts with the organic molecules resulting in well defined crystals of uniform shape, size and orientation [Khaner, 2007; Weiner & Addadi, 1997]. During post trauma osteo-regeneration both types of biomineralization processes are observed however the involvement of BCM is more dominant. Features common to bone mineralization are also seen in the biomineralization of many non skeletal tissues and cells and an examination of those properties helps in understanding the mechanism behind skeletal tissue mineralization.

3.1 Non-skeletal biomineralization

The biomineralization process in non skeletal cells and tissues generates very complex, diverse and interesting mineral forms and this process can be observed in almost in all organisms [Ozawa & Hoshki 2008; Veiss, 2005]. An evolutionary break through about this process was achieved in a report on the formation of magnetites in magnetotactic bacteria which indicated the commonality of biomineralization mechanisms in different biological forms and it also highlighted that this process is regulated by highly complex control systems that are operational even in simple organisms. Several examples of non skeletal biomineralization in multicellular organisms are observed in nature along with the more common unicellular mineral producers. Some of these include silica spicule producing sponges, diatoms and actinopoda; synthesis of amorphous calcium carbonate in ascidians and formation of layered aragonite platelets in the nacreous layer of mollusk shells,few of such examples has been shown in Figure 4 below. [Sarikaya, 1999].

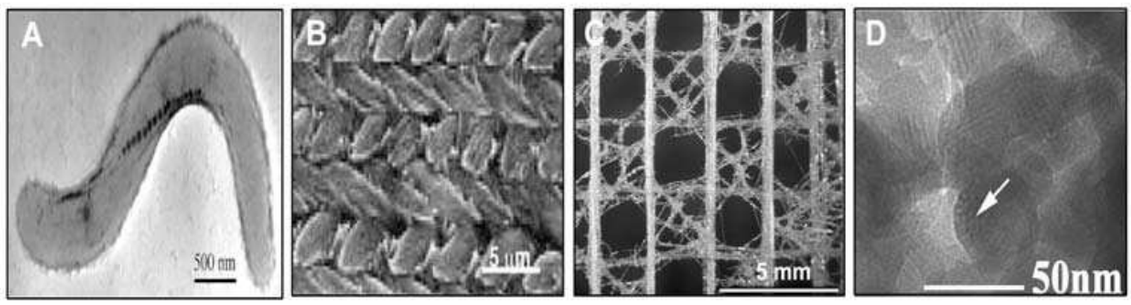


Fig. 4. Biologically controlled mineralization of hierarchical structures observed in A) magnetospirullum magnetium bacteria B) TEM of organic lattice of nacreous shell found in atrina C) finely organized enamel rod structures of mouse tooth D) ordered structures in siliceous skeleton lattice.[Atsushi et al., 2008; Yael et al.,2001;Sarikaya, 1999; James et al., 2007]

3.2 Biomineralization in skeletal tissue

As indicated above, the biomineralization process in the bone tissue is different from what is exhibited by nonskeletal cells and tissues, because in skeletal cells it is primarily cell dependent i.e. it is controlled by BCM mechanisms. At the sub-cellular level biomineralization in bones is mediated by the formation of matrix vesicles (MV) which are membrane encased vesicles of size 20-200nm that are formed by a special exocytic membrane

budding process in polarized and differentiatiating osteoblasts/osteocytes of the long bones and also in the hypertrophic chondrocytes of the cartilage and odontoblasts of the growing teeth [Anderson, 2003]. After being secreted out of the cell, the MVs begin to deposit calcium phosphate/apatite crystals within the lumen of the vesicle itself or are specifically transported through the vesicular membrane into the matrix and they mineralize in conjunction with matrix collagen [Ciancaglini, 2006]. This process can thus be divided into 2 phases - in phase I intra-luminal deposition of amorphous calcium phosphate, octa-calcium phosphates and HAp crystals is seen and in phase II seepage of HAp crystals occurs through the MV membrane into extracellular fluid resulting in nucleation of the crystals within collagen fibrils as calcified nodules [Guido & Isabelle, 2004; Kazuhiko et al, 2009]. Type-1 collagen acts as a template for initiating the crystallization of secreted calcium hydroxyapatite crystals **[**Vincet, 2008**]** which subsequently gets associated with other ECM components such as proteins, polysaccharides, proteolipids and proteoglycans to support activities such as cell adhesion, transport of ionic molecules, cell signaling etc. Understanding the steps of matrix biomineralization and its degeneration is therefore necessary in order to develop synthetic analogs that would mimic the matrix components that aid in the regeneration of new tissue [Joshua et al., 2009; Alves et al., 2010**;** Veiss, 2005].

3.3 Steps in bone modeling and remodeling

As mentioned earlier and shown in Figure 3 the process of bone modeling and remodeling is a homeostatic process where the bone formation and resorption processes are observed simultaneously. The two processes are regulated by independent but related controls but since basic steps are very different from one another they need to be understood sperately in order to design materials to replace this integral component of the bone tissue.

3.3.1 Bone modeling

As mentioned above the bone modeling process in long bones is dependent mainly upon the calcification of the collagenous matrix of the bone mass. This process of physiological mineralization of collagen is controlled by the balance of enzymes, such as metalloproteinases, transporters, such as type III Na/Pi co-transporter, and channels, such as the annexin channels, which together aid to efficiently export the mineralizing molecules from the MVs into the matrix. In a recent study, using proteo-liposomal vesicles, it has been shown how to reconstruct a model that would mimic the MV microenvironment and would help us in better understanding the MV microenvironment [Simao et al., 2010]. In addition to the MV associated enzymes, transporters and channels some other molecules in the matrix such as tissue nonspecific alkaline phosphatase (TNAP), the group of docking proteins ankyrins and nucleotide associated inorganic phosphate, that influence the transport of MV pyrophosphate into the matrix and thereby regulate its calcification [Ellis, 2009, Robert, 2001]. These matrix associated molecules exert their effects by directly controlling the amount of free inorganic phosphate in the ECM which in turns determines the transport PPi from the MVs [Ellis, 2009]. The effective role of matrix associated TNAP in controlling vesicle mineralization is highlighted in a disease named hypophosphatasia where TNAP activity is decreased because of a mutation in this gene the mobility of PPi from MVs to the matrix is very high [Robert, 2001]. Mineralization initiation in matrix vesicles is a function of several inhibitors, promoters that needs a proper balance between the elements that maintain them.

In addition to Type I collagen there are some other proteins in the matrix that also associate with the mineralized collagen and then further enhance or inhibit the mineralization process. Some of these proteins observed in bones and teeth are shown in Table 1. Osteopontin[OPN] and Bone Sialoprotein[BSP] are acidic proteins with high affinity for Ca^{2+} ions are localized within the collageneous matrix found adjacent to mineralization front that are involved in determining calcification. BSP are found to be initiator of mineralization whereas OPN affinity for apatite crystal founds to inhibit the crystal maturation process [Hunter et al ., 1996; Bernards et al., 2008].

Bone	Dentin	Enamel
Osteocalcin (OC)	dentin matrix protein 1	Enamelin
Osteopontin (OPN)	dentin sialo-phospho protein	Matrix extracellular phospho-glycoprotein (MEPE)
Osteonectin (ON)	-	-
Bone sialoprotein (BSP)	-	-

Table 1. Major non-collageneous proteins that associate with mineralized ECM in different bone tissues

3.3.2 Bone remodeling

In contrast to the matrix modeling process the remodeling of the mineralized matrix is more complex because it can be controlled by many different mechanisms. In the case of normal bone homeostasis we observe a balance between the calcification and decalcification reactions in the bone matrix where the decalcification of the matrix is facilitated by the removal of the dead osteocytes and discharged MVs from the matrix. This process is primarily carried out by osteoclasts which arise from the endosteum. However, the decalcification process can be disturbed due to several reasons which could be either related to blockages or total stoppage of the calcification process or due to pathological changes in the tissue such as migration of cancer metastatic cells, activation of osteoporotic reactions etc.

The modeling and remodeling of the matrix thus represent the two orthopedic interfaces of the bone which are generated at periosteum and endosteum respectively and their mineralizing and de-mineralizing functions overlap in the matrix as shown in Figure 3.

4. Materials and methods for the mimicry of bone components

Based upon the details of the natural processes that lead to mineralized bone formation and its degradation, as described above, there are several reports in the literature that describe strategies to generate materials *in vitro* that are similar to the *in vivo* physicochemical and/or biological properties of the bone components. In fact bone biomimetism remains as one of the most actively pursued and financially a very rewarding area of human tissue engineering. A brief summary describing the different types of materials and processes that are currently in use to generate bone like materials, for their use as bone implants or substitutes, is provided here.

4.1 Materials useful as substrates or modifiers in bone implants and/or bone substitutes

The choice of materials that can be used to repair or replace a damaged or deformed bone is very wide. An overriding factor in choosing a base material for this purpose is its bioactivity and biocompatibility *in vivo*.

Materials	References
Metals	
Stainless steel AISI 316L, Co–Cr–Mo alloy	Yeung et al.,2007; Aksakal et al., 2008; Seligson et al.,1997; Marti 2000
Ti and its alloys	
Ti6Al4V, TNZT alloys (Ti–Nb–Zr–Ta), Ni Ti, TiNbZr	Aksakal et al., 2008; Chakraborty et al., 2009; Yeung et al.,2007; Banerjee et al., 2004; Banerjee et al., 2006; Niinomi 2003; Ning et al., 2010; Seligson et al.,1997
Ceramics and Bioglass	
α-Al_2O_3, high alumina ceramics, PSZ (partially stabilized zirconia), 45S5 BG, S45P7	Kapoor et al., 2010; Christel et al., 1988; Gorustovich et al., 2010; Yuan et al., 2001
Polymers	
Polyethylene (PE), Polymethacrylic acid (PM MA), polyglycolic acid (PGA), poly lactic acid (PLA), polycarbonate (PC), polypropylene(PP)	Andersson et al., 2004; Reis et al., 2010; Oral et al., 2007; Butler et al., 2001; Athanasiou et al., 1998; Smith et al.,2007; Geary et al., 2008; Shalumon et al., 2009; Jayabalan et al., 2001
Composites	
Mg–Zn–Zr, HA-PEEK poly (aryl-ether-ether-ketone), Polyphospha zenes, BG-COL-HYA-PS (glass-collagen hyaluronic acid-phosphatidylserine)	Ye et al.,2010; Kurtz et al., 2007; Sethuraman et al., 2010; Xu et al., 2010

Table 2. A list of materials in use as base/substrate material in bone implants

Since there is no material available that can per se become a bone substitute, several modifications on the original material are required to make it biocompatible. The aim to do these modifications is that the new material should be nontoxic and biologically inert but yet it should show orthopedic bioactivity and its production should be cost effective. The biocompatibility of the material is also dependent upon certain host factors such as general health, age, tissue perfusion and immunological factors [Wooley et al., 2001] and therefore only certain types of materials have been used so far for this purpose. A list of such materials currently in use is given in Table 2.

Each of the listed materials in the Table has some unique quality that qualifies it to be used as the base material or the substrate of an orthopedic implant. Cationic metals for example can form ionic bonds with non-metals and can be easily converted into alloys which have good ductile properties and heavy load bearing strength. Among the nonmetals, ceramics are interesting because their inter-atomic bonds are either totally ionic or predominantly

ionic and they can be covalently bonded to a number of compounds including proteins. Among the polymers for orthopedic use, plastics and elastomers have been the main choice but because of their limited weight bearing capacities their use is restricted. The composites are useful because they can combine the properties of two or more compounds making it a more versatile material to get a functional hierarchy of substances needed to make a bone like substance.

Besides the substances which are used as substrates for making biocompatible materials, there are many other unique elements of bone structure which lend themselves to be mimicked by manmade materials as functionalizing compounds of the substrates. One of the most commonly mimicked biomaterial for this purpose is apatite which is the most abundant phosphate mineral on earth found in mineralizing vertebrates. Among all the calcium phosphate minerals available hydroxyapatite (HAp) is found to be the most thermodynamically stable bioceramic material at physiological environment which helps in faster osteointegration. Hence the most sought after properties that material scientists and bone tissue engineers look for in their apatite are bone bonding ability and osteo-conductivity in addition to their general biocompatibility and bioactivity. The starting compounds used for making HAp is generally calcium phosphate and based on some solution parameters like super saturation, other ionic products and pH we can get many other apatite phases apart from HAp. These non-naturally occurring apatite phases can be more useful than naturally occurring ones.

MINERAL NAME	**Ca/P ratio**	**Abbreviation**
Monocalcium phosphate monohydrate	0.5	MCPM
Monocalcium phosphate:dihydrate	0.5	MCPD
Dicalcium phosphate: dehydrate mineral brushite	1.0	(DCPD)
Anhydride mineral monetite	1.0	(DCPA)
Octacalcium phosphate	1.33	(OCP)
α-tricalcium phosphate	1.5	(αTCP)
β-tricalcium phosphate	1.5	(β-TCP)
Whitelock mineral	1.29	
Hydroxyapatite O- HAp	1.67	OHAp
Calcium-deficient hydroxyapatite	1.5-1.67	(CDHA)
Fluorapatite	1.67	(FAp)
Chloroapatite	1.67	(ClAp)
Carbonated apatite TYPE A	1.67	(CO3Ap)
Tetracalcium phosphate, mineral hilgenstokite	2.0	(TTKP or tetcp)

Table 3. Different types of calcium phosphates obtained during preparation of HAp

A list of the various types of apatite phase that can be obtained from different calcium phosphates is given in Table3. Besides using calcium phosphate, a combination of various salts is also used to generate HAp. This process is more close to the natural process because the constituents of starting material are based upon the constituents of the natural body fluid such as blood plasma. The solution that most represents the similarity with blood plasma is referred to as simulated body fluid or SBF and its many constituents have been described elsewhere Tadashi and Hiroaki 2006 and Jalota et al 2006.

		Na^+	K^+	Ca^{2+}	Mg^{2+}	$HCO3^-$	Cl^-	$HPO4^{2-}$	$SO4^{2-}$	Ca/P	Ph
Blood Plasma		142	5	2.5	1.5	27	103	1	0.5	2.5	7.4
SBF Range		127-734	5-10	2.5-12.5	1.5-7.5	4.2-35	111-724	1-5	0.05-1	0-2.5	7.25-7.4
TYPE-1		142	5	2.5	1.5	4.2	148	1.8	**----**	1.4	7.25
TYPE-2		142	5	2.5	1.5	27	147.8	1	0.5	2.5	7.4
TYPE-3	**c-SBF2**	142	5	2.5	1.5	4.2	147.96	1	0.5	2.5	7.4
	c-SBF3	142		2.5	1.5	35.23	117.62	1	0.5	2.5	
	SBF-JL1	142	5	2.5	-	34.9	111	1	-	2.5	
	SBF-JL2	142	**---**	-	-	34.88	109.9	1.39	-	0	
TYPE-4	**SBF**	142	5	2.5	1.5	4. 2	147.8	1	0.5	2.5	7.25
	d-SBF	142	5	1.6	0.7	4.2	144.1	1	0.5	1.6	7.25
TYPE-5		142	5	2.5	1.5	4.2	148	1	0.5	2.5	7.4
TYPE-6	**SBF**	142	5	2.5	1.5	4.2	147.8	1	0.5	2.5	7.4
	5XSBF	714.8	**----**	12.5	7.5	21	723.8	5	**----**	2.5	7.6
TYPE-7		127	10	12.5	3	35	123	5	**----**	2.5	7.4
TYPE-8		142	5	2.5	1.5	4.2	147.8	1	0.05	2.5	7.4
TYPE-9	**SBF-1**	142	5	2.5	1.5	4.2	148	1	0.5	2.5	7.4
	5XSBF	213	7.5	3.8	2.3	6.3	223	1.5	0.75	2.53	
	SBF-2	142	5	2.5	1.5	4.2	148.8	1	0.5	2.5	
TYPE 10	**SBF-a**	714.8	**----**	12.5	7.5	21	723.8	5	-	2.5	7.4
	SBF-b	704.2	**----**	12.5	1.5	10.5	711.8	5	-	2.5	
TYPE-11		142	5	2.5	1.5	4.2	148.8	1	0.5	2.5	7.4
TYPE-12		142	5	2.05	1.5	4.2	148	1		2.05	7.4
TYPE-13		142	5	2.5	1.5	4.2	148.5	1	0.5	2.5	7.4
TYPE-14	**1XSBF**	142	5	2.5	1.5	4.2	147.8	1	0.5	2.5	7.5
	3CaP	109.5	6	7.5	1.5	17.5	110	3	-	2.5	
	SBF										
TYPE-15	**SBF(N)**	142	**5**	2.5	1.5	27	123	1	0.5	2.5	7.2
	SBF(O)	142	**5**	2.5	-	-	123	1	0.5	2.5	
TYPE-16		109.5	6	7.5	1.5	17.5	110	3	0		6.65-6.71 6.55-6.65 6.24-6.42

Table 4. Recipes for making different types of Simulated Body Fluids for biomimetic preparation of Apatite

[Reference for the above Table are a-Liu et al.,1998; b-Kokubo & Kim, 2004; c-Marc & Jacques,2009; d-Chikara et al., 2007; e-Kokubo,1996; f-Bharati et al.,2005; g-Qu & Mei,2008; h-De Medeiros et al., 2008; i-Tsai et al.,2008; j-Habibovic et al.,2002; k-Hyun et al.,1996; l-Silvia et al.,2006; m-Xin et al.,2007; n-Yajing et al.,2009; o-Kapoor et al.,2010; p-Haibo & Mei 2008]

Over the years the constitution of SBF has undergone so many modifications that would be compiled into a list of different SBFs that can used to obtain bone like apatite for bone remodeling purposes. This compilation is shown in Table 4. The original SBF was intended to study mainly the bone-bonding ability of the apatite and it lacked in sulfate

ions in relation to original plasma constituents. The SBF constitution was later upgraded with major variations done in chlorine and bicarbonate compositions and to a lesser extent in sulphate ions. SBF with higher Cl^- and lower HCO3- concentrations and variations in buffer systems and pH are found to be in equilibrium with the blood plasma. The physiological pH is maintained in this in vitro system using tris (hydroxymethyl) amino methane (Tris)/HCl.

4.2 Methods for preparing substrates and modifier materials

While the base substrate materials are prepared by conventional metallurgical methods, their bioactivity is induced by functionalizing them with many modifier materials. The modifier materials include proteins, enzymes and most importantly the different types of apatites. There is an endless list of techniques by which apatite deposition can be carried out on orthopedically selected substrates, but the successful methods are those which give high bone bonding ability and good osseointegration. Among the different available techniques, plasma spray, sol-gel synthesis and biomimetic methods are the most successful. Some salient features of the first two and details of the biomimetic approaches are provided here.

4.2.1 Plasma spray

Plasma spray coatings on to metal substrates have gained interest during the past decades due to its high deposition rate and its large scale efficiency. This method is compatible with various platforms including ceramic composites apart from metals. Numerous studies have been carried out on the bone bonding behavior of these coatings with the substrates. The thickness of the coating is of few microns size. The precursor is mainly fed in the form of powder which is released into a plasma gun. A high voltage argon gas generates plasma where the powder gets partly melted and is directed towards the substrate followed by rapid cooling further impelling the substrate thus depositing a coat. This method has been used to deposit different functionalized materials on either metal or non-metal surfaces. [Chen et al., 2008; Chen et al., 2006; Culha et al., 2010]

But the major concerns regarding this process a) is the instability of the coatings therefore poor binding of the coating with the substrate or implant .This necessitates them for further processing to increase the mechanical interlocking of the coating-substrate system. b) High processing temperatures involved lead to changes in CaP phases resulting in the formation of less stable phases thereby reducing the bonding strength between the substrate and the coating. c) These coatings are largely amorphous with less homogeneity over the entire substrate resulting in structures of low crystallinity which signifies that the substrates are not bioactive enough to induce the required bone attachment. Many functionalized scaffolds have been developed by this technique and there biocompatibility was checked *in-vivo* so that these implants can be used for various orthopedic applications [Heimann et al., 2004; Wu et al., 2009]

4.2.2 Sol-gel synthesis

This technique is one of the oldest in developing thin film coating having varied applications like protective coatings, passivation layers, sensors and membranes. The methodology involves the fabrication of materials by using a chemical solution (sol) which acts as the precursor for a specialized integrated network (gel) of either particles or network oligomers/polymers. The unique property of this method is that the kinetics of the reaction

can be controlled by monitoring the particle size, porosity and thickness of coating. Hence the fabricated materials can be obtained in the form of films, powders, fibers, processed at a lower temperature which differentiates it form the conventional processing strategies [Podbielska and Ulatowska-arza 2005].

The starting materials used are inorganic or metal-organic precursors (alkoxides). The chemistry of this process involves basically two reactions like hydrolysis and polycondensation. When metal- alkoxides are used the alkoxide is dissolved in alcohol and hydrolyzed by the addition of water, whereas in case of metalloids, acid or base catalyst is added which replaces the alkoxide ligands with hydroxyl groups. In case of inorganic precursors like salts, hydrolysis proceeds by the removal of a proton to form a hydroxo (-OH) or oxo (=O) ligand. Therefore subsequent condensation reactions in case or organic and inorganic produces oligomers or polymers composed of M-O-M or M-μ(OH)-M bonds.

The coating is generally done by depositing the precursor on to the substrate either by dip coating or spin coating, later the samples are dried at high temperature which results in shrinkage and also increases the density of the deposited precursors. The coating thickness is a function of withdrawal speed, concentration and viscosity of the solution hence the porosity of the gel is dependent on the rate at which the solvent is removed. The simplicity of this procedure develops uniform coatings of high homogeneity [Klein, 1988].Many biocompatible, bioactive and stable metals/non-metals and bioglass scaffolds are deveopled by this technique by depositing HAp, various bioactive proteins in the form of thin films and nanoparticles[Weng et al., 2003; Wang etal.,2008; Vijayalakshmi et al.,2008] for hard and soft tissue replacement[Kim et al.,2005; Nguyen et al.,2004; Sepulveda et al.,2002; Zheng et al.,2009].

4.2.3 Biomimetic process

Since the theory of biomimetic process proposed by Kokubo, the study of bioactivity using SBF has been reviewed by many research groups all these years. Why these studies are at a faster pace and what makes this process so challenging from other technologies in predicting bone bioactivity *in vivo*. This process aims at mimicking the blood plasma compositions in acellular conditions using SBF [Tadashi & Hiroaki, 2006]. For natural bone to bond with the implants there must be specific appropriate response which it feels that it can be accepted, is mainly achieved by depositing apatite on to these surfaces termed as bioactivity/bone-bonding ability. Bones ability to deposit calcium phosphate defines its characteristic property as a hard connective tissue. Several results have been obtained using this procedure and they have been summarized in Table 6.

Bio-mimetic Coating Method used to Functionalize Ti-6Al-4V and α-Al_2O_3

Our lab is also developing functionalized scaffolds which can be in long run used for bone engineering applications.

We are working with metal (Ti and its alloys like (Ti-6Al-4V, TiZr, and TiNb), non-metals (Ceramic like α-Al_2O_3) and glass, functionalizing them in order to check the cell behavior *in vitro* and also check there bio-compatibility properties *in vivo*.

There are many methods to functionalize the metal/non-metal surface by using HAp/calcium phosphate which can be done by various methods like plasma spray method, sol-gel coating method, dip coating methods but the most easy and efficient way to mimic the natural component of bone is by Biomimetic coating method, hence we have utilized this process to develop an even, functionalized HAp coating on a titanium alloy (Ti-6Al-4V) and

Cell culture studies and materials used	Objectives	Results	References
Apatite and apatite/ collagen composite coatings on PLLA using Saos-2 osteoblast like cells	Cell attachment, proliferation and differentiation	Biomimetic apatite/collagen coating found to exhibit higher proliferation and differentiation in comparison to apatite coatings	Chen et al., 2008
Biomimetic and electrolyti -cally deposited carbonate apatite on Ti alloy using MC3T3-E1 cells.	Cellular proliferation and differentiation	Higher proliferation and OC and BSP mRNA expression on biomimetically coated substrates than electrolytically deposited method.	Jiawei et al., 2009
Chemically pretreated CP Ti immersed in SBF for 2 and 14 days and tested using human osteoblasts (MG-63) cells.	Cell spreading, proliferation and differentiation	A well spread morphology was observed both functionalized surfaces. TiCT and TiHCA surfaces rendered increased expression of collagen 1 and ALP at 7 and 14 days.	Barbara et al ., 2008
HA deposition on negatively charged SAM coated glass cover slips by culturing human mature OC of bone cell tumor for 24hrs	Osteoclastic activity through F-Actin ring formation, calcium release and formation of resorption pits	Osteoclast were able to attach and resorb on coated glass cover slips	Asiri et al., 2009
Biomimetic apatite deposition on hyaluronic acid (HA)-based polymer scaffold	Osteogenic induction of mesenchymal stromal cells (h-MSCs)	At higher mineralization on HA-based scaffold.	Cristina et al ., 2010
Incorporation of bisphosph -onate sodium clondrate into biomimetically coated apatite on to starch based scaffold using human osteoblast-like cell line (SaOs-2)	Effect of BP on osteoclastic activity and cell morphology, attachment and proliferation	Osteoblastic activity was simulated with bisphopshonates at dose dependent concentration of 0.32mg/ml by enhanced cell viability	Oliveira et al., 2010
BMP-2 into biomimetic apatite coatings using Rat bone marrow stromal cells for 8days on Ti implants	Osteogenic activity	Protein incorporated CaP coatings enhanced the alkaline phophatase activity	Yuelian et al., 2004

Table 6. Cellular responses to biomimetically prepared substrates and coatings

on a bioinert ceramic substrate (α-Al_2O_3). In our method, the metal /ceramic substrates were incubated in simulated body fluid (SBF) at 25°C for different time points with prior treatment with globular protein BSA (bovine serum albumin) [Chakraborty et al., 2009; Kapoor et al., 2010]. This process leads to the formation of HAp coating exhibiting bone like apatite growth on the surface. It may further be noted that bone, a natural composite comprises non stoichiometric calcium hydroxyapatite (HAp) precipitated in a controlled reaction environment of a highly aligned, anisotropic organic template. It differs from stoichiometric hydroxyapatite (HA) in composition, crystallinity and other physical and mechanical properties developed artificially through various methods.

The surface treatment and coating of these materials had shown a better cellular response *in vitro* and also a good biocompatibility property *in vivo* when compared with untreated and uncoated materials. The surface treatment by globular protein i.e., BSA might provide a functionalized template comprising of charged amino-acids which resulted in more nucleation sites [Chakraborty et al., 2009] hence led to the even coverage of HAp (about 280-300μm) by immersion of the materials in SBF at desired temperature of 25°C between the pH range of 5-7, which resulted in the formation 30-40 nm albumin globules, under specified conditions, on both ceramic and Ti-6Al-4V alloy substrates. In comparison with the untreated substrates the coverage of HAp was very much poor(less than 200μm), hence BSA treatment has led to the development of nano-sized globules after HAp coating which have led to the better cellular-activity *in-vitro* which is due to "cooperativity" reaction [Chakraborty et al., 2009] between protein molecules and the charged surface of HAp, depending on the concentration of the protein molecules in the coating [SBF] solutions.

We have done a comparative study of biological properties of the unique coating of HAp developed on both metal and non-metal which is less reported. Based on the methodology of functionalizing these materials we have generated many substrates of Ti and Ceramic which showed a different structural variation and these specific morphological structures of protein and HAp has led to good fibroblast [NIH-3T3] cell response. The Ti-6Al-4V which is BSA treated and coated for 4 days has shown a nano-sized globules (as indicated by arrows) due to globular protein treatment has shown a better *in-vitro* and *in-vivo* activity which can be seen in Figure 1 panel c in comparison with the bare Ti-6Al-4V panel a, BSA treated Ti-6Al-4V panel b and coated Ti-6Al-4V for 4 days without prior treatment with BSA panel d which did not show nano-sized HAp globules.

The unique structural property of HAp coating on Ti-6Al-4V treated with BSA and coated for 4 days is shown in Figure 2 where panel a shows the inter and intra connection of HAp fibers into plates which can be seen in higher magnification in panel b. Panel c shows the femur bone like growth of HAp fibers [Kapoor et al., 2010] which represents the unique methodology in mimicking the bone like components by generating a highly functionalized scaffold for *in-vivo* applications.

On the contrary, micron sized globules of HAp [Figure. 3(c)] were observed on the BSA treated and coated for 2days ceramic substrate surface. This may be attributed to the enhanced hydrophilicity of the BSA treated ceramic substrate (it already has intrinsic hydrophilicity) that accumulates –OH groups throughout the mechanically roughened (grit blasted) surface, on immersion in simulated body fluid (SBF), aqueous medium. These act as nucleation sites and induce Ca^{2+} ions from SBF to be coordinated to the above –OH groups on the substrate, by electrostatic force of attraction. Hence nucleation of a large number of HAp globules takes place and they grow fast into micron sized globules owing to the high surface energy as mentioned, resulting in a dense coverage of substrate surface. Hence due

to large deposition of micron-sized HAp globules the NIH-3T3 cellular response was much better on this ceramic substrate in comparison to the bare ceramic (panel a), BSA treated ceramic(panel b) and untreated and coated for 2 days panel d which showed a much bigger HAp deposition.

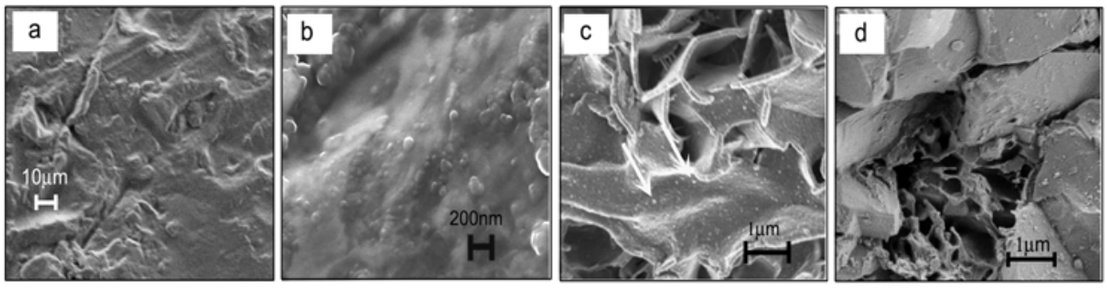


Fig. 4. SEM images of different Ti-6Al-4V where (a)Bare Ti-6Al-4V (b)BSA Treated Ti-6Al-4V (c)BSA Treated and Coated for 4 days Ti-6Al-4V (d) Coated for 4 days Ti-6Al-4V.(Image generated from Kapoor et al., 2010).

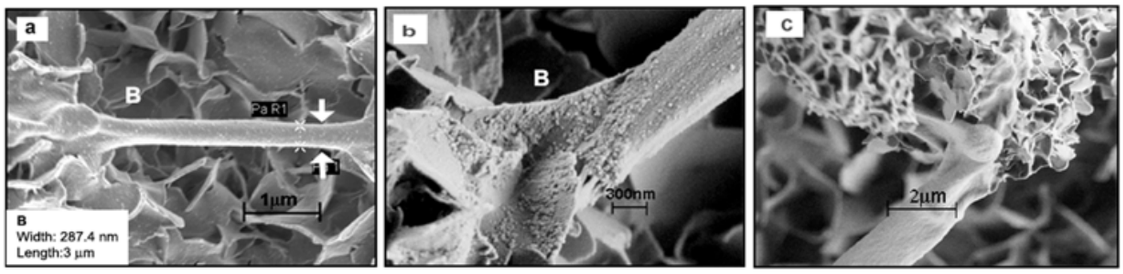


Fig. 5. SEM Images of Ti-6Al-4V substrate which is BSA treated and coated for 4 days where (a) Inter- and intraconnection of the HAp fiber in the crystal plates of 4-day coated substrate. (b) Higher-magnification image of B showing the fiber merges into the crystal plates of the HAp coating. (c) Femur bone-like structure obtained in B4. (Image generated from Chakraborty et al., 2009).

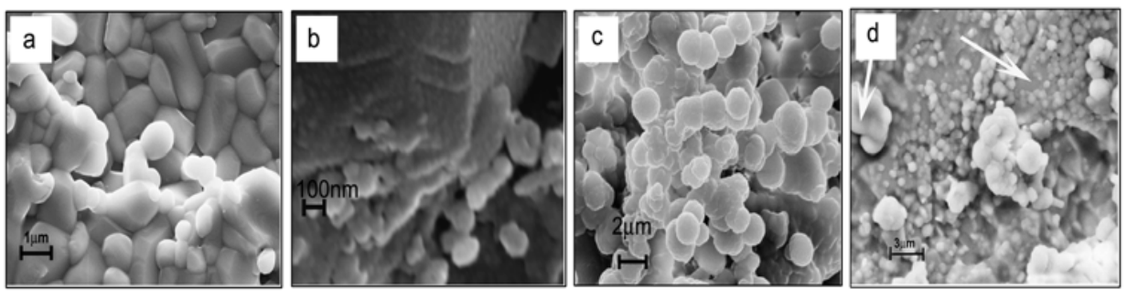


Fig. 6. SEM images of α-Al_2O_3 where (a)Bare α-Al_2O_3 (b)BSA Treated α-Al_2O_3 (c)BSA Treated and Coated for 2 days α-Al_2O_3 (d) Coated for 2 days α-Al_2O_3.(Image generated from Kapoor et al., 2010).

Our *in vivo* experiments also proven that metal/nonmetal implants which are protein treated and coated are more bioactive as they showed no negative response in term of any kind of inflammatory responses.

This comparative assessment of metal/non-metals structural and biological properties showed that metal when treated with protein and biomimtically coated for HAp can be used as a scaffold for many biomedical applications especially for osteoconduction. In modification for the method proposed, many biologically active molecules like osteogenic agents and growth factors can be co-precipitated with apatite crystals onto metal implants for the better osteogenic behavior as this biomimetic coating can be readily absorbed *in-vivo*.

5. Orthopedic challenges

As new methodologies for making functional components of human tissues to rectify a deformity or for developing new treatments of disease and trauma get developed we realize the limitations of the techniques and principles of biomimetic tissue engineering in facing up the real challenges of this approach. While many new methodologies have become available for the management of orthopedic disease and trauma, the computability of the manmade materials in this area is far from ideal. We describe here some of the unmet challenges of this field.

5.1 Biocompatibility and stability of *in-vivo* scaffolds

One of the most important aims of biomimetic design and production of materials for bone implants is to make them stable and compatible to the local bone tissue. Since there is considerable diversity in the details of local anatomies of specific bones the presently available general implant materials are prone to infection, extensive inflammation, and poor osteointegration. Besides their life span is less than 15 years which clearly shows the inability to mimic the longetivity of the molecular components of bone [Harold 2006, Porter 2009]. The implant failure is mainly attributed to acute complications, host responses, prosthesis dislocations and surgery failures seen at initial stages after surgery, and also after several years post surgery when implant loosening, osteolysis, implant wear and tear, instability, infection and fractures are observed.

In order to increase implant life it would be advisable to seed them with young osteoblasts which would sustain the production of bone mass on the implants (Xynos et al., 2001). It would also be useful to use bioactive agents in the coatings that would activate pathways related to cell survival, proliferation and differentiation. Thus it is clear that in order to increase the life of the implanted material it would be advisable to shift the focus of material production from a purely material science outlook to a cell biological and molecular biological approach.

5.2 Materials for osteoporotic applications

Osteoporosis a major health threat to bone degenerations due to decreased bone quality, are characterized by reduction in bone mass and disordered skeletal micro-architecture and are susceptible to fracture risks at sites of hip, spine and wrist [Borges & Bilezikian, 2006]. Much of the concerns regarding this are found in older populations where treatment becomes possible to an extent through regular controlled diet activities. Since the loss in bone mass can be directly attributed to the abnormal remodeling process therefore biomimetic tissue engineering approaches could offer alternate approaches to reduce the hyperactive bone resorption process. One of the targets for this could be the receptor for nuclear factor kappa B which seems to be involved in osteoblast–osteoclast coupling mechanisms.

6. Conclusion

We have shown in this chapter how one can use biomimetic approaches to simulate the osteoregenerative (periosteal surface) and osteo-degenerative (endosteal surface) interfaces of appendicular bones. These processes include novel tissue engineering strategies that combine developments in the field of material science with the cell and molecular biological pathways that are seen in the natural differentiation of osteoblast and osteoclast. We hope that some of these strategies would lead to the better management of trauma and age related degeneration of bone tissues.

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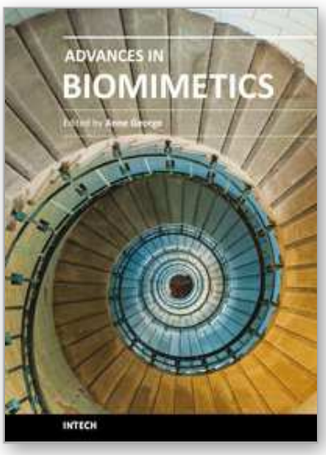
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