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ΣΧΟΛΗ ΜΗΧΑΝΙΚΩΝ
ΤΜΗΜΑ ΜΗΧΑΝΙΚΩΝ ΒΙΟΪΑΤΡΙΚΗΣ

Bone Tissue Engineering & Regenerative Medicine

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Ο κάτωθι υπογεγραμμένος Θρασύβουλος-Παναγιώτης Παπαγεωργίου του Αντωνίου, με αριθμό μητρώου 48012059 φοιτητής του Πανεπιστημίου Δυτικής Αττικής της Σχολής Μηχανικών του Τμήματος Μηχανικών Βιοϊατρικής τεχνολογίας, δηλώνω υπεύθυνα ότι:

«Είμαι συγγραφέας αυτής της πτυχιακής/διπλωματικής εργασίας και ότι κάθε βοήθεια την οποία είχα για την προετοιμασία της είναι πλήρως αναγνωρισμένη και αναφέρεται στην εργασία. Επίσης, οι όποιες πηγές από τις οποίες έκανα χρήση δεδομένων, ιδεών ή λέξεων, είτε ακριβώς είτε παραφρασμένες, αναφέρονται στο σύνολό τους, με πλήρη αναφορά στους συγγραφείς, τον εκδοτικό οίκο ή το περιοδικό, συμπεριλαμβανομένων και των πηγών που ενδεχομένως χρησιμοποιήθηκαν από το διαδίκτυο. Επίσης, βεβαιώνω ότι αυτή η εργασία έχει συγγραφεί από μένα αποκλειστικά και αποτελεί προϊόν πνευματικής ιδιοκτησίας τόσο δικής μου, όσο και του Ιδρύματος.

Παράβαση της ανωτέρω ακαδημαϊκής μου ευθύνης αποτελεί ουσιώδη λόγο για την ανάκληση του πτυχίου μου».

Ημερομηνία

07/10/2021

Ο Δηλών

Περίληψη

Στην παρούσα διπλωματική εργασία γίνεται εκτενής ανάλυση του ταχέως εξελισσόμενου πεδίου της ιστομηχανικής με κατεύθυνση την ιστομηχανική των οστών. Επιπρόσθετα, παρουσιάζονται και αναλύονται όλα τα βοηθητικά επιστημονικά πεδία τα οποία μας παρέχουν πολύτιμα υλικά/εργαλεία για την επίτευξη του επιθυμητού αποτελέσματος, την γρήγορη και πλήρη αναδόμηση του εκάστοτε ιστού. Αφού αναφερθούμε στην ανατομία, την φυσιολογία καθώς και τους αντίστοιχους μηχανισμούς που ρυθμίζουν την ομοιόσταση και την φυσιολογική αναδόμηση των οστών παρουσιάζονται τα μέρη απο τα οποία αποτελείται κάθε εφαρμογή ιστομηχανικής (κύτταρα, ικριώματα, αυξητικοί παράγοντες/βιομόρια). Ακολούθως, αναλύονται η χρονολογική ανάπτυξη και εξέλιξη του πεδίου της ιστομηχανικής απο τις πρώιμες πειραματικές και εστιασμένες εφαρμογές μέχρι τις πιο σύγχρονες λύσεις, την ανάπτυξη εμπορικών προϊόντων τα οποία βασίζονται στις αρχές της ιστομηχανικής και δημιουργούν μία νεά αγορά. Στην συνέχεια, γίνεται εκτενής αναφορά και ανάλυση των διαφόρων κυττάρων, των αντίστοιχων υλικών για την κατασκευή των ικριωμάτων (scaffolds), καθώς και διαφόρων βιομορίων ή παραγόντων ανάπτυξης οι οποίοι ενσωματόνονται σε εφαρμογές ιστομηχανικής για βελτιστοποίηση του τελικού αποτελέσματος.

Abstract

Tissue Engineering comprises an interdisciplinary field that combines the principles of engineering and life sciences to develop biological substitutes that maintain, restore, and improve tissue function. The fundamentals of tissue engineering involve a reliable cell source, proper 3D constructs for tissue growth and support and various biomolecules that accelerate the whole procedure. This field possesses an alternative approach that overcome limitations of current organ transplantation procedures. In this direction, Bone Tissue Engineering holds prominent role as overcomes limitations of conventional clinical treatments such tissue morbidity, immune rejection, and pathogen transfer. The scope of this thesis is to discuss the fundamentals of bone tissue engineering field and analyze early and current approaches of this field.

Keywords: bone tissue engineering, scaffolds, stem cells, growth factors, biomaterials, 3D-printing, regenerative medicine, bone scaffolds, vascularization, bone healing, polymers, ceramics, rapid prototyping techniques, biomolecules

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Contents

1. Bone: Anatomy, Physiology and Remodeling

1.1. Human skeleton

Human skeleton consists of three main parts: **bones**, **cartilages,** and **joints**.

- **Bone** comprises kind of connective tissue that offer support to vital organs, a good environment for bone marrow and storage of minerals, whereas participate in both calcium and acid-base balance (Taichman, 2005). It offers the ability to the skeleton to withstand weight, while it is also giving the needed strength to human skeleton.

- **Cartilage** is another type of connective tissue that consists of special cells, called chondrocytes along with glycosaminoglycans, proteoglycans, collagen fibers and, sometimes, elastin. It doesn't contain blood vessels or nerves, while nourished through diffusion. Three types of cartilage can be found in human skeleton, namely, **Hyaline cartilage** (e.g., long bones), **Fibrous cartilage** (e.g., intervertebral discs or knee meniscus) and **Elastic cartilage** (e.g., ear, epiglottis, respiratory tube etc.).

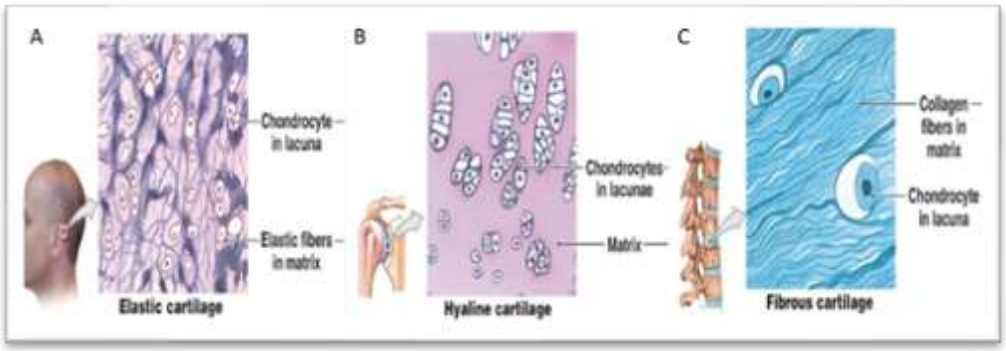

Image 1-1: The three types of cartilage found in human body: A) Elastic cartilage with limited intercellular space. This cartilage type can be found in external ear flaps and parts of the larynx. B) Hyaline cartilage with greater cellular and intercellular part compared to elastic one. Hyaline cartilage forms internal structures of nose, ears, and trachea. C) Fibrous cartilage has the biggest intercellular-to-cells ratio.

1) **Joints** can occur between several bones, bone and cartilage and cartilages making the human skeleton mobile. Also, they are classified either by structure (as **fibrous**, **cartilaginous**, and **synovial joints**) or by function (as **synarthroses**, **amphiarthroses** and **diarthroses** according the amount of movement allowed).

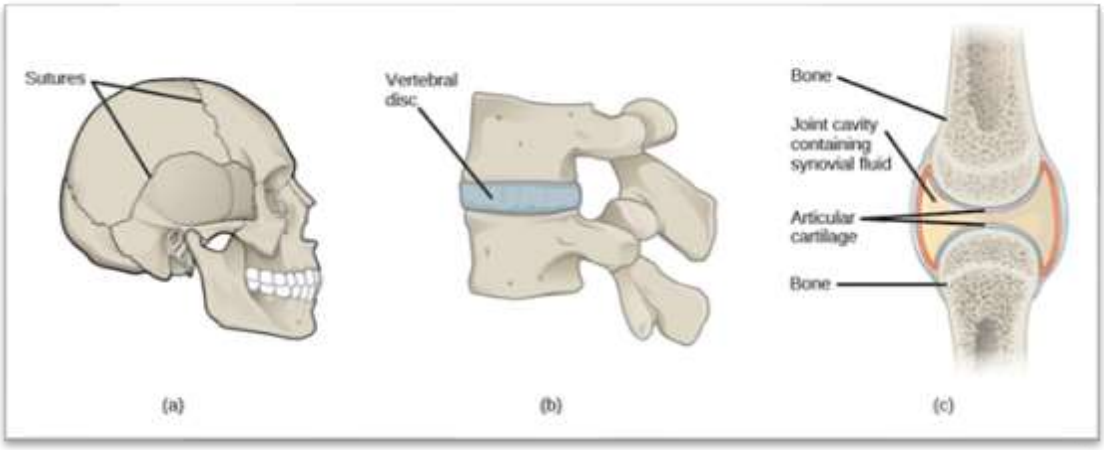

Image 1-2: Joint types according to their structure (L to R): Fibrous joint, Cartilaginous joint, and Synovial joint.

Human skeleton consists of **306 bones** at birth, but this number decreases down to 206 mainly due to the fusion of some bones until adulthood. The skeleton is subdivided in axial and appendicular parts. The **axial skeleton** includes bone of head, vertebrae, and chest (80 bones). On the other hand, the **appendicular skeleton** includes all remaining bones of the human skeleton.

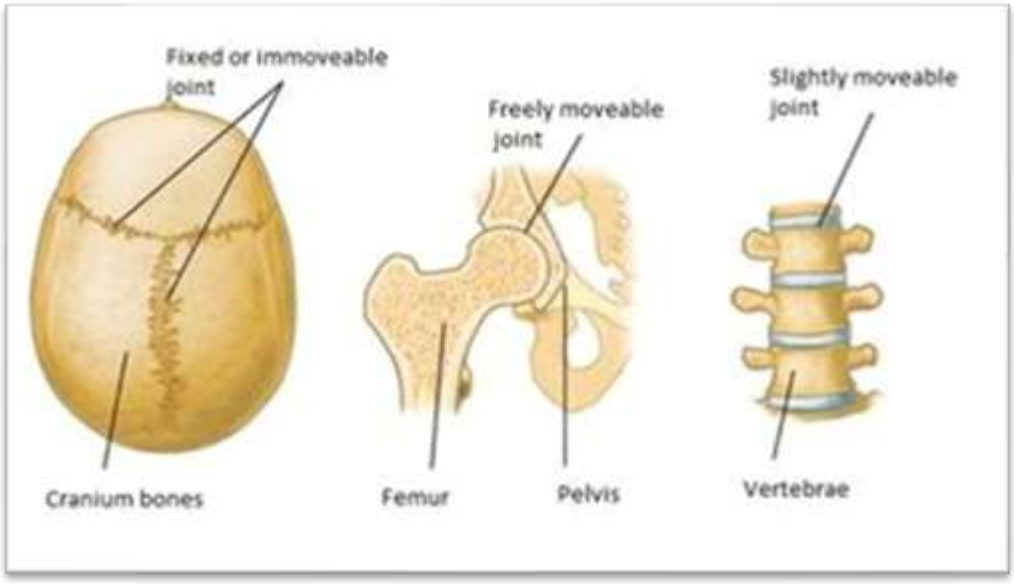

Image 1-3: Functional classification of joints (L to R): Synarthrosis (immovable), Diarthrosis (freely movable), and Amphiarthrosis (slightly movable).

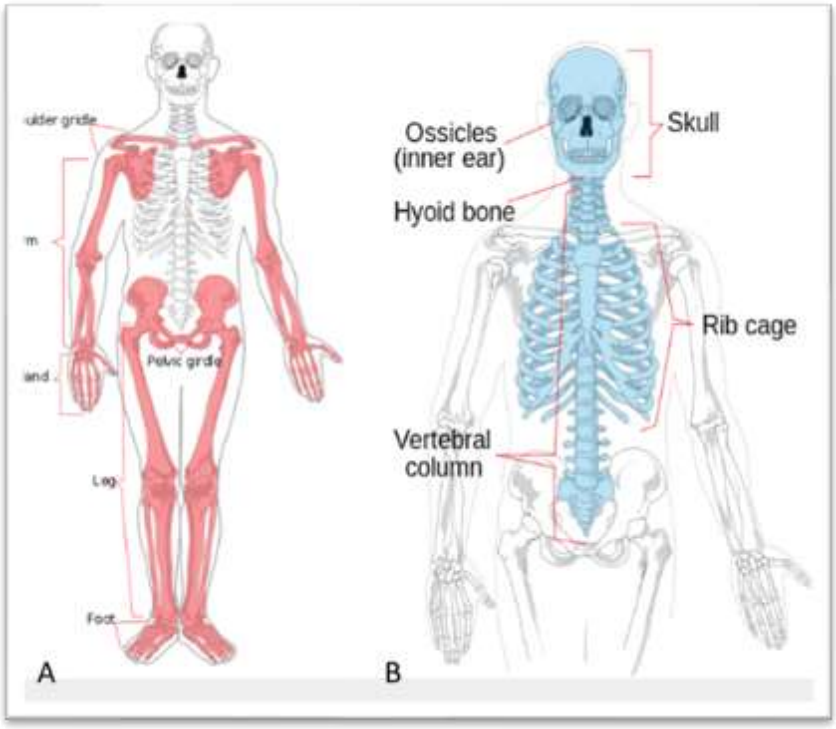

Image 1-4: Axial and Appendicular Skeleton (L to R). Adapted from: (http://pediaa.com/difference-between-axial-and-appendicular-skeleton/) [Accessed 17 Mar. 2019]

1.2. Bone parts and classifications

Bone characterized by its ability of regeneration and repair throughout the life. It is highly anisotropic being able to change over time according to the specific needs of tissues around it.

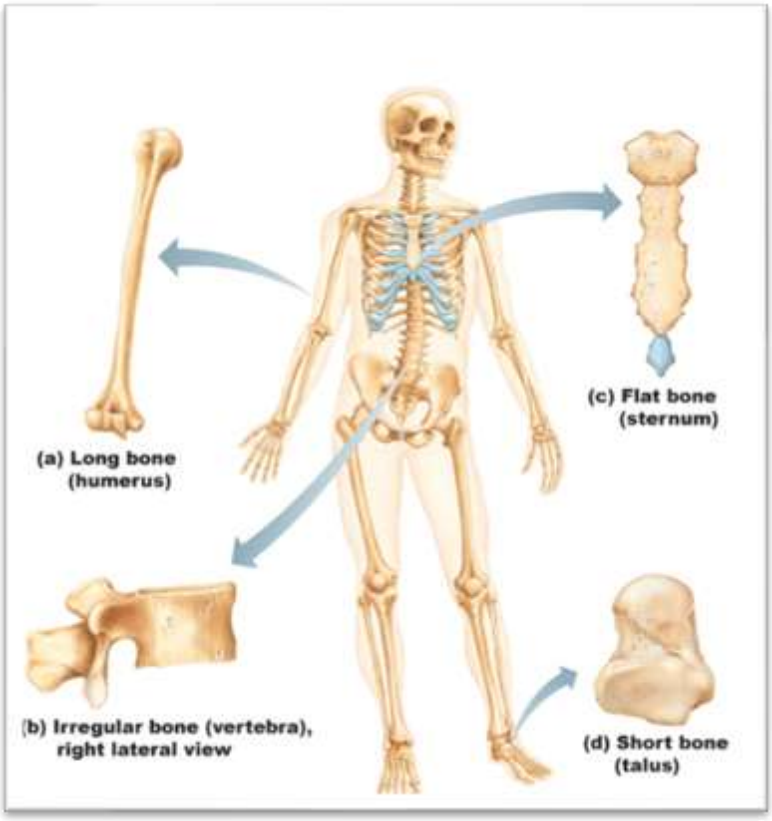

Image 1-5: Bone classification according to shape. Source: (https://sites.google.com/site/ehsanatomyphysiologyaa/chapter-7-skeletal-system/1-bone-structure) [Accessed 17 Mar. 2019])

Long bones found in human skeleton (e.g., femur, tibia etc.) consists of three distinct parts: **diaphysis** that forms the central shaft of the long bones whereas accommodate bone marrow, and **epiphysis** that forms both rounded ends/extremities of this type of bones (Image 1-6).

Bone (osseous) tissue has two main structural components: **cortical bone** (outer part) and **trabecular or cancellous bone** (inner part) that will be analyzed below.

Cortical bone

The cortical bone is rigid, dense (less than 10% porosity), surrounds bone marrow, accounts for 80% of the total human bone mass and, as its name implies, forms the external shell (cortex) of bones. Moreover, it is responsible for the white-like color of most bones due to high concentration of calcium phosphates. Internally, cortical part consists of microscopic columns called **osteons** interconnected via blood vessel canals (**Volkmann's canals**) with a typical porosity less than 5% (Clarke, 2008). Each osteon is approximately 400mm long and 200 mm wide and facilitates a canal, called **Haversian canal**, and numerous layers of osteoblasts and osteocytes cells.

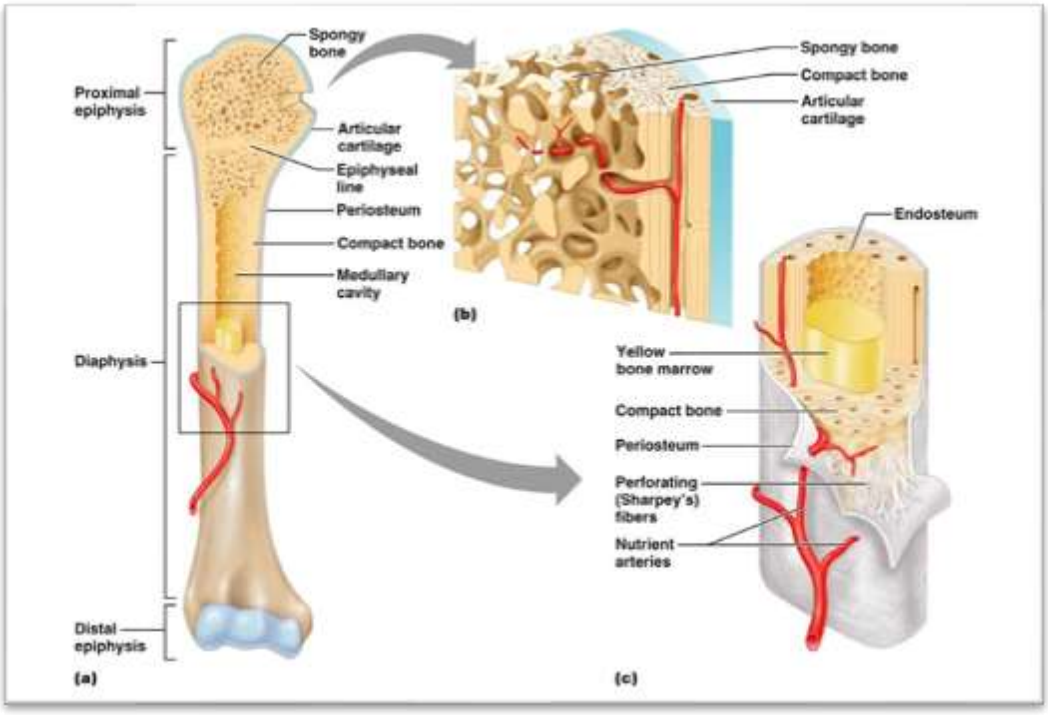

Image 1-6: Structures of cortical and trabecular parts of human bone. Source: (https://www.slideshare.net/MissReith/lecture-bone-structure-markings [Accessed 19 Mar. 2019])

Trabecular bone

Trabecular (or cancellous) part of bone is honeycomb-like (spongy) and composed of trabecular plates and rods 50-400 mm thick (Eriksen et al., 1994). It is like a cell porous network which facilitates bone marrow and hematopoietic stem cells. Trabecular bone accounts for 20% of total bone mass having 50–90 % porosity, total area of approximately 7 m^2, and 10x the surface area of cortical bone. The bone porosity is highly crucial affecting the mechanical properties of tissue. Thus, cortical bone, having higher mineral content, is stiffer than trabecular part, while trabecular bone can withstand more strain before fracturing (Hall, 2012). The different bones have different ratios of cortical/trabecular bone depending on their function (e.g., cortical to trabecular bone ratio in vertebra is 25:75 while in radial diaphysis is 95:5).

Except from cortical and trabecular parts, bone has also an outer part which is called periosteum and one inner membrane called endosteum. **Periosteum** contains a fibrous layer which accommodates fibroblasts, and an osteogenic layer contains progenitor cells that in case of bone healing developed into osteoblasts. On the other hand, **endosteum** is a thin membrane surrounding the medullary cavity being in contact with trabecular bone. It has a smaller total area than periosteum and facilitates blood vessels, osteoblasts, and osteoclasts (Clarke, 2008).

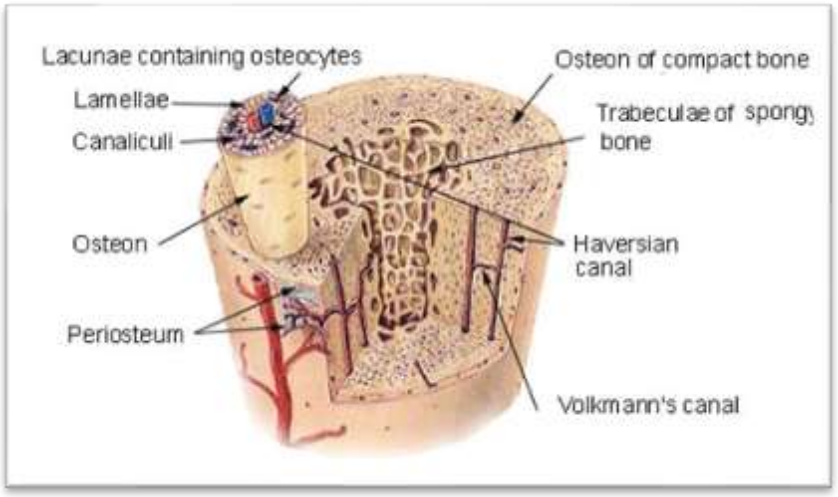

Image 1-7: Cortical (compact) and cancellous (spongy) parts of bone. (Source: https://www.wpclipart.com/medical/anatomy/bones/bones_2/structure_of_compact_and_spongy_bone.png.html [Accessed 13 Jun. 2017])

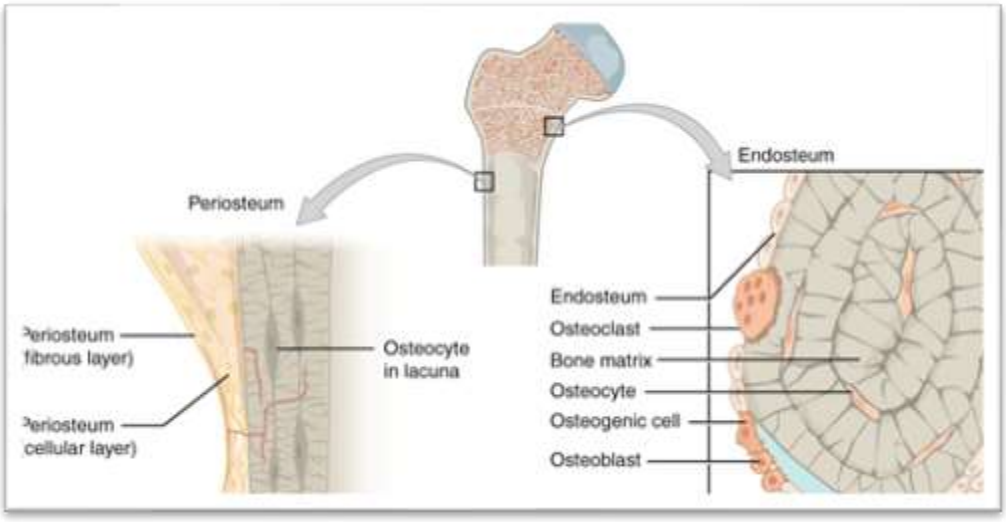

Image 1-8: Anatomy of periosteum and endosteum (Available at: https://en.wikipedia.org/wiki/Endosteum#/media/File:607_Periosteum_and_Endosteum.jpg [Accessed 21 Jun. 2017])

1.3. Bone components

Bone tissue, just like any other tissue in human body, consists of living cells (osteoblasts, osteoclast etc.) embedded in a mineralized organic matrix. This matrix, called **extracellular matrix (ECM)**, consists of:

- **Inorganic/mineral component** (50-70%) that mainly made of **Hydroxyapatite** [**Ca10(PO4)6(OH)2**], the major salt (99%) founded in bone matrix, calcium, phosphate, hydroxyl and other ions. The basic difference between geologic hydroxyapatite crystals and bone hydroxyapatite crystals is that the latter is very small (approximately 200 Å) and more soluble allowing mineral metabolism. Need to mention, that

bone hydroxyapatite constitutes about a quartet of total bone volume and half of the normal bone mass (Kini & Nandeesh, 2012).

- **Organic component** (20-30%) which is mainly composed by type I collagen (80-90%) that give bone its tensile strength and other non-collagenous proteins (10-20%) such as proteoglycans (Cohen, 2006).
- **Water** (5-10%)
- **Lipids** (<3%)

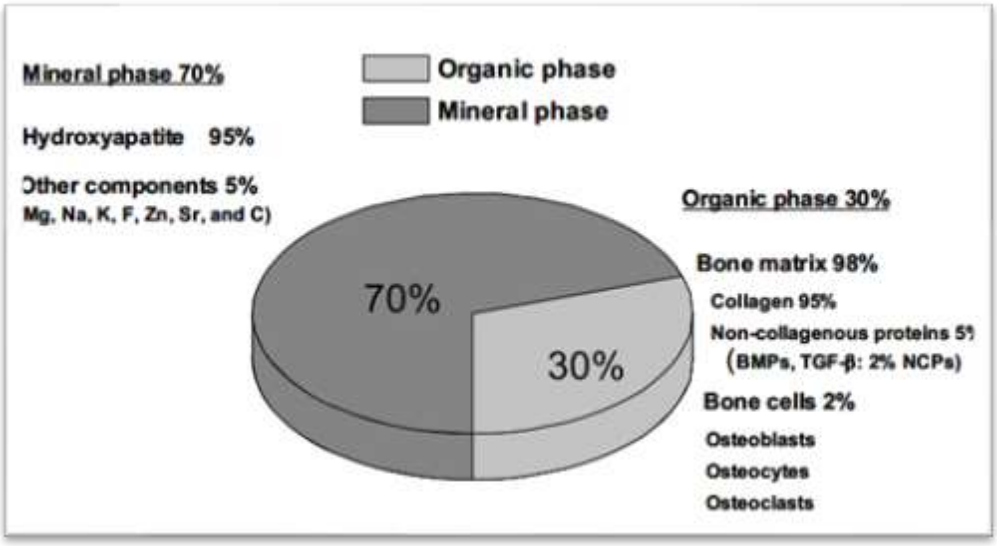

Image 1-9: Bone and ECM composition. Adapted from: (Alvarez and Nakajima, 2009)

1.4. Bone cells

Cellular bone part consists of numerous different types of cells such as **osteoblasts**, **osteoclasts,** and **osteocytes** with different roles, respectively.

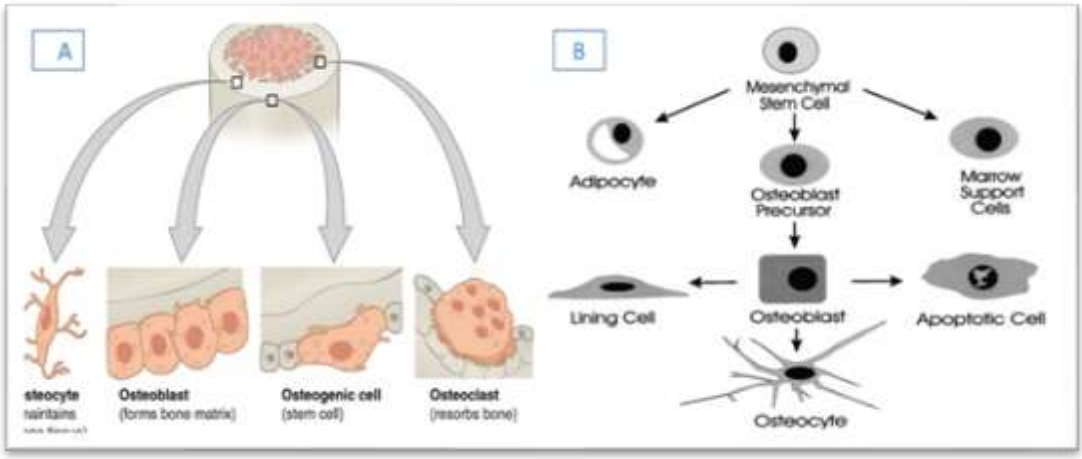

Image 1-10: A) Cell types find in bones (Available at: http://www.outlanderanatomy.com/tag/bones/ [Accessed 2 Jun. 2017]) B) Bone cells lineages Source: (Raisz LG, 1999)

Osteoblasts

Osteoblasts are end-differentiated mesenchymal stem cells entrapped in bone marrow and periosteum. Osteoblasts synthesize extracellular matrix (ECM) proteins (e.g., type I collagen) and mineralization of bone (Mackie, 2003). Mesenchymal stem cells differentiation requires the existence of some Wnt-proteins and canonical Wnt/β-catenin pathway.

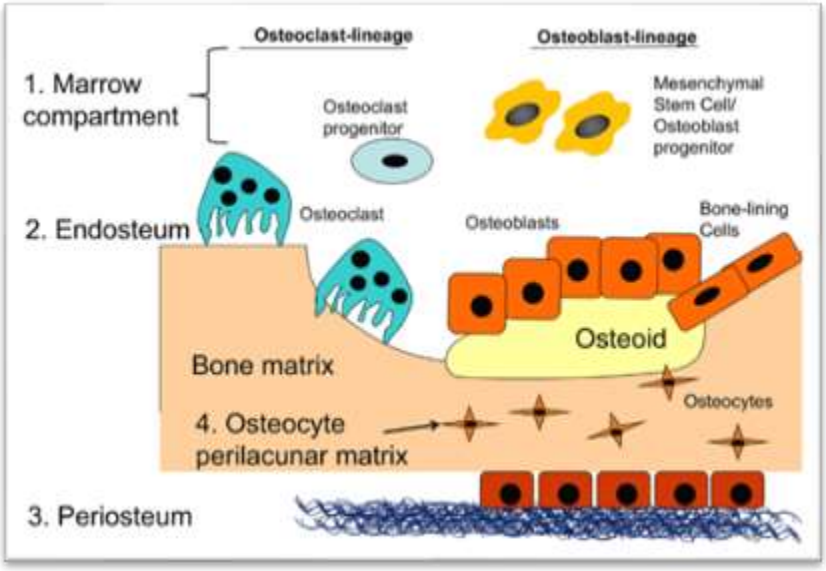

Image 1-11: Overview of the cells involved in bone remodeling and the matrix compartments of bone. Adapted from: (Alford et al., 2015)

Osteoblasts possess main role in regulation of bone resorption through activation of RANK ligand (RANKL). Moreover, osteoblasts secrete a receptor called OPG (osteoprotegerin) that stops RANK/RANKL interaction leading to differentiation delay (Caetano et al., 2007). Therefore, the ratio between RANKL and OPG determines osteoclasts' function.

Osteoclasts

Osteoclasts are multinucleated (typically five nuclei) cells coming from hemopoietic progenitor cells, while they typically resorb mineralized tissues (Lee, 2010). To become multinucleated mature osteoclasts, mononuclear precursor cells fuse together via different procedures. Mature osteoclasts resorb bone via the acid secretion which is triggered exclusively from oxidative phosphorylation of glucose taken up by GLUT2 transporter in mitochondria (Image 1-12) and accomplished by very high expression of vacuolar electrogenic H^+-ATPase and cathepsin K enzyme ([Blair et al., 1989; Blair, 1998). Acid secretion (pH 3-4) of H^+ ions is deposited into Hydroxyapatite (Image 1-14), making it to release calcium (Blair, 1989). As a result, hundred milligrams of calcium are deposited and resorbed from bone hydroxyapatite on a daily basis.

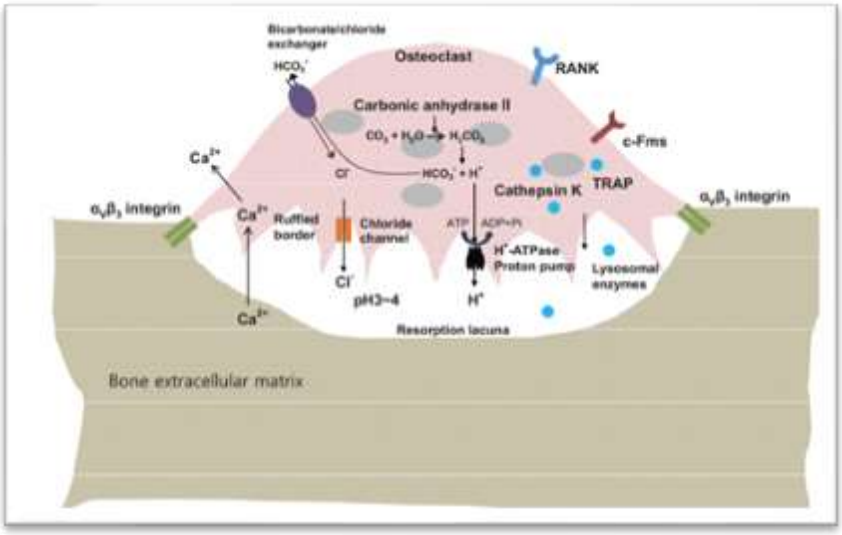

Image 1-12: The acid-transport pathway of the osteoclast for bone resorption
Source: (Lee, 2010)

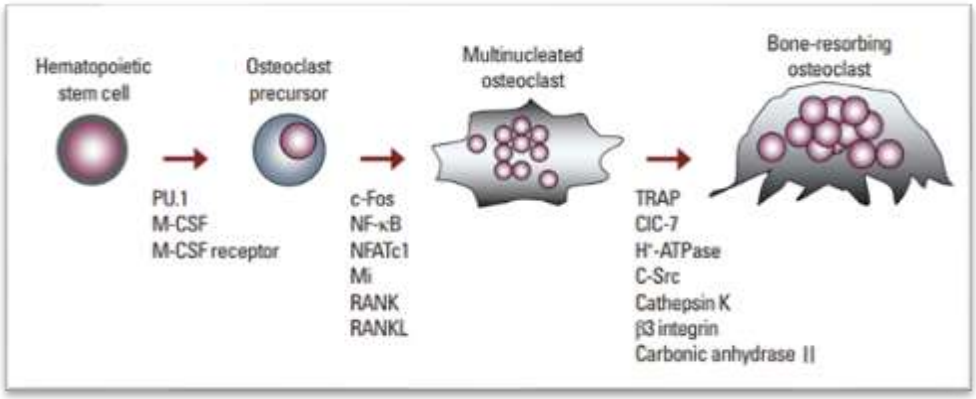

Image 1-13: Osteoclast differentiation procedure and its critical molecules
Source: (Lee, 2010)

$$[Ca_3(PO_4)_2]_3Ca(OH)_2 + 8\,H^+ \leftrightarrow 10\,Ca^{2+} + 6\,HPO_4^{2-} + 2\,H_2O$$

Hydroxyapatite (*solid*) (*solution*)

Image 1-14: Liberation of Hydroxyapatite calcium via acid secretion of H+ ions
Source: (Blair, 1998)

Osteocytes

Osteocytes comprise the majority of cells found in human skeleton (approximately 95% in adult bone), having dendritic shape. They communicate with their surrounding cells through cytoplasmic processes and gap junctions, called **canaliculi**, composed mainly from connexin (Aarden et al, 1994). Osteocytes have reduced cell organelles compared to osteoblasts (30% less volume for osteocytes and 70% for mature

osteocytes) (Palumbo, 1986). They possess multiple roles for bone tissue: 1) ensuring communication between different sites in the bone and extraosseous matrix 2) building mineral surface part while 3) offer repair capacity for regions deep inside on the bone (Aarden et al., 1994).

Growth factors

Growth factors (GFs) are proteins produced either by the bone cells or extra-osseous tissues, regulating various cellular functions and activities. Bone contains several growth factors, among bone morphogenetic proteins (**BMPs**), transforming growth factors beta (**TGFs-β**), insulin-like growth factors I and II (**IGF-I** and **IGF**-II), platelet derived growth factors (**PDGFs**), basic and acidic fibroblast growth factors (**bFGF** and **aFGF**), epidermal growth factors (**EGFs**), and tumor Necrosis Factors (**TNFs**) (Solheim, 1998). Most of growth factors are released as high molecular weight precursors that will produce active factors of lower molecular weight via a process called **proteolysis**. Growth factors bind to various receptors in cell surface where activate a **protein kinase** which in turn triggers the transcription of a gene into mRNA in order to be finally translated into proteins (Solheim, 1998). Some GFs contribute to bone formation while some others stimulate bone resorption as seen in Table 1.

Cytokines

Cytokines are also polypeptides produced in the lymphocytic and monocytic cells, supporting all cellular functions (e.g., immunological response and inflammation) (Kini & Nandeesh, 2012).. The most important cytokines in bone remodeling process given below (Table 1).

Table 1: Cytokines and growth factors (GFs) take part in bone remodeling process (Source: Kini and Nandeesh, 2012)

	Bone formation stimulators	Bone resorption stimulators
Growth factors	BMP-2, BMP-4, BMP-6, BMP-7,IGF-I, IGF-II TGF-b, FGF, and PDGF	TNF, EGF, PDGF, FGF, M-CSF, and GM-CSF
Cytokines	IL-4,IL-13, IFN, and OPG	IL-1, IL-6, IL-8, IL-11, PGE 2, PGE1, PGG2, PGI2, and PGH2

1.5. Bone Modeling and Remodeling

Bone is a highly metabolic tissue of human skeleton that changes throughout life. Approximately 10% of bone tissue is replaced each year

with complete renewal every 10 years. Normally, there is a balance between bone modeling and remodeling but if this balance staggers it gives rise to various consequences especially in bone matrix synthesis. Modeling may be increased in various bone remodeling abnormalities such as hypoparathyroidism, renal osteodystrophy, and treatment with anabolic agents. On the other hand, **bone remodeling** (or bone metabolism) is a process where mature old bone is removed, and new bone tissue is formed (via endochondral or intramembranous ossification).

1.6. Bone diseases, injuries and abnormalities

Bone fractures

A **fracture** can occur when the bone cannot withstand outside force and falls.. Fractures are classified in open and closed ones, where closed fractures are those in which the skin is intact, while open fractures involve wounds that communicate with the fracture. Also, fractures (both open and closed) can be further classified in 7 types as seen below (Image 1-15)

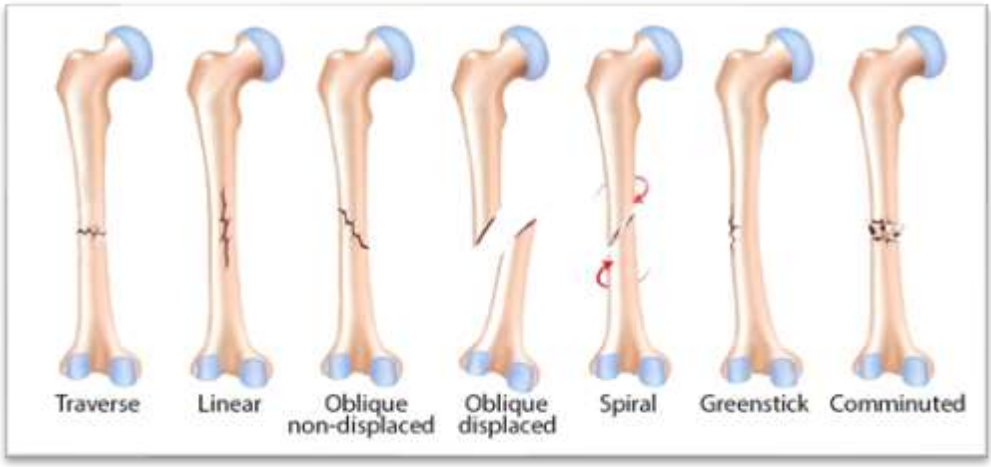

Image 1-15: Image 1 16: Classification of bone fractures (L to R) traverse fracture (fracture at a right angle to the long axis), linear fracture (fracture that runs parallel to the long axis of a bone), oblique fracture (occurs at an angle to the long-bone axis), spiral fracture (runs around the axis of the bone), greenstick (incomplete) fracture (only one side of the bone is broken), and comminuted fracture (a break or splinter of the bone into more than two fragments). Source: (https://orthopedic-institute.org/fracture-care/types-of-fractures/) [Accessed 21 Mar. 2019]

Osteoporosis

Osteoporosis is a metabolic bone disorder that defined as a skeletal disease, characterized by low bone mass, reduced bone mineral density (BMD), micro-architectural deterioration of bone tissue and alterations in the amount and variety of non-collagenous proteins inside bone tissue. Consequently, trabecular bone acquires a honeycomb-like shape with big holes increasing bone fragility and susceptibility to fracture.

Osteoporosis can be roughly categorized in primary osteoporosis which called postmenopausal of senile and secondary osteoporosis that occurs as a result of taking medicines known to cause bone matrix breakdown (e.g., corticosteroids), insufficient nutrition and chronic diseases. Bone densitometry or **DEXA** (dual-energy x-ray absorptiometry) imaging is the technique of choice to diagnose osteoporosis and monitor the response to treatment.

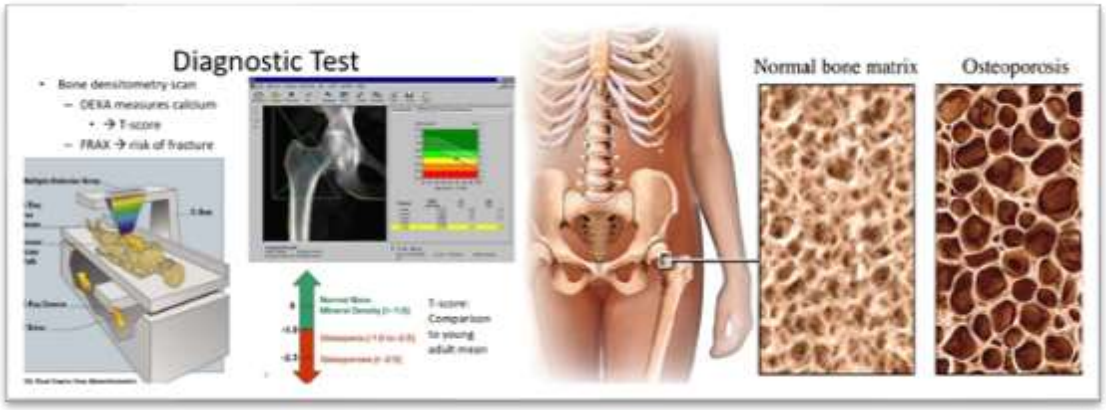

Image 1-16

Image 1-16: A) Bone densitometry (DEXA) in the assessment of osteoporosis (Available at: https://www.slideshare.net/lukelightning/osteoporosis-therapy-overview [Accessed 18 Jun. 2017]). B) Osteoporotic bone vs Healthy bone(Available at: https://www.researchgate.net/Image/The-decalcifi-ed-osteoporotic-bone_fig2_41485468 [Accessed 20 Mar. 2019])

Paget's disease

Paget's disease is a remarkable disorder of bone remodeling procedure. In this bone disorder, the osteoclasts become abnormally activated, mainly due to viral infection, led to excessive bone resorption followed by increased bone formation and drive into woven bone formation (Image 1-17). Paget disease is the second most common bone disorder (after osteoporosis) in elderly persons and estimated to occur in 2-3% of individuals in the U.S over age 60 (Siris, 1998). Paget disease may involve a single bone but frequently is more multifocal. Paget disease it is more common in axial skeleton bones, and the skull.

Image 1-17: Paget's disease of the bone (Available at: http://docteur.top/la-maladie-de-paget-obtenir-des-faits-sur-ce/ [Accessed 18 Jun. 2017)

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	Bone resorption	Bone formation
Osteoporosis	↑ ↑ [a]	↑
Glucocorticoid osteoporosis	↑	↓ ↓
Hyperparathyroidism	↑ ↑	↑ ↑
Hyperthyroidism	↑ ↑	↑ ↑
Paget disease[b]	↑ ↑	↑ ↑
Inflammation	↑ ↑	↓
Osteopetrosis[c]	↓ ↓	↑
Immobilization	↓	↓ ↓

[a] ↑ ↑, definitely increased; ↓ ↓, definitely decreased; ↑, transiently or variably increased; ↓, transiently or variably decreased.

[b] Some lesions may be largely osteoclastic, but most show increased osteoblastic activity as well.

[c] Increased formation is responsible only in rare cases.

Image 1-18: Bone remodeling in various bone disorders. Adapted from: (Raisz, 1999)

Osteomalacia/Rickets disease

Osteomalacia is a disorder marked by defective mineralization of the skeleton, resulting in incomplete mineralization of osteoid and as a result in soft or fragile bones. Finally, there is a decrease in Ca/PO_4 ratio, increase in alkaline phosphatase, and decrease in calcium excretion (Kini & Nandeesh, 2012).This disorder happens either due to insufficient amounts of vitamin D in the diet or there is any malabsorption of vitamin D. When the disease occurs in children, it is called **Rickets disease** and tends to produce intense skeletal deformities (bowed legs).

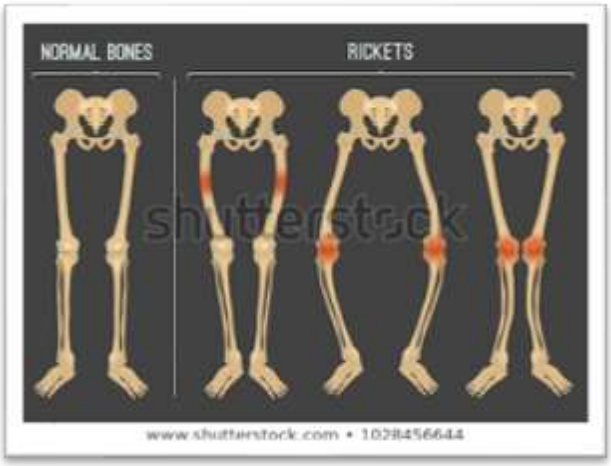


Image 1-19: Rickets disease. Source: (https://www.shutterstock.com/image-vector/normal-bones-versus-rickets-osteomalacia-types-1028456644 [Accessed 18 Aug. 2017])

Osteosarcoma

Osteosarcoma (**osteogenic sarcoma)** is the most common type of bone cancer. The cancer cells in this bone tumor are similar to bone cells, but the created bone tissue has not adequate strength and mechanical properties. Patients with osteosarcoma usually die from pulmonary metastatic disease.

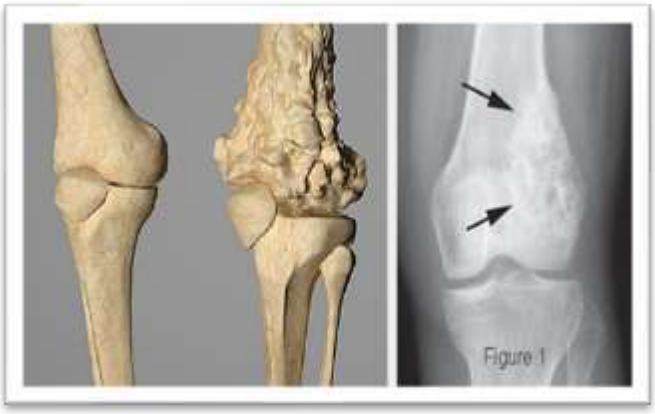


Image 1-20: Bone osteosarcoma images.

2. Tissue Engineering and Regeneration Medicine

2.1. Background and history

The artificial generation of tissues and organs, has been a field of intense study for all scientific fields. The first mention to “Tissue Engineering” is attributed to Fra Angelico, a renowned early Italian Renaissance painter. Fra Angelico in its famous painting entitled “Healing of Justinian” depicts Saints Damien and Cosmas transplanting a Homograft limb into a patient (Image 2-1).

Image 2-1: Fra Angelico’s painting “The Healing of Justinian by Saint Cosmas and Saint Damian”. Source: https://uploads0.wikiart.org/images/fra-angelico/the-healing-of-justinian-by-saint-cosmas-and-saint-damian-1440.jpg

In modern era especially in 80’s the term “tissue engineering” was attributed to the use of prosthetic devices and the surgical manipulation of human tissues (Vacanti, 2006). The term "**Tissue engineering**" had been firstly defined at a National Science Foundation workshop as "**the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes that restore, maintain, or improve tissue function**" despite the fact that this did not cover all aspects and discipline of tissue engineering. It was back in 1991 when the term of tissue engineering was first mentioned in an article entitled “Functional Organ Replacement: The New Technology of Tissue Engineering” in “Surgical Technology International” journal from Robert Langer and Joseph A. Vacanti. In the following years, more precisely in 1993, Langer & Vacanti

are the first who defined Tissue Engineering (TE) as "**an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function**" (Langer & Vacanti, 1993). This latter term is the most similar to tissue engineering as it is known today.

The purpose of tissue engineering is to establish a new clinical technology that makes possible medical treatment for diseases that have been too difficult to be cured by existing methods (Ikada, 2006). This field has also combined many discrete areas as a new therapeutic means that may overcome the limitations of current methods for artificial organs and transplantation methods applied.

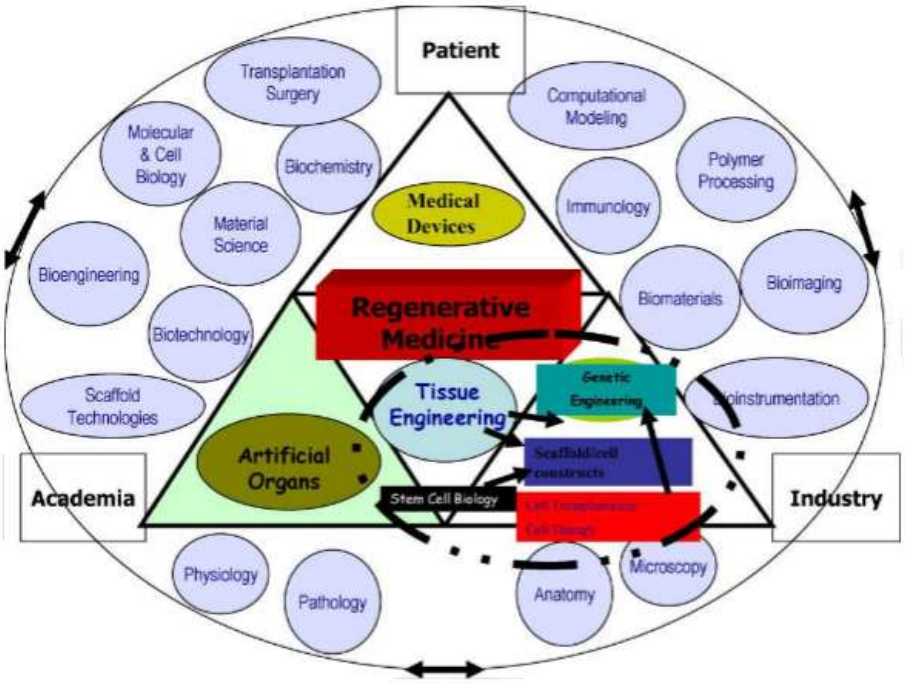


Image 2-2: Overview of multidisciplinary field of Tissue Engineering and Regenerative Medicine.

2.2. Early trials & applications of Tissue Engineering

The first experiments were made in 1970's by a pediatric surgeon at the Children's Hospital named W. Green. He tried to generate new cartilage using chondrocytes seeded onto spicules of bone and implanted in mice (Green, 1977). Despite the fact that, these experiments are not successful Dr. Green correctly assumed the importance of biocompatible materials in the generation of new tissue by seeding viable cells onto appropriate scaffolds. In 1981, Burke et al. tried to produce bilayer artificial skin composed of a proper collagen matrix to facilitate the growth of dermal fibroblasts. The artificial skin was a membrane made of distinct epidermal and dermal portions developed through extensive experiments for 10 years while the function of each portion physiologically resembled its counterpart in normal human skin (Burke et al., 1981). In 1991, a young patient suffer

from Poland Syndrome, a congenital malformation of the ribcage leads to the absence of sternum, became the first human to receive a tissue-engineered implant composed of a synthetic polymeric scaffold seeded with autologous chondrocytes. The whole procedure was held at the Children's Hospital in Boston and was performed from 3 out of 8 members of the founding governing board of the Tissue Engineering Society, Dr. J. Upton and Drs. J. and C. Vacanti (Vacanti, 2006). In 1998, Drs. Shuffelberg and J. Vacanti utilized a autologous cell-seeded coral scaffold in order to regenerate the distal phalanx of an amputated thumb (Santin, 2009). Despite the importance of all above mentioned examples, the settlement of tissue engineering as today known is attributed to Joseph Vacanti of the Children's Hospital and Robert Langer of MIT (Langer & Vacanti, 1993).

Image 2-3: (Left) Joseph Vacanti and (Right) Robert Langer

These two renowned scientists had the innovative idea not to seed cells directly into naturally formed scaffolds, characterized by unpredictable outcomes, but to design appropriate scaffolds for each case using biomaterials (Vacanti, 1988). Moreover, their mutual paper published in Science journal in 1993 is considered as the keystone of multidisciplinary field of Tissue Engineering.

Langer and Vacanti in their article discussed the foundations and future challenges of tissue engineering and its attempts to provide viable solutions to problems such as loss and failure of human organ or tissue. They divided tissue engineering in three main strategies (Langer & Vacanti, 1993):

1. **Isolated cells or cells substitutes** where native tissue cells having a specific function. This strategy shows numerous limitations including failure of infused cells and immunological rejection
2. **Tissue-inducing substances** where large-scale signal molecules, such as growth factors, are produced and delivered in target points inside human's organs or tissues
3. **Cells placed within properly formed matrices**

They also subdivided the third strategy in two approaches, called **open** and **closed bioreactor systems**. In the first case, closed systems, cells and matrix are kept isolated from external human environment by a membrane, called bioreactor, which facilitates the proper biochemical processes and

the perfusion of nutrients and wastes but at the same time protects the transplant from immunological rejection (e.g., from antibodies) as seen in (Image 2-4). As a result, viability of tissue transplant is assured.

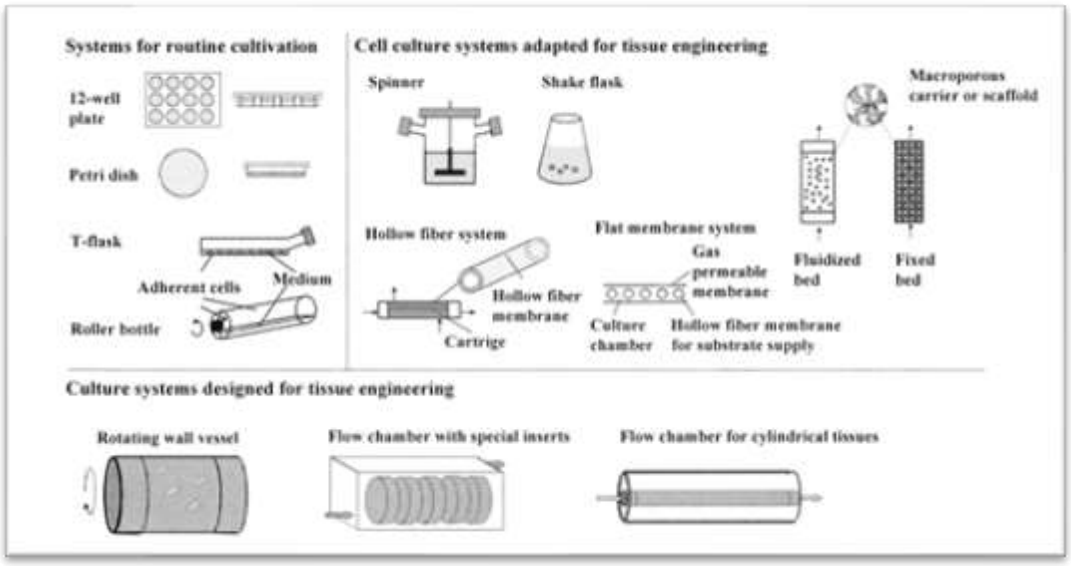


Image 2-4: Cell culture systems used in tissue engineering. Adapted from: (Pörtner et al., 2005)

In the second case, called open systems, cells are attached to matrices such as culture-dish systems or flasks and then incorporated into human's body (Image 2-5). The latter procedure is characterized by higher risk of cross-contamination, limited usefulness, while requires individual manual handling for medium exchange and cell seeding (Meyer et al., 2009). Despite the huge effectiveness of artificial bioreactors there are extensive limitations in creating the best micro-environmental conditions. As a result, the ultimate goal of true tissue regeneration will require the use of human body.

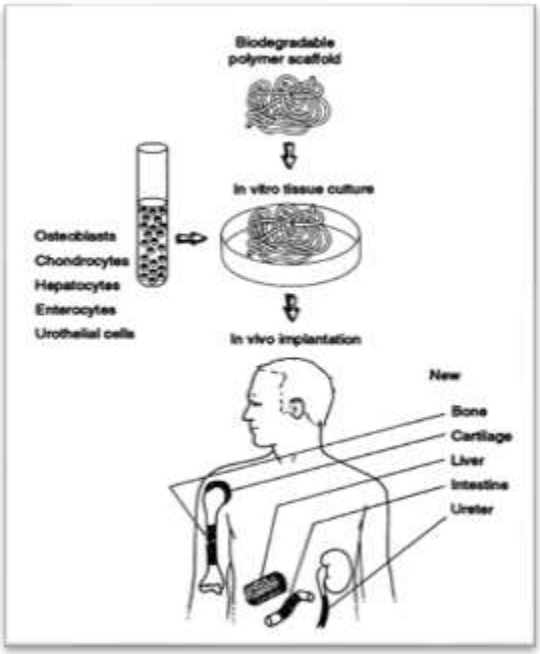


Image 2-5: Basic steps of open bioreactor system implants. Source (Langer, R. & Vacanti, J., 1993)

Tissue engineering was first introduced to the forefront of public awareness with a BBC broadcast from Dr. Charles Vacanti's laboratory at the University of Massachusetts where Vacanti and his colleagues created the renowned **Vacanti mouse**, fondly referred to as "**auriculosaurus**" (Image 2-6). This broadcast explored the potential of tissue engineering in generating new tissues and organs, which was considered impossible until then. Since that time, Tissue Engineering has been considered one of the most promising biomedical technologies of 21st century (Nerem, 1991).

Image 2-6: The infamous Vacanti mouse created by Charles Vacanti and his colleagues in the University of Massachusetts Medical Center and published in 1997. Source: (Cao et al., 1997)

2.3. The basic pillars of Tissue Engineering

The three key elements of every Tissue Engineering are: **cells**, **scaffolds** and various biomolecules e.g., **growth factors**.

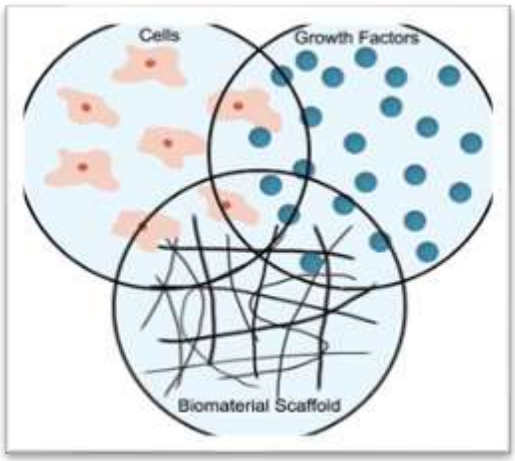


Image 2-7: The three basic pillars (cells, scaffolds, and growth factors) of every tissue engineering application.

Cells

One of the key elements in most TERM approaches is the use of a cell population in order to induce creation of new tissue through the interaction with the resident cells. The most important issue for the success of a tissue engineering application is the source of harvested cells. In TE there are numerous cell sources among them **stem cells**, **progenitor stem cells** and more contemporary **human embryonic stem (ES) cells** and **induced pluripotent stem cells (iPS cells)**.

Stem cells can be expanded indefinitely in culture and directed to differentiate into a particular cell type of interest depending on each application (e.g., bone cells, dermal cells). Adult stem cells are among the most thoroughly studied through the past years despite their limited expansion and high donor-dependency (Lanza et al., 2013). Progenitor cells differentiate from stem cells being able to generate only tissue cells of the same type. The difference between stem cells and progenitor cells is that the former can replicate indefinitely, whereas progenitor cells can divide only a limited number of times. Except from above mentioned cell types, in state-of-the-art tissue engineering applications we can find more types of cells (e.g., mesenchymal stem cells, hematopoietic stem cells, and adipose stem cells) which will be thoroughly described in a next chapter.

Scaffolds

Scaffolds are in sense biodegradable templates that offer the proper support acting as temporary substitutes of extracellular matrix (ECM) for cell growth/differentiation and finally tissue regeneration. Through the

last decades several biomaterials have been investigated including **polymers**, **ceramics** and **composites**. The desirable properties of scaffolds used in tissue engineering applications and techniques or scaffold fabrication and construction will be discussed thoroughly in the following chapters.

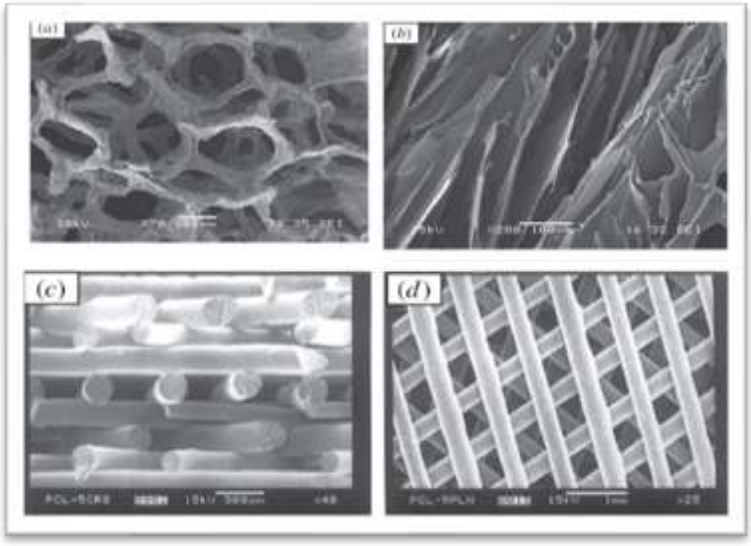


Image 2-8: SEM images of various biodegradable scaffolds

Growth factors

The aim of growth factors is to facilitate and promote cell differentiation, provoke vascularization and finally regenerate new tissue. However, the direct injection of growth factors into the regeneration site is not much effective. For this reason, properly formed carriers (e.g., scaffolds) can be used for the controlled release of growth factors at the desired site. Except from their supporting role, scaffolds can be used to deliver growth factors or drugs to the sites of repair expediting the recovery process and tissue remodeling.

2.4. Major scientific fields of tissue engineering in Biomedicine

Cardiac Tissue Engineering

Cardiovascular diseases (CVD) and more precisely heart infarction (HI) remains the leading cause of morbidity and mortality in developed Western-world countries (Hennekens, 1998; Maher et al., 1997). According to the American Heart Association, heart failure rate has increased from 5.7 million (2009-2012) to 6.5 (2011-2014) in adults Americans. In the same direction, the total costs of CVD diseases are expected to increase (approximately doubled) in the next two decades (Table 2).

In the UK, CVD accounts for 238 000 annual deaths, approximately 39% of all deaths per annum generating substantial socioeconomic costs. Heart attacks comprise the main cause of death in patients with CVDs. Heart attack or myocardial infarction (MI) denoted as the occlusion of one or more blood vessels supplying the heart with nutrients and oxygen. These vessels called coronary arteries (right coronary artery-RCA and left coronary artery-LCA). Cardiac tissue cells, called cardiomyocytes, are terminally differentiated, so they are unable to replicate after infarction or injury (Anversa et al., 2002), leading to the creation of scar tissue. The scar tissue created by infarcted zone finally loses its contractile, mechanical and electrical properties that had previously.

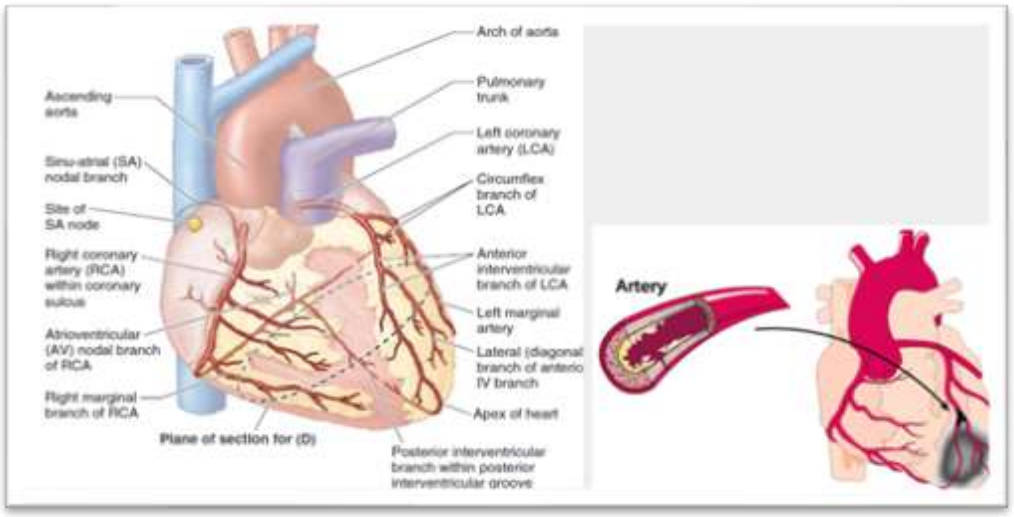
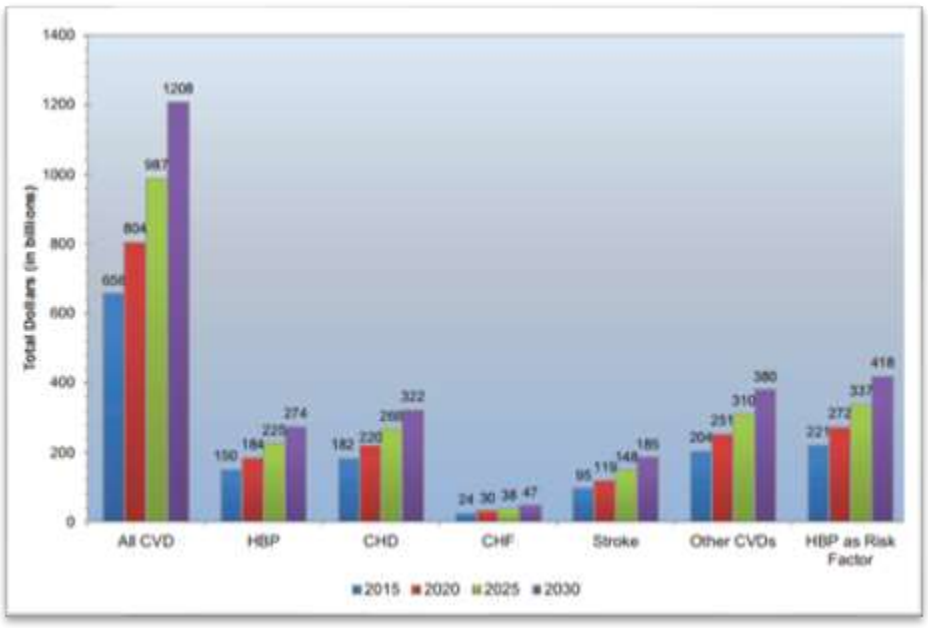


Image 2-9: Heart anatomy and infracted coronary artery. Adapted from: (Moore et al., 2015)

Table 2: Projected total costs of CVD, 2015 to 2030 (2012 dollars in billions) in the United States.



CHD indicates coronary heart disease; CHF, congestive heart failure; CVD, cardiovascular disease; and HBP, high blood pressure (Source: Heart Disease and Stroke Statistics—2017 Update: A Report from the American Heart Association)

One more challenge in cardiovascular diseases treatment is the controversial efficacy of current pharmaceuticals or device treatments and the severe shortage of heart donors increasing the gap between supply and demand for heart implant. Thus, there is an urgent demand for new methods to repair damaged heart tissue. A very promising alternative to current drug treatments is the implantation of exogenous cells with a procedure called cell-based therapy.

In the heart, cellular-based repair strategies can include: 1) direct transplantation of cells into damaged part of the heart 2) Tissue engineering techniques for tissue repairing and 3) therapies that invoke heart tissue regeneration. In the first approach specific types of healthy cells (principally pluripotent stem cells e.g., ESCs and iPSCs) are transplanted into scar heart tissue to repopulate the injured myocardium and offset the approximately 1 billion cardiomyocytes lost during myocardial infarction (Liau et al, 2012). The second approach based on tissue engineering principles as they have already denoted, it gives rise to a new field called cardiac tissue engineering. **Cardiac Tissue Engineering** (**CTE**) aimed to repair or regenerate a damaged section of the heart through the synthesis of a scaffold or patch made from a biomaterial combined with a specific type of cells (Leor et al., 2005).

Cardiac Tissue Engineering strategies can also be classified as in vitro and in vivo approaches. In vitro Tissue Engineering includes a proper culture dish or a bioreactor where the engineered cardiac graft, usually

a cell-seeded scaffold, is cultivated before the implantation into the scar cardiac tissue. This approach provides good control on shape, dimensions and properties of engineered grafts. On the other hand, in vivo cardiac tissue engineering approaches (or in situ cardiac generation) include the direct construction of replacement cardiac tissue in the natural environment of human body. Thus, this procedure is simpler, although the outcome is not the same satisfying due to the poor control on graft development.

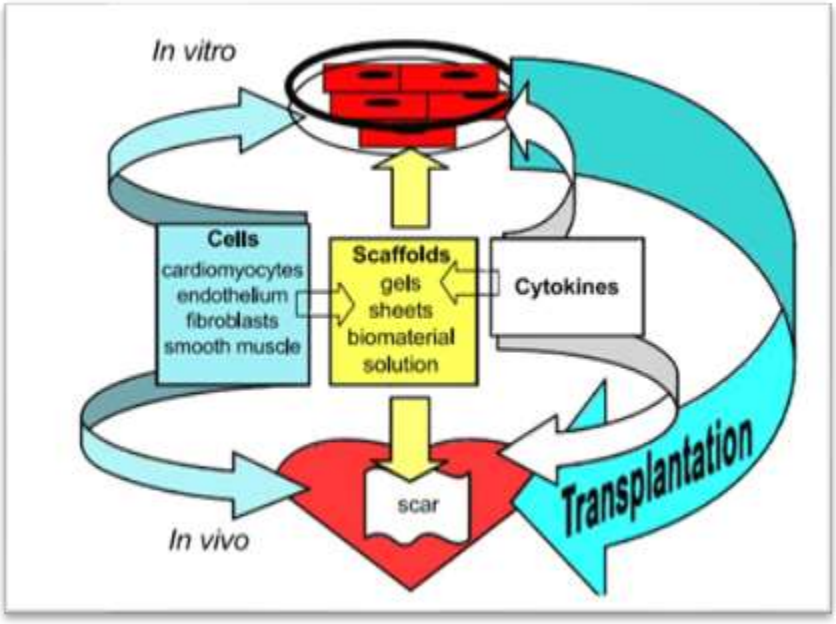


Image 2-10: In vitro and in vivo approaches in Cardiac Tissue Engineering (CTE). Adapted from: (Leor et al., 2005)

The cells used in cardiac tissue engineering are usually stem cells mainly because they offer unlimited proliferation capacity, while they can be easily expanded in culture-dish environment. On the other hand, the most important drawback of stem cells in CTE is their allogeneic character which require continuous immunosuppression. Among various types of stem cells used in CTE, the most significant sources for cells are **multipotent** and **pluripotent stem cells (ESCs, iPSCs)**. Unlike multipotent adult stem cells, pluripotent stem cells are theoretically capable of proliferating indefinitely inany kind of tissue type found in human body (Liau et al., 2012). Nevertheless, safety concerns have already raised for numerous types of cells.

Last but not least, one more significantly important aspect of cardiac tissue engineering is the properties of cardiac tissue constructs. Cardiac tissue replacement should exhibit phenotypic cardiac properties. The **preferred properties** include **proper vascularizatio**n after implantation, **flexibility**, **electrophysiological stability**, **robust mechanical characteristics**, ability to **contraction**, **stability** after

implantation, **non-immunogenicity** (Jawad et al., 2007 & Zimmermann et al., 2004).

	Autologous	Easily obtainable	Highly expandable	Cardiac myogenesis	Clinical experience	Safety concerns
'etal cardiomyocytes	No	No	No	Yes	No	No
'mbryonic stem cells	No	No	Yes	Yes	No	Yes teratoma
'keletal myoblasts	Yes	Yes	Depend on age	Debated	Yes	Yes arrhythmias
'rude bone-marrow cells	Yes	Yes	Depend on age	Debated	Yes	Yes calcification
'esenchymal stem cells	Yes	No	Depend on age	Yes	No	Yes Fibrosis calcificatio
'ematopoietic stem cells	No	Yes	Yes	Debated	Yes	No
'ibroblasts	Yes	Yes	Yes	No	No	No
'mooth muscle cells	Yes	Yes	Yes	No	No	No

Image 2-11: Advantages and limitations of various cell sources already utilized in CTE. Adapted from: (Leor et al., 2005)

Cartilage Tissue Engineering

Cartilage is a smooth elastic tissue that can be found as structural component at joints in the end of long bones (femur, tibia etc.). Yet, cartilage tissue exists in the trachea, bronchi, nose, ears, larynx and intervertebral disks. Due to its elasticity cartilage tissue is proper as skeletal tissue substitute in fetuses. Cartilage tissue is classified in three types: **hyaline**, **elastic** and **fibrocartilage** depending on the amount of intercellular space and proportion of specified cells.

Due to its lack of sufficient blood supply, articular cartilage has limited capacity of repair and remodeling in case of injury. As a result, even minor injuries may lead to progressive damage and gradual joint degeneration. Major repair techniques for cartilage injury include resection of damaged tissue, mosaicplasty, marrow stimulation, autologous chondrocyte implantation (ACI) and total joint arthroplasty as a technique of choice in patients with diffuse articular cartilage injury.

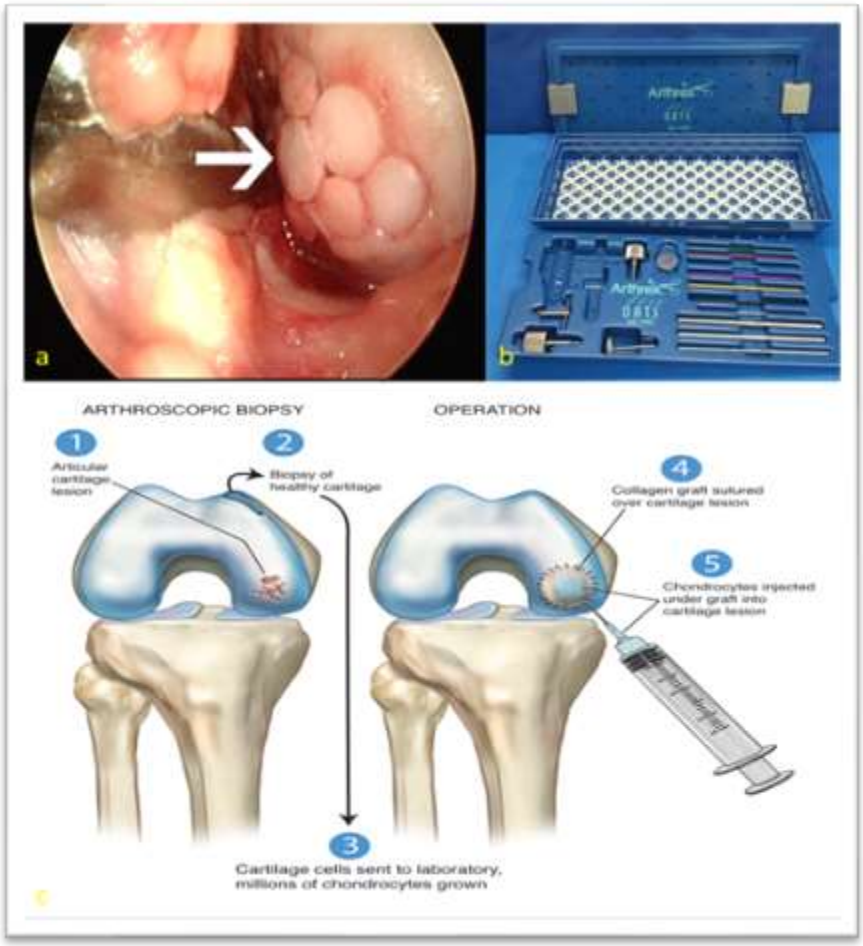


Image 2-12: A) Mosaicplasty cartilage repair technique using B) Arthrex Osteochondral Autograft Transfer System (OATS®) C) Autologous chondrocyte implantation (ACI) technique in cartilage defect site.

Chondrocytes, the resident cells in articular cartilage, are metabolically active cells and their shape vary among the different regions of articular cartilage (e.g., chondrocytes found in the surface zone are flatter than ones in deep articular zone).

In the last decades Cartilage Tissue Engineering (CTE) field has emerged as a viable solution for cartilage limited capacity of self-repair. In order to accomplish repair of cartilage, scientists incorporate cells from various cell sources into proper biomaterial scaffolds, with or without growth factors, which can provide structural support in the defective cartilage area. Among the most well-studied cell sources we can find **chondrocytes**, **mesenchymal stem cells** (**MSCs**) from different sources, **induced pluripotent stem cells** (**iPSCs**) even **fibroblasts**, though the two most clinically applicable cell types in cartilage tissue engineering are chondrocytes and MSCs (Liu et al., 2017). The use of chondrocytes in tissue engineering application shows some limitations such as the need for culture expansion because the harvested cells are insufficient for repair purposes, morbidity at donor sites due to injury of harvested tissue etc.

On the other hand, MSCs can effectively alleviate the limitations of chondrocytes. MSCs can be harvested from a number of sources (bone marrow, adipose tissue, periosteum and perichondrium) and differentiate into numerous cell types such as chondrocytes, fibro-chondrocytes and hypertrophic chondrocytes depending on application's needs.

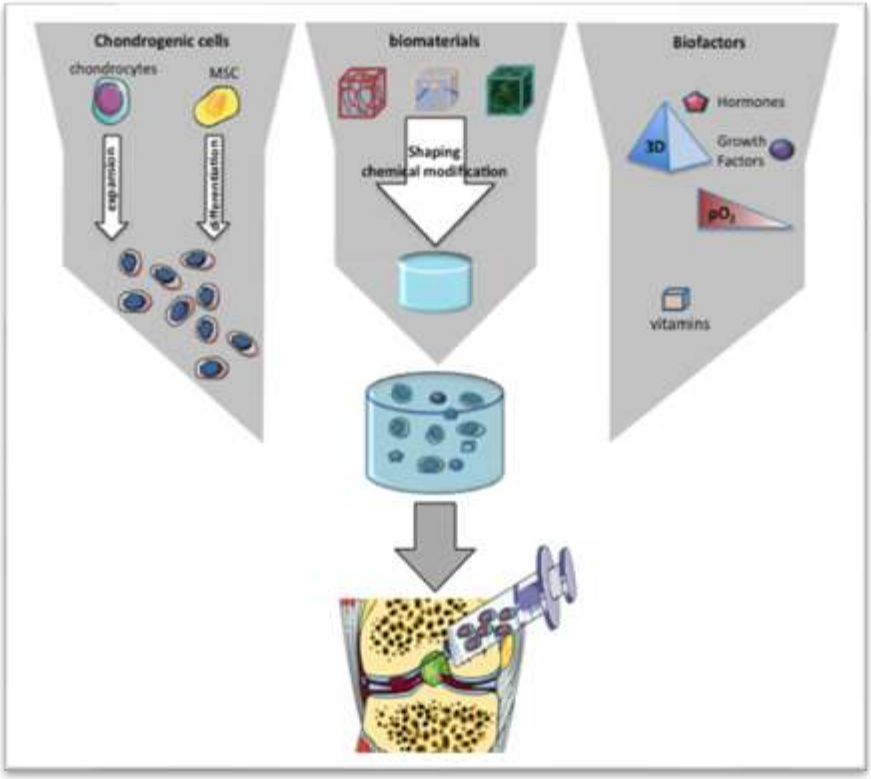


Image 2-13: Basic pillars of Cartilage Tissue Engineering and their collaboration to cartilage repair process Adapted from: (Vinatier & Guicheux, 2016)

As regards, scaffolds or biomaterials used in Cartilage Tissue Engineering, they should meet some criteria: 1) mechanical properties consistent with those of existing cartilage 2) gradual integration with adjacent cartilage 3) durability throughout the whole lifespan and as any other scaffold they should be 4) biocompatible and 5) biodegradable at similar rate as ECM deposition to the site (Bernhard et al., 2016).

Cartilage Tissue Engineering Scaffolds can be classified according to their matter state into **hydrogel scaffolds** and **solid scaffolds**. The use of Hydrogel scaffolds in CTE has already shown numerous advantages such as similar mechanical behavior to native articular cartilage, proper viscoelasticity for cartilage mechanical loading etc. (Liu et al., 2017).

On the contrary, solid scaffolds are subdivided into natural and synthetic scaffolds according to their source. **Natural scaffolds** such as

collagen-based scaffolds, chitosan, agarose, alginate, fibrin glue, hyaluronic acid and small intestinal submucosa (SIS) have already extensively studied appearing the benefits of nontoxicity and proper biofunction. Yet, they are characterized by limitations like potential pathogen transfer. **Synthetic scaffolds** show an advantage over natural scaffolds due to their flexibility in design, tunable mechanical and chemical properties, and absence of possible disease transmission. Nevertheless, synthetic scaffolds such as PLA[1], PGA, PLGA, PCL, PEO and PEG are characterized by poor biocompatibility and probable inflammatory response to the host site.

Material source	Examples	Advantages/disadvantages
Natural	Collagen based	Advantage: providers of molecular cues to the cells, stimulating them to produce more collagen. Disadvantage: poor mechanical properties and can undergo contraction due to interactions with cells when not combined with other materials.
	Hyaluronic acid	Advantage: bioactive properties, with the ability of interacting with chondrocytes (via CD44). Disadvantage: poor mechanical properties of the unmodified hyaluronic acid; the combination or engraftment with other materials, such as polyethylene glycol or dextran, allowed optimization of the biomechanical properties of the hyaluronic acid-based scaffolds.
	Fibrin glue	Advantage: extensively used for wound healing and, additionally for its use as fixative for scaffolds to native tissue. It can also be used as a matrix. Disadvantage: enhancement of cartilage repair is limited.
	Chitosan, agarose and alginate	Advantage: used as either hydrogels, sponges or pads. Disadvantage: still not available for clinical applications.
Synthetic	Polylactide, polyglycolide, polyethylene glycol and polyurethane	Advantage: no batch variation. Disadvantage: possible inflammatory response against degradation products.

Image 2-14: Principal materials and their properties used in Cartilage Tissue Engineering. Adapted from: (Moreira-Teixeira et al., 2011)

Last but not least, numerous cartilage tissue engineering applications have utilized growth factors to stimulate the development and homeostasis of articular tissue. Among various growth factors that have already been used in cartilage tissue engineering processes, IGF-I[2], TGF-b1, BMP-2, -7 and FGF-18 have been shown anabolic activity in the production of extracellular matrix (ECM). On the contrary, growth

[1]Polyglycolic acid (PGA), Polylactic acid (PLA), Poly(lactic-co-glycolic acid) (PLGA), Polyethylene glycol (PEG), poly-3-caprolactone (PCL) polyethylene oxide (PEO)

[2] Transforming growth factor-b1 (TGF-b1), Bone morphogenetic protein (BMP), Insulin growth factor I (IGF-I), fibroblast growth factor (FGF), Platelet-derived growth factor (PDGF)

factors such as TGF-b2, have been found to have both stimulating and inhibitory effects on cartilage remodeling (Fortier et al., 2011).

Bone Tissue Engineering

As already mentioned, bone is responsible for body support and protection of vital internal organs . Bone consists of an internal part called cancellous bone with high porosity (50-90%) and an outer part called cortical bone that characterized by higher mechanical strength and lower porosity (10-30%). Bones in human body can be categorized into long (e.g., femur, tibia, ulna, etc.), short (e.g., phalanges, carpus, tarsus, etc.), flat (e.g., skull, sternum, hip, ribs, etc.) and irregular (e.g., vertebrae, sacrum, mandible, hyoid, etc.). Bone tissue has the potential to regenerate and repair itself after various bone injuries and damages. Yet, in pathological fractures or large bone defects bone self-repair is insufficient, so alternative bone reconstruction techniques are needed.

Today's gold standard technique for repair of large bone defects involves bone grafts. Bone grafts are implanted materials that harvested from various bone sources (iliac crest, fibula, distal radius etc.) promoting bone healing process. It was estimated that five hundred thousand surgical cases with bone grafts are performed annually around the world with total costs more than $2.5 billion (Amini et al., 2012). As any other biomaterial used in human's body environment, bone grafts should ideally fulfill the characteristics of osteogenesis, osteoinductivity, osteoconduction and osseointegration.

- **Osteogenesis** comprises the production of new bone by the osteoblasts through differentiation of native bone osteoprogenitor cells.
- **Osteoinduction** referred to the ability of grafts to promote bone-forming procedures via differentiation of multipotent mesenchymal stem cells (MSCs) of the surrounding host tissues. After that, MSCs produce osteoprogenitor cells (preosteoblasts) followed by development of osteoblasts. Growth factors of different families (like BMP, TGF, FGF, IGF and PDGF) has already established for their osteoinduction properties.
- **Osteoconduction** denoted as the property of a biomaterial (e.g., bone graft) act as permanent and resorbable scaffold, but simultaneously permits bone growth on its porous surface (Albrektsson & Johansson, 2001). Finally, osseointegration was defined as the direct contact, in microscope level, between living bone and bone graft without any intervention of fibrous tissue (Brydone et al., 2010).

Bone grafts are classified into **autografts**, **allografts,** and **xenografts** according to their harvesting source as seen below (Image 2-15).

Autografts or autogenous bone grafts remain the gold standard for bone graft techniques due to their proper osteogenic, osteoinductive and osteoconductive properties and lack of immunogenicity which enhance the chances of graft incorporation into defect site. The limitations of them concern donor site morbidity, pain and need for additional surgery (Oryan et al., 2014). On the contrary, **allografts** characterized by osteoinductive and osteoconductive properties, lack of donor site morbidity and availability in various sizes and shapes. Yet, they present drawbacks such as the lack of osteogenic property, probable disease transmission, increased cost and low heal rate.

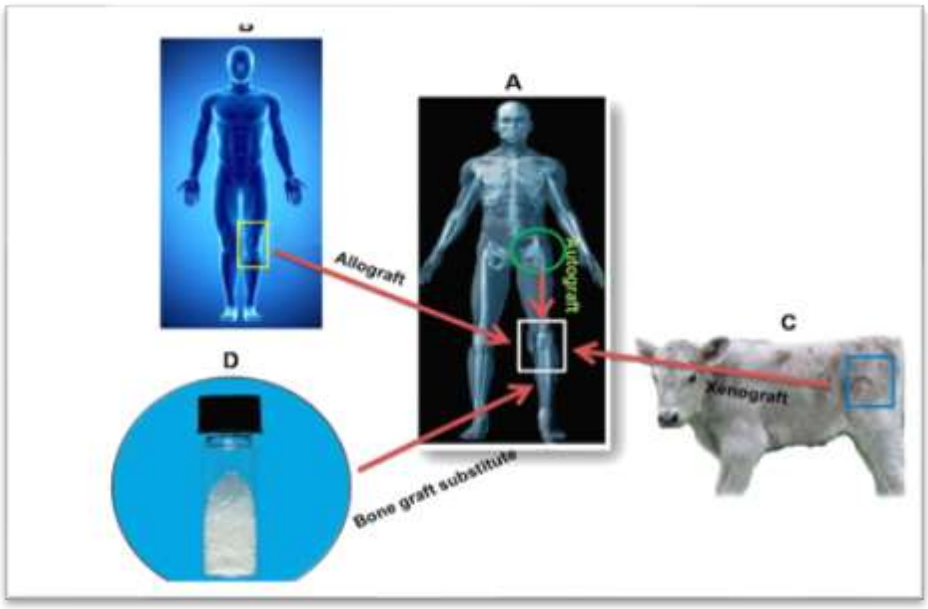


Image 2-15: Various bone graft sources. Adapted from (Oryan et al., 2014)

Finally, **xenografts** or xenogeneic grafts are harvested from a donor of one species (e.g., bovine, porcine etc.) and grafted into a recipient of another species (e.g., human). This kind of grafts presents benefits like high availability and low cost, while they are linked with limitations like transmission of zoonotic diseases and poor osteogenic ability (Oryan et al., 2014). Others common bone repair techniques are distraction osteogenesis, bone cement fillers and stand-alone bone morphogenetic proteins (BMPs) (Amini et al., 2012). Despite the importance and wide use of all previously mentioned techniques in bone defects repair, none possess all ideal characteristics e.g., high osteoinductivity, angiogenesis, biological safety, low cost, high availability and no size restrictions.

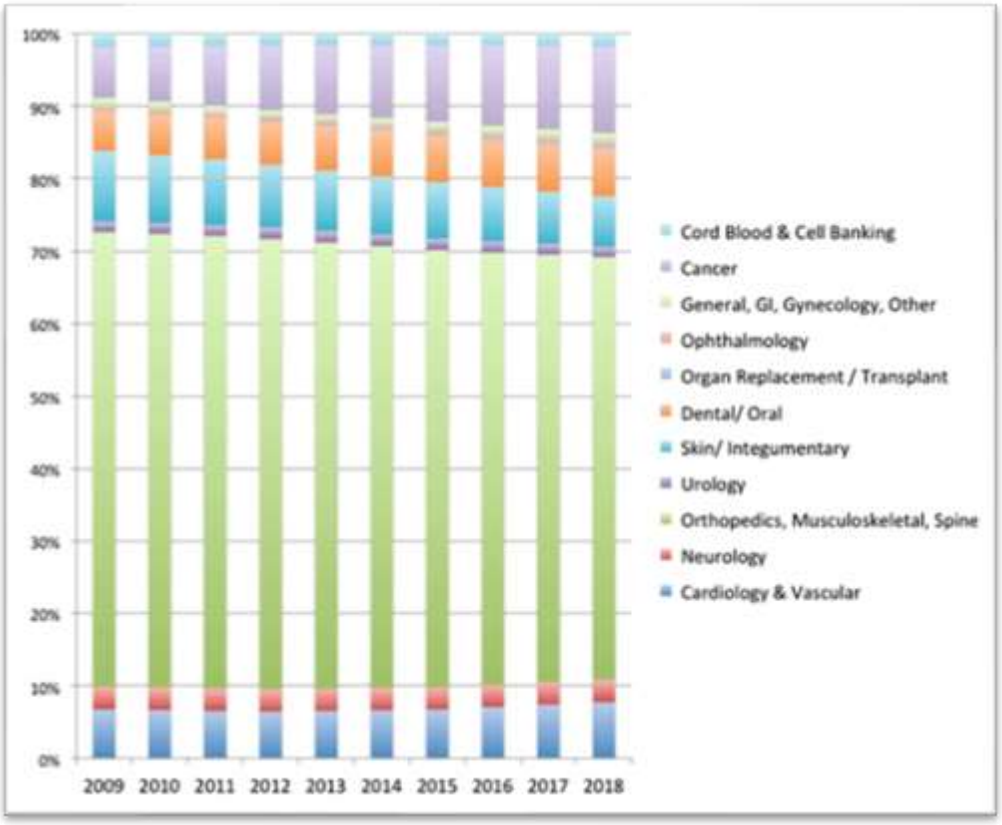


Image 2-16: Distribution of Cell therapy & Tissue Engineering revenues by clinical are for years 2009-2018. Adapted from: (Li & Mai, 2017)

Bone Tissue Engineering (BTE) possesses a potential alternative, alleviating all previously mentioned issues and limitations. This field **aims to induce new functional bone tissue through the synergistic combination of biomaterials, cells and healing promotive factors** (Amini et al., 2012). More specifically, a biomaterial scaffold mimicking the natural bone extracellular matrix (ECM) ,while provides the proper support for bone remodeling. These scaffolds should meet some criteria like **high porosity**, **suitable pore size** and **shape, hydrophilicity and hydrophobicity** where needed (Oryan et al., 2014).

Scaffolds in BTE applications are classified in two big families: natural or organic and synthetic or artificial biomaterials that will be thoroughly examined in following chapters. One more vital issue in Bone Tissue Engineering are the cells which enhance the bone regeneration procedure and finally lay down fresh-formed bone matrix in the defect site. Various types of stem cells comprise the most promising sources for bone tissue engineering. Last but not least, morphogenetic signals (growth factors) are used to direct the cells to the phenotypically desirable type and accelerate the whole bone-heal process. In the following chapters the basic pillars of Bone Tissue Engineering scientific

field (cells, scaffolds and growth factors) are thoroughly described and analyzed.

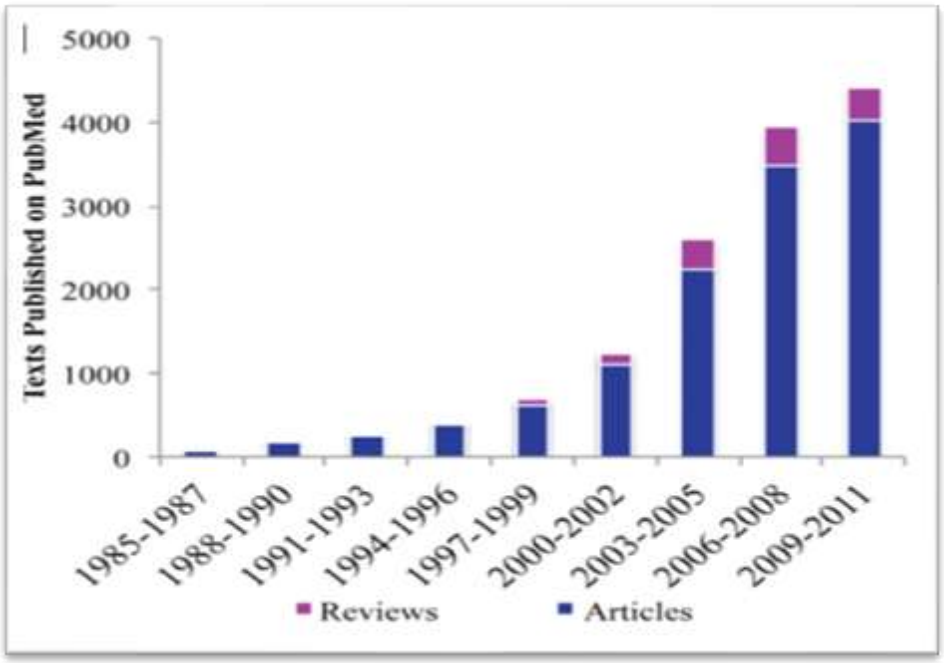


Image 2-17: Published articles on Bone Tissue Engineering scientific field from mid-1980s until 2011 in PubMed database presents the high potential of BTE in bone healing processes. Adapted from (Amini et al., 2012)

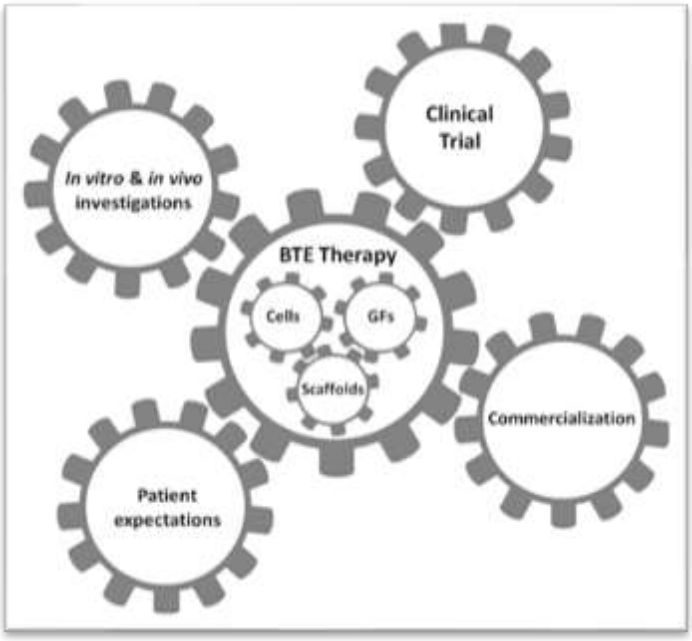


Image 2-18: Schematic representation of challenges in Bone Tissue Engineering applications. Adapted from: (Roseti et al., 2017)

Pancreas Tissue Engineering

Pancreas is a human organ consists of two separated functional units, the exocrine and endocrine pancreas. The exocrine part of pancreas occupies approximately 95% of pancreatic tissue mass and composed of acinar and ductal cells responsible for the synthetization of proper digestive enzymes and secretion of them into duodenum through a tube-like network of pancreatic ducts.

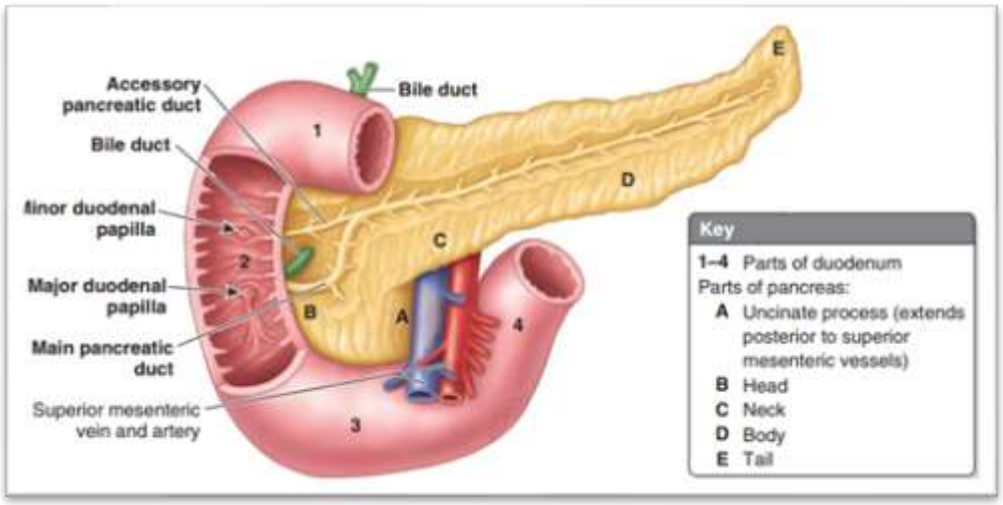


Image 2-19: Sectional Anatomy of pancreas. Adapted from (Moore et al., 2015)

Endocrine part of pancreas consists of various pancreatic polypeptide cells that form islets called **islets of Langerhans** after their discovery in 1869 by German pathological anatomist Paul Langerhans. Approximately 80% of exocrine pancreas cells found in each islet, are β-cells that secrete blood sugar-regulating hormones into the bloodstream. The most important β-cell secreted hormone called insulin and regulates the glucose uptake. Despite the fact that other pancreatic cell types (α, γ, δ and ε) are found in minor quantities, they also secrete important hormones like glucagon (alpha cells), somatostatin (delta cells) and pancreatic polypeptide (pancreatic polypeptide cells) (Nir and Dor, 2005).

The destruction of β-cells by the immune system causes an autoimmune disease known as Diabetes Type 1 or Type 1 diabetes mellitus (T1DM). **Diabetes Type 1** can be found only in 5-10% of people having diabetes disease. On the contrary, **Diabetes Type 2** is not an autoimmune condition, while is associated with peripheral resistance to insulin and impaired insulin secretion (Nir & Dor, 2005). According to the American Diabetes Association, approximately 1.4 million Americans are diagnosed with diabetes each year, while the total number of diabetic people worldwide is expected to rise to 366 million in 2030, so a viable treatment for diabetic patients is crucially important. Existing therapeutic techniques for diabetic patients include direct insulin injection, pancreatic islets transplantation, autologous stem cell transplantation and finally whole pancreas transplantation (Takahashi et al., 2016). However, all previously mentioned repair techniques are associated with numerous limitations. For example, insulin injection despite it is among the most used therapies for diabetic patients, it also has limitations like hypoglycemic attacks and skin stiffness. Pancreatic islets or whole pancreas transplantation possess viable solutions to diabetes disease but, they suffer from limitations like severe lack of donors, high cost and high risk of immunosuppressive agents that used to prevent transplant rejection (Naftanel & Harlan, 2004).

As a result, tissue engineering may be a promising way to alleviate all above mentioned limitations. As any other tissue engineering application, **pancreas tissue engineering (PTE)** includes proper cells and biomaterial scaffolds with or without growth factors.

Cells that used in PTE application include stem/progenitor cells of the ductal epithelium, adult ductal or acinar cells, bone marrow stem cells and hepatic cells. However, the most promising cell sources are, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells harvested either from mouse or human. Scaffolds that are utilized in pancreatic TE applications in order to construct 3D cultures divided into: natural ones (e.g., collagen, chitosan and Matrigel™) and synthetic scaffolds (e.g., acrylonitrile copolymers, PEG and fibrin hydrogel). Among the most commonly used scaffolds for such applications,

Matrigel™ is a gelatinous protein mixture that is extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma and composed of approximately 60% laminin, 30% collagen IV, and 8% entactin, while it is commercially available by Corning Life Sciences. Additionally, to scaffolds, numerous pancreatic tissue engineering applications use proper biomolecules or growth factors (e.g., VEGF) to accelerate the pancreatic tissue remodeling process and promote the functionality of fresh-formatted tissue.

Vascular Tissue Engineering

Blood vessels consist the basic element of the circulatory system of human body. They are served as conduits delivering oxygen and nutrients to, and waste products away from, tissues while maintain a balance in blood distribution. Blood vessels range in size including micro vessels (<1mm), small vessels (1-6 mm) and large vessels (>6 mm) (Chang & Niklason, 2017). The major types of blood vessels that can be found in human body are: 1) arteries that carry oxygenated blood from heart 2) capillaries that perform the exchange of substances between blood and cells and 3) veins that carry deoxygenated blood from the capillaries back to the heart. Blood vessels consist of three distinct layers: an inner layer called **Tunica Intima**, an intermediate layer called **Tunica Media** and the outer layer called **Tunica Adventitia**.

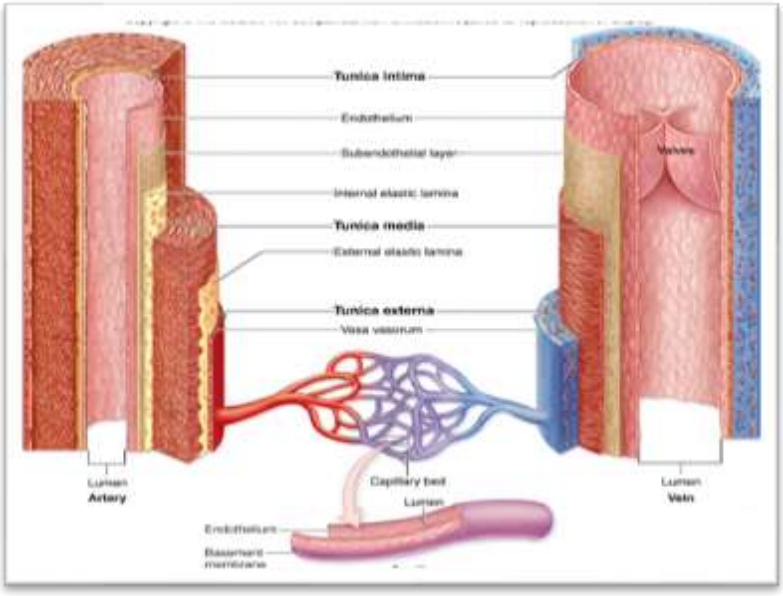


Image 2-20: Anatomy of human body's blood vessels. Source: (http://mysciencevirtualclass.blogspot.com/2011/02/blood-vessels.html [Accessed 22 Mar. 2019])

Despite we referred to **Vascular Tissue Engineering** field as a distinct field, it is usually integrated in almost all tissue engineering applications (bone, cardiac, muscle, skin etc.). The creation of proper microvascular network plays crucial role in every tissue engineering application because the freshly formatted tissues, especially those beyond 200 μm

(oxygen diffusion limit[3] in tissues), require the formation of new blood vessels to maintain cell viability providing nutrients and oxygen, while remove metabolites and other wastes.

Vascularization of tissues occurs via two distinct mechanisms: **vasculogenesis** and **angiogenesis**.

Vasculogenesis is de novo formation of blood vessels through the migration and differentiation of endothelial precursor cells (angioblasts) in response to local cues (such as growth factors and extracellular matrices) during early embryonic development.

On the other hand, **angiogenesis** referred to the creation of small vessels (capillaries) utilizing existed blood vessels.

The major **strategies** that utilized in order to accomplish the proper **vascularization of engineered tissue** are:

1) **scaffolds with ideal properties** (pore size, pore interconnectivity etc.),
2) 2) **direct delivery of angiogenic factors to the damaged site** and
3) 3) **in-vitro** or
   4) **in-vivo pre-vascularization** (Castells-Sala et al., 2013).

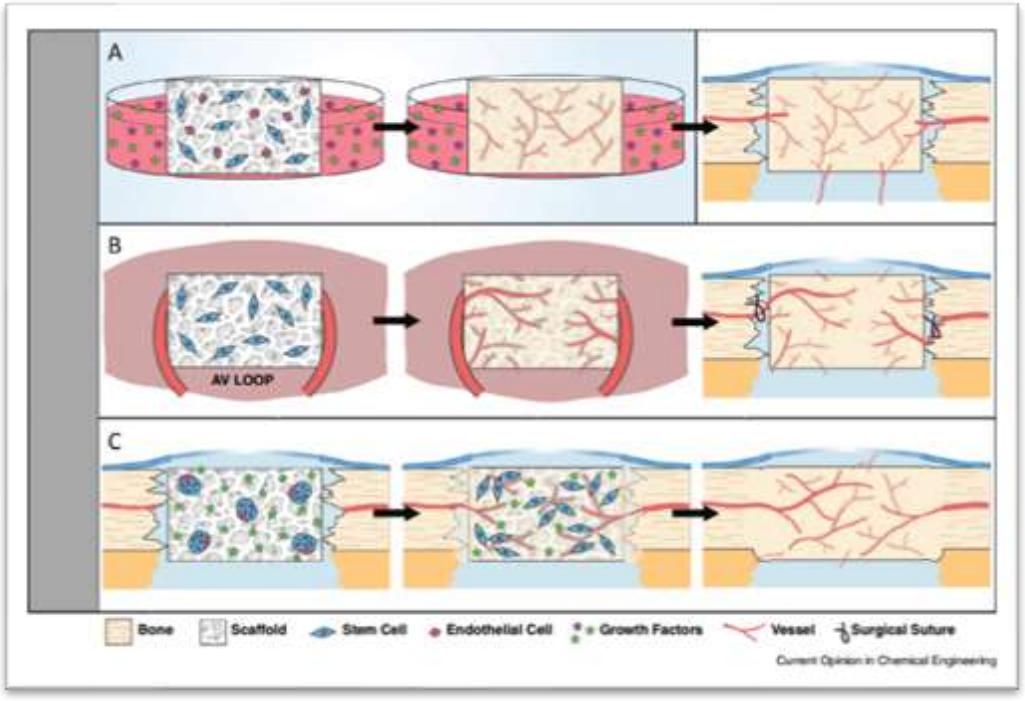


Image 2-21: Vascularization approaches for tissue engineering. A) In vitro pre-vascularization B) In vivo pre-vascularization C) scaffold-based vascular-induced

[3] Diffusion limit is denoted as the maximum distance from the nearest capillary in which oxygen it is able to diffuse. This value for oxygen in human tissue vary between 100-200 μm

technique with proper angiogenic factors adherent to them. Adapted from: (Hutton & Grayson, 2014)

Scaffold-based strategies include the use of 3D scaffolds with ideal properties such as pore size for faster blood-vessel ingrowth and pore interconnectivity that induce cell migration (Rouwkema et al., 2008). The most well-studied biomaterials are natural ones like decellularized matrices, collagen sponges and synthetic products (PGA, PCL and other widely used polymers in TE).

On the other hand, the delivery of angiogenic factors or molecules directly to the site of interest stimulates the mobilization of endothelial (progenitor) cells and thus accelerates the angiogenesis in native tissue. The most promising molecules that have thoroughly studied are VEGF[4], bFGF, PDGF, and BMP-2, -4 or -6. Another strategy for enhancing vascularization in tissue engineering applications called in-vivo prevascularization (Image 2-22). This strategy consists of the implantation of a tissue-engineered construct into a highly vascularized bed, such as muscle or within an arteriovenous (AV) loop. In the following weeks (called vascularization period), a microvascular network which, supplied with blood by the arteriovenous loop, will be formatted around the engineered construct. This technique shows advantages such as the direct perfusion after implantation and limitations like the need of two separate surgeries, one to implant the construct at the vascularization site and another one to implant the construct at the final defect site.

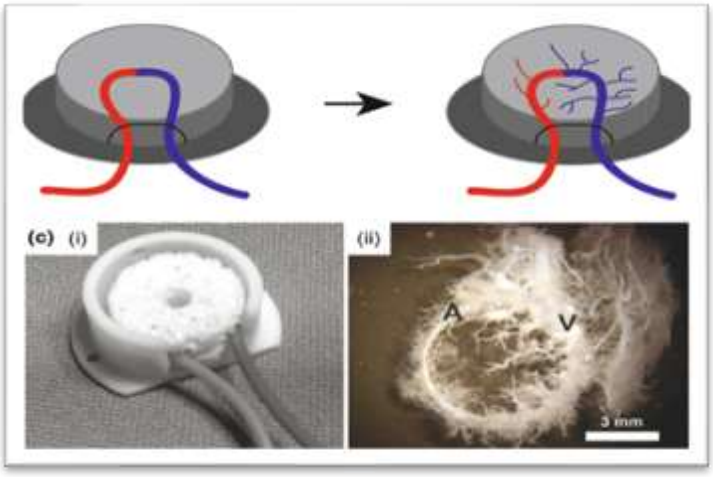


Image 2-22: In vivo prevascularization technique. (Top) schematic illustrations of construct vascularization around the arteriovenous loop. (Bottom) highly vascularized construct that was obtained 8 weeks after implantation. Adapted from: (Rouwkema et al., 2008) and (Chang & Niklason, 2017)

[4] Sonic Hedgehog Homolog (SHH), basic fibroblast growth factor (bFGF), Platelet-derived growth factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Placental growth factor (PIGF)

The last technique, called in vitro prevascularization, is based on the observation that endothelial cells are able to form prevascular structures under the right culture conditions in vitro. The main advantage of this technique is the fact that it is not rely on vessel ingrowth of host, avoiding the need for extra surgery. On the contrary, the main limitation is the slower rate of anastomosis compared to in vivo prevascularization strategy.

2.5. Regenerative medicine background and history

In the late 1990s, **Regenerative medicine** has been defined as **"the process of replacing or regenerating human cells, tissues or organs to restore or establish normal function"** (Mason & Dunhill, 2008). Regenerative medicine is a field emerged from the interdisciplinary activities of tissue engineering community and research in stem cell biology (Santin, 2009). Although regenerative medicine and tissue engineering seems to belong in the same field, the restoration of tissue is accomplished in very different ways respectively.

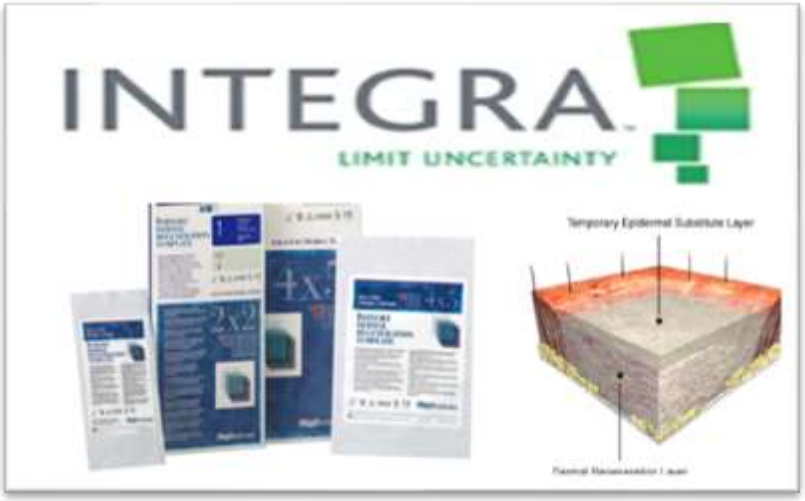
Regenerative medicine based on strategies, such as **cell-based therapies**, **gene therapy, and immunomodulation**, **gene therapy and nanomedicine**, in order to provoke organ restoration and regeneration. Moreover, it includes methods and strategies that may utilized non-traditional tools used in the field of tissue engineering (Santin, 2009).

Due to similar objectives tissue engineering and regeneration medicine have been merging in the following years creating the broad field of **Tissue Engineering and Regenerative Medicine** (**TERM**). As previously mentioned, cells (especially somatic, adult-stem or embryo-derived) have played a crucial role in the progress of regenerative medicine through past years, while hold great promise for the future of it. On the other hand, numerous ethical and technical issues raised, as regards the use of human embryo-derived cells for regenerative medicine purpose (Mason & Dunnill, 2008).

2.6. Market products and commercialization efforts

Around 90s stem cells research field has already been in public awareness, so TERM industry began to emerge possessing a viable state-of-the-art solution for bioindustry. Although small compared with medical device and pharmaceutical industries TERM industry, as called, holds its own market share since 1994, including hybrid products with characteristics of both drugs and devices (Lysaght, 1995). The first widely available tissue engineering products were skin substitutes and more precisely living skin replacements. The first product called Integra Dermal Regeneration Template manufactured by Integra Life Sciences

and cleared by FDA in 1996. Next in turn was **Transcyte®** marketed by Advanced Bio Healing Inc. and introduced as an acellular dermal substitute product cleared for medical use by FDA back in 1997. It is typically used to treat second degree burns that are expected to heal on their own.



Dermagraft® on the other hand was a dermal equivalent made from dermal fibroblasts derived from foreskin, also manufactured by Advanced Bio Healing Inc., but it also used to treat diabetic foot ulcers. Dermagraft® finally cleared by FDA in 2001. One more skin substitute product named **Apligraf**® was made with collagen including both a dermal equivalent and an epidermis by human keratinocytes manufactured by Organogenesis and cleared by FDA for venous leg ulcers in 1998 and diabetic foot ulcers applications in 2001. Last but not least, Vericel Corp. manufactured **Carticel**® for cartilage replacement and **Epicel**® for skin replacement cleared in 1997 and 2007 respectively. Except from all above-mentioned market products there are also many more as seen in Image 2-25.

From cumulative number of units manufactured and patient treated we can estimate the current value of regenerative medicine cell therapy market in the order of **100-200 million** dollars **per annum** with growth rate approximately 22.5% (Mason & Manzotti, 2010). Among all above mentioned TERM market products, the largest contributors in today's market value of scientific field are: Apligraf® and Infuse®, a graft-shaped recombinant bone morphogenetic protein product produced by Medtronic (Image 2-23).

As regards the private sector activity, by 1994 there were $246 million, 40 business units mainly in skin substitute area and 1500 employees working there. By 1997, there was approximately no change in active companies and business units compared to former data, but private sector activity had almost doubled to $453 million and employee number

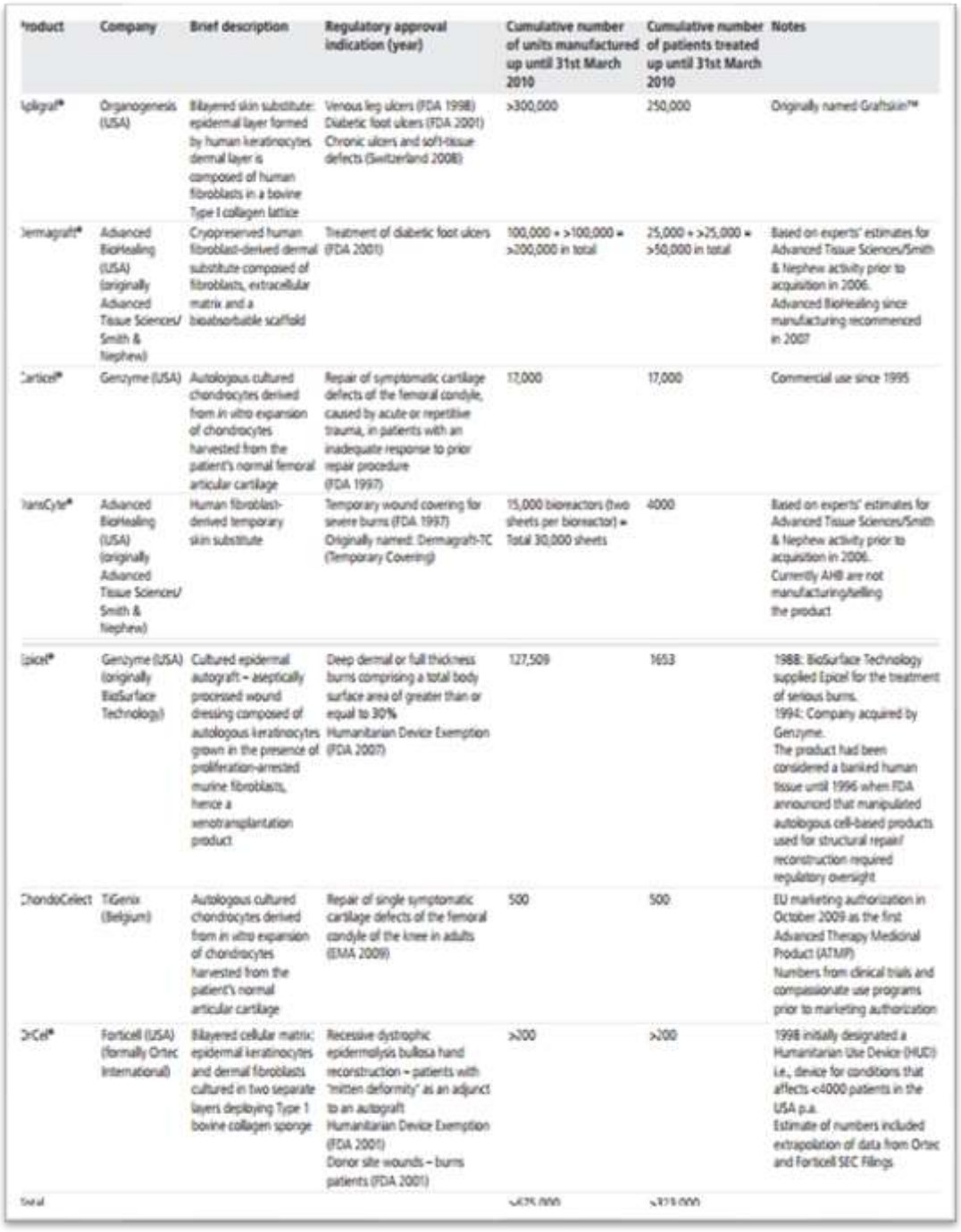
increased to nearly 2380 (58.6% raise). As recently as 2000, the total private sector activity had increased to $610 million mainly due to the emergence of stem cell scientific field which lead to further increase in business unit number (approximately 70), employee number (more than 3000) and modification of tissue engineering field into tissue engineering and regenerative medicine (TERM).

Image 2-23: Some renowned tissue engineering products available in market.

The continuously expanded tissue engineering product market is meant to be staggered in 2003 when capital value of publicly traded TE companies decreased from $2.6 billion in 2000 to $0.3 billion in 2003 (almost 90% decrease). On the contrary, business units increased from 73 to 89, but employee number reduced from 3080 to 2610. The 2007 data (Lysaght et al., 2008) provided evidence that the field of tissue engineering was now back to years of “great expectations”, as total private section activity had soared from $487 million in 2003 to $2.4 billion in 2007. Of the $2.4 billion in private sector activity in 2007, more than half came from the sale of products, a total more than $1.3 billion. Of this, the main contributor was Infuse product marketed by Medtronic.

Image 2-24: Growth of tissue engineering and regenerative medicine (TERM) field through past years (Source: Lysaght, M., Jaklenec, A. and Deweerd, E., 2008)

Year	1994	1997	2000	2003	2007
total private sector activity (in millions $)	246	453	610	487	2400
number of business units	40	40	73	89	171
number of employees	1500	2380	3080	2610	6100
capital value of regenerative medicine companies (in billions $)	1.7		2.6	0.3	4.7



Product	Company	Brief description	Regulatory approval indication (year)	Cumulative number of units manufactured up until 31st March 2010	Cumulative number of patients treated up until 31st March 2010	Notes
Apligraf®	Organogenesis (USA)	Bilayered skin substitute: epidermal layer formed by human keratinocytes dermal layer is composed of human fibroblasts in a bovine Type I collagen lattice	Venous leg ulcers (FDA 1998) Diabetic foot ulcers (FDA 2001) Chronic ulcers and soft-tissue defects (Switzerland 2008)	>300,000	250,000	Originally named Graftskin™
Dermagraft®	Advanced BioHealing (USA) (originally Advanced Tissue Sciences/ Smith & Nephew)	Cryopreserved human fibroblast-derived dermal substitute composed of fibroblasts, extracellular matrix and a bioabsorbable scaffold	Treatment of diabetic foot ulcers (FDA 2001)	100,000 + >100,000 = >200,000 in total	25,000 + >25,000 = >50,000 in total	Based on experts' estimates for Advanced Tissue Sciences/Smith & Nephew activity prior to acquisition in 2006. Advanced BioHealing since manufacturing recommenced in 2007
Carticel®	Genzyme (USA)	Autologous cultured chondrocytes derived from in vitro expansion of chondrocytes harvested from the patient's normal femoral articular cartilage	Repair of symptomatic cartilage defects of the femoral condyle, caused by acute or repetitive trauma, in patients with an inadequate response to prior repair procedure (FDA 1997)	17,000	17,000	Commercial use since 1995
TransCyte®	Advanced BioHealing (USA) (originally Advanced Tissue Sciences/ Smith & Nephew)	Human fibroblast-derived temporary skin substitute	Temporary wound covering for severe burns (FDA 1997) Originally named: Dermagraft-TC (Temporary Covering)	15,000 bioreactors (two sheets per bioreactor) = Total 30,000 sheets	4000	Based on experts' estimates for Advanced Tissue Sciences/Smith & Nephew activity prior to acquisition in 2006. Currently AHB are not manufacturing/selling the product
Epicel®	Genzyme (USA) (originally BioSurface Technology)	Cultured epidermal autograft – aseptically processed wound dressing composed of autologous keratinocytes grown in the presence of proliferation-arrested murine fibroblasts, hence a xenotransplantation product	Deep dermal or full thickness burns comprising a total body surface area of greater than or equal to 30% Humanitarian Device Exemption (FDA 2007)	127,509	1653	1988: BioSurface Technology supplied Epicel for the treatment of serious burns. 1994: Company acquired by Genzyme. The product had been considered a banked human tissue until 1996 when FDA announced that manipulated autologous cell-based products used for structural repair/ reconstruction required regulatory oversight
ChondroCelect	TiGenix (Belgium)	Autologous cultured chondrocytes derived from in vitro expansion of chondrocytes harvested from the patient's normal articular cartilage	Repair of single symptomatic cartilage defects of the femoral condyle of the knee in adults (EMA 2009)	500	500	EU marketing authorization in October 2009 as the first Advanced Therapy Medicinal Product (ATMP) Numbers from clinical trials and compassionate use programs prior to marketing authorization
HyCel®	Forticell (USA) (formally Ortec International)	Bilayered cellular matrix: epidermal keratinocytes and dermal fibroblasts cultured in two separate layers deploying Type 1 bovine collagen sponge	Recessive dystrophic epidermolysis bullosa hand reconstruction – patients with "mitten deformity" as an adjunct to an autograft Humanitarian Device Exemption (FDA 2001) Donor site wounds – burns patients (FDA 2001)	>200	>200	1998 initially designated a Humanitarian Use Device (HUD) i.e., device for conditions that affects <4000 patients in the USA p.a. Estimate of numbers included extrapolation of data from Ortec and Forticell SEC Filings
Total				>435,000	>335,000	

Image 2-25: Tissue Engineering & Regenerative Medicine (TERM) commercially available products under FDA approval until March 2010.

3. Cells in Bone Tissue Engineering

As we have already mentioned, Bone Tissue Engineering (BTE) requires a reliable cell source. Cells have an enormous influence on every tissue engineering application comprising one of the three basic pillars of tissue engineering (collaborating with scaffold, and growth factors). They induce new tissue formation through interaction with resident cells of the host tissue (e.g., bone). Cells can also be classified into **autologous** (patient's own), **allogenic** (human other than patient) and **xenogeneic** (animal origin) according to their harvesting specie.

Autologous cells comprise the 'gold standard' in tissue engineering due to their high activity potential and compatibility that lead to no need for immunosuppressive actions. However, they demonstrate limitations such as difficulty in harvesting of a sufficient number of cells for therapy and morbidity incidents. This fact led to the need for proper culture expansion, a time-demanding procedure.

On the other hand, **allogenic cells** demonstrate an alternative option that suffers from limitations like poor immunocompatibility, transmission of various diseases, high cost and lower incorporating properties. Finally, **xenogeneic cells** has already been accused of transmission of several animal diseases (Oryan et al., 2014).

3.1. Stem cells in Tissue Engineering

In human body we can find more than 200 different types of cells with specific roles and functions. **Stem cells** are undifferentiated cells (a characteristic referred to as stemness) having unique ability of self-renewal and plasticity, a feature that enable them to differentiate into any cell type we want according to every application.

After egg fertilization of a sperm "zygote" is created. The **zygote** referred as totipotent stem cell due to its potential of unlimited plasticity. After the fertilization of the egg, more precisely after 4-6 days, the cells form a kind of a bubble called **"blastocyst"**. The cells composing the inner cell mass (ICM) of blastocyst called **embryonic stem cells (ESCs)** and can developed into all cell types (ectoderm, mesoderm, and endoderm) as seen in (Image 3-1):

• Cells in **endoderm** form the epithelial lining on human body

• Cells in **mesoderm** fill the space between endoderm and ectoderm with cells like those in muscle tissue, cartilage tissue, and bone tissue

• Cells in **ectoderm** compose the outermost layer of tissues such as nervous system, tooth enamel and epidermis

These stem cells called "**pluripotent**" and can be evolved in any type of 200 cell types hosted in human body.

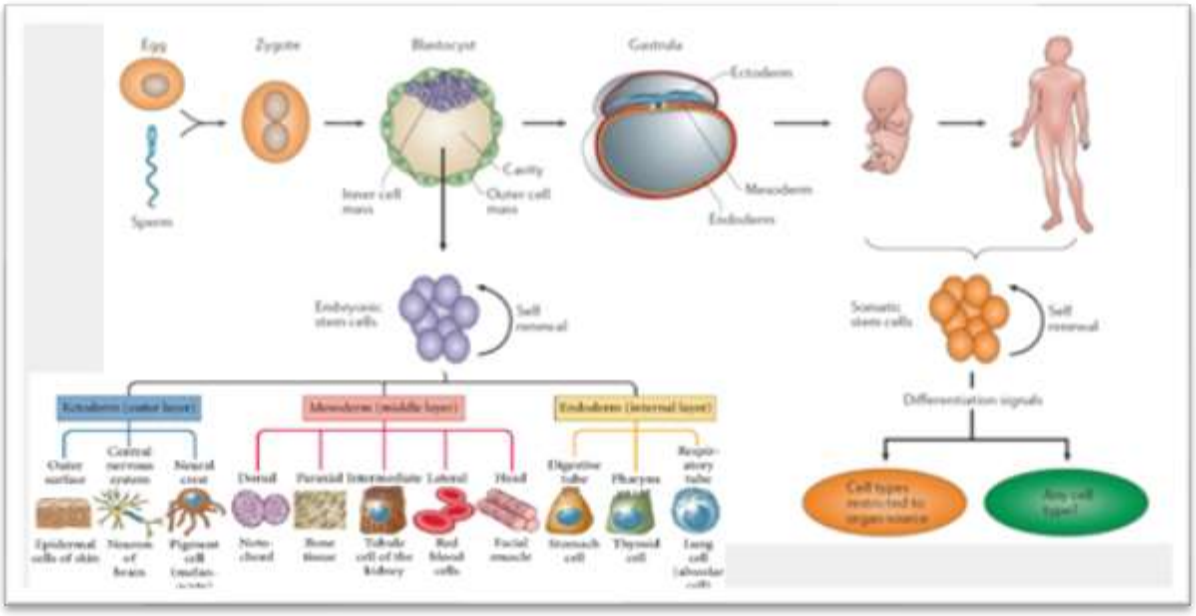


Image 3-1 Stem cell differentiation diagram. Adapted from: http://www.nature.com/scitable/content/ne0000/ne0000/ne0000/ne0000/58394/10.1038_nrg1829-f1_large_2.jpg

During the differentiation of stem cells, a population of undifferentiated cells, called **adult/somatic stem cells**, remains among the specialized cells. **Adult stem cells** are capable of making identical copies of themselves and transformed into end-differentiated cells with dedicated roles. Adult stem cells can be found in several sites throughout the human body in miniscule quantities presenting difficulty for identifying and isolating them to use in therapy.

Stem cells that would be used in tissue engineering application should display the following characteristics (Gimble et al., 2007; Lanza et al., 2013):

1. easy acquisition in sufficient quantities (billions of cells)
2. safe transplantation to either an autologous or allogeneic host
3. differentiation into multiple cell lineages
4. manufacture based on Good Practice guidelines

3.2. Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) derived from inner part of blastocyst, called inner cell mass (ICM). ESCs are stem cells with high pluripotency and considered as the **"gold standard"** for TERM applications. In 1998, the first human ESC lines were isolated, holding a great advance in regenerative medicine (Thomson, 1998). As regards the bone tissue engineering, ESCs are capable of differentiating into all cell types found in bone tissue. On the other hand, to bypass ethical and regulatory considerations that raise, sometimes extra embryos developed in vitro (Amini et al., 2012). Despite their promising properties, additional concerns include the possibility for teratoma formation

when implanted in vivo and the fact that tumorigenicity probability of donor ESCs should be addressed before their use for tissue engineering applications (Yousefi et al., 2016).

3.3. Adult Stem Cells

Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells are multipotent stem cells first isolated from marrow stroma where support and nurture the hematopoietic functions of the bone marrow. Due to their multipotency, MSCs are able to differentiate into cartilage, fat, and bone cells of mesoderm lineage and transdifferentiate[5] into muscle cells, neurons, epithelial cells etc. of the rest lineages (ectoderm and endoderm). MSCs in bone marrow stromal compartment have been the most studied stem cells after they first studied by Friedenstein in 1974 (Friedenstein et al., 1974). He suggested the use of bone marrow-derived mesenchymal stem cells (BM-MSCs) in order to transfer the hematopoietic microenvironment to ectopic sites.

Except from bone marrow cavity, MSCs have been already isolated from other tissues i.e., cord blood (CB), peripheral blood (PB), placenta, umbilical cord blood (UCB), synovial membrane, skin, deciduous teeth, pancreas, lung, and dental pulp.

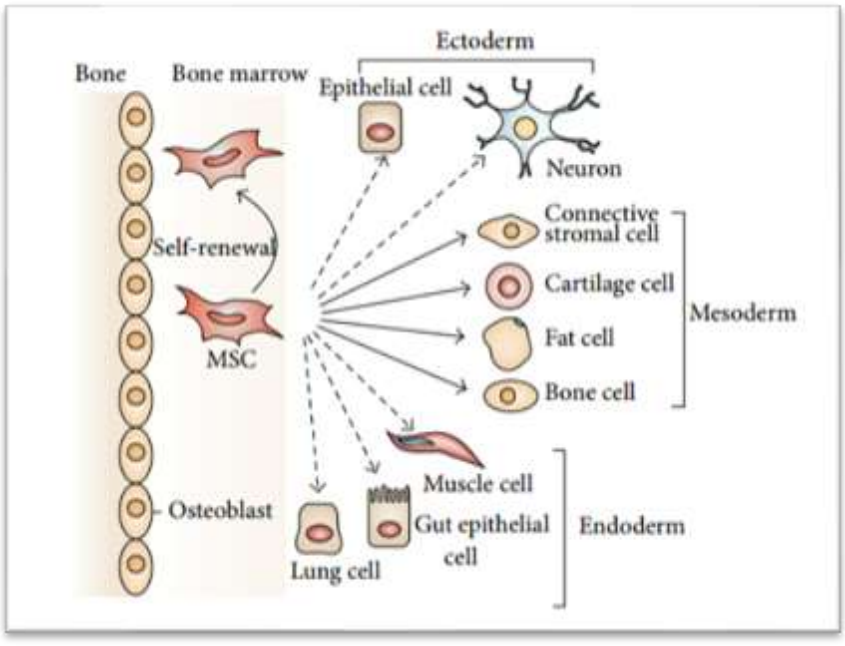


Image 3-2: The ability of BM-MSCs to self-renew and to differentiate into cells of all lineages. Source: (Uccelli et al., 2008)

[5] Transdifferentiation: the direct conversion of one differentiated cell type to another

Mesenchymal stem cells are able to limit the host immune response to foreign cells due to their hypoimmunogenic characteristics that make them suitable also for allogenic transplantation. Thus, they can be extracted, expanded, and stored for future use and applications. Recent studies have shown that MSCs are not only non-immunogenic, but also show ability to suppress immune responses and inhibit T-cell responses both in vitro and in vivo. Moreover, MSCs comprise the most extensively studied cells amongst the variety of autologous stem cells utilized in bone tissue engineering (BTE) applications due to their ability to promote osteogenic procedures. Given the ease of their isolation and their extensive proliferation rate (e.g., 30-50 PD[6] for BM-MSCs) MSCs show a great potential for medical or commercial applications.

On the other hand, MSCs are quite rare and make up about 0.001-0.01% (1 MSC per 10^5 adherent stromal cells) of all nucleated cells in human bone marrow cavity, so in vitro expansion is essential before implantation and therapy.

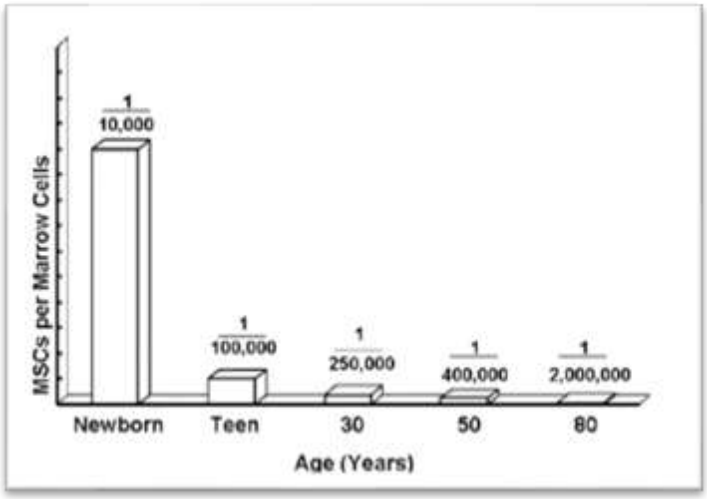


Image 3-3: Estimate of Bone marrow-derived MSCs (BM-MSCs) using colony forming units-fibroblastic (CFU-f) assay, showing that MSCs decline with age. Adapted from: (Caplan, 2007)

As regards the bone tissue engineering, amongst the various sources of MSCs, **bone marrow-derived mesenchymal stem cells** (**BM-MSCs**) comprise the most extensively studied type. However, due to their low abundancy and difficult harvesting procedure, the utilization of different sources is necessary.

[6] Population doubling (PD) accurately assesses the cell growth of a colony

Tissue/Cell Type	*In Vitro*	*In Vivo*
Bone/Osteoblasts	Yes	Yes
Cartilage/Chondrocytes	Yes	Yes
Fat/Adipocytes	Yes	N.D.
Heart/Cardiomyocytes	Yes	Yes
Heart/Purkinje Cells	N.D.	Yes
Liver/Hepatocytes	Yes	Yes
Pancreas/β Cells	Yes	N.D.
Brain/Astrocytes	Yes	Yes
Brain/Neurons	Yes	Yes
Brain/Oligodendrocytes	Yes	Yes
Hematopoietic Cells	N.D.	Yes
Skeletal Muscle/Myocytes	Yes	Yes
Endothelium	Yes	Yes
Kidney	N.D.	Yes

N.D. Not yet demsonstrated

Image 3-4: Differentiative capacity of MSCs into cells of all lineages (ectoderm, mesoderm, endoderm) Source: (Porada et al., 2006)

Umbilical cord blood-derived mesenchymal stem cells (**UCB-MSCs**) are proposed as a possible alternative source for bone tissue engineering-utilized cells. Umbilical cord comprises a 60-80 cm conduit between the fetus and the mother during pregnancy. Umbilical cord consists of two umbilical arteries (UCAs) and one umbilical vein (UCV). UCB possesses an interesting source of MSCs because, in contrary to BM-MSCs, the collection process is morbidless and painless. Additional benefits of UCB-MSCs are: 1) higher cell yields comparing to BM-MSCs and 2) absence of reports of teratomas production and ethical considerations that ESCs have. Umbilical cords despite their reach content in MSCs, have been discarded in most of the times after birth. Nowadays, there is an alternative option for parents to store umbilical cords in private biological for future use (Vernon et al., 2012).

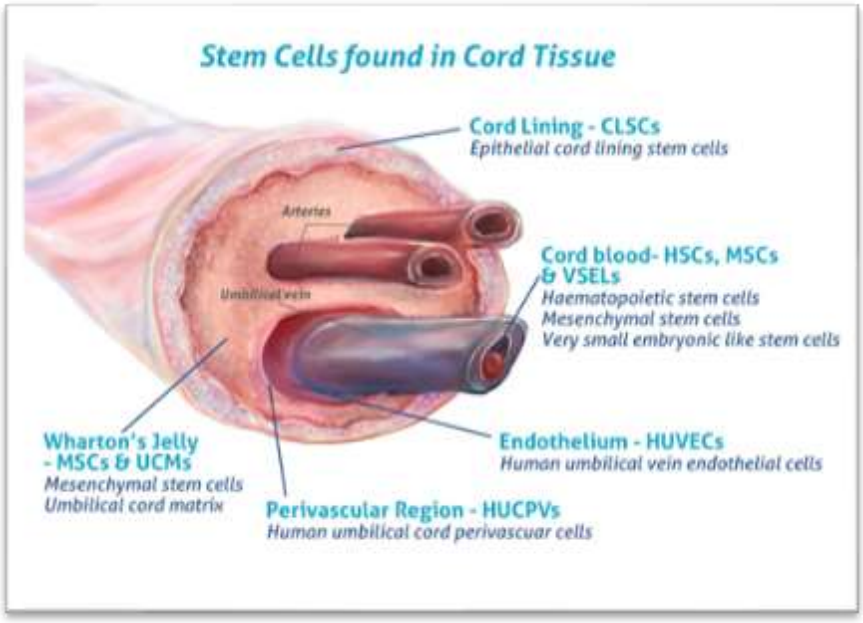


Image 3-5: Compartments of umbilical cord. (Available at: http://cells4life.com/2016/08/umbilical-cord-tissue-stem-cells-more-valuable-than-you-think/ [Accessed 26 Jun. 2018])

Adipose-derived stem cells (ASCs)

Adipose tissue possesses a source of adult stem cells that can differentiate into multiple tissue cells (e.g., osteogenic, myogenic, adipogenic, etc.). The procedure to collect Adipose-derived stem cells (ASCs) called **liposuction** (also referred as suction-assisted lipectomy), where small amount of adipose tissue (typically 100-200 ml) is obtained under local anesthesia (Mizuno, 2009).

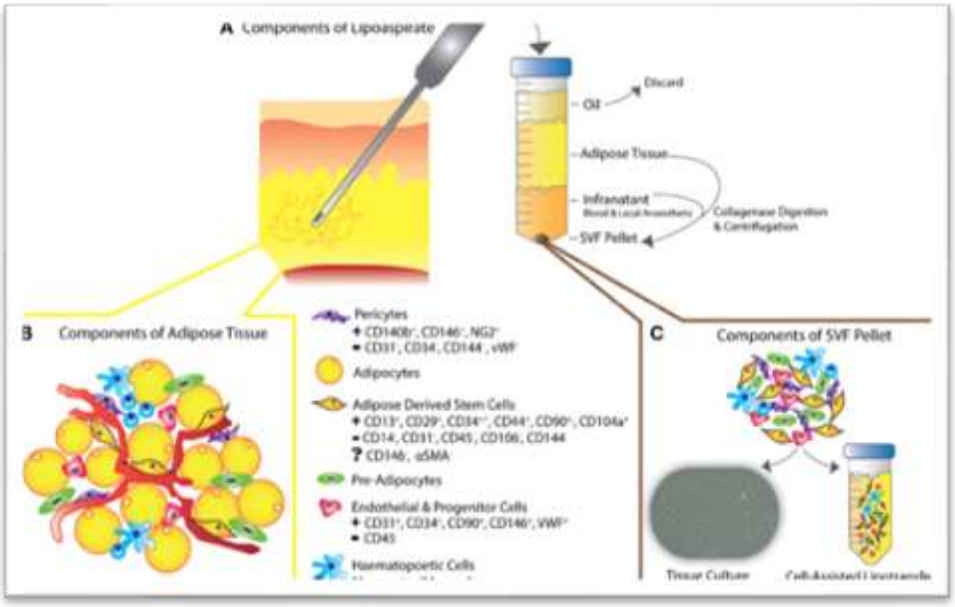


Image 3-6: Schematic diagram of liposuction procedure and adipose tissue components. Adapted from: (Shukla et al., 2015)

The amount of ASCs per gram of adipose tissue is approximately $5x10^3$ stem cells (1-5% of isolated cells), which is 500-fold greater of isolated cells). Moreover, liposuction produces less patient discomfort and pain than other procedures i.e. bone marrow aspiration. On the other hand, ASCs need more study to test their use in bone tissue engineering (BTE) applications (Yousefi et al., 2016). Last but not least, the efficacy of cell harvesting procedure is highly affected by the harvesting.

Dental Pulp Stem Cells (DPSCs)

Dental Pulp Stem Cells (**DPSCs**) are cells isolated from human dental pulp of mature teeth. Dental pulp is divided into four histological layers: 1**) odontoblast layer** (external layer) 2) **cell poor zone** (second layer), rich in extracellular matrix (ECM) 3**) cell-rich zone** (third layer), containing dental pulp stem cells (e.g., DPSCs) and 4) **inner layer** (pulp core), that consists of the vascular area and nervous plexus (Image 3-7B) (d'Aquino et al., 2008).

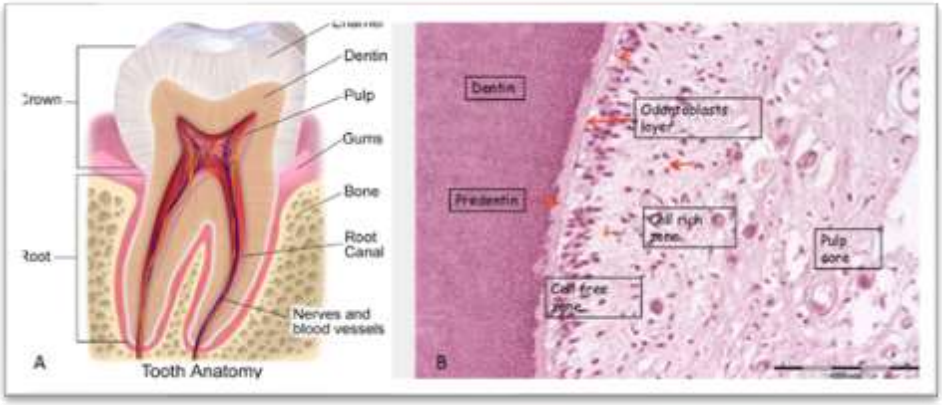


Image 3-7 A) Tooth anatomy. Available at: https://upload.wikimedia.org/wikipedia/commons/thumb/9/99/Blausen_0863_ToothAnatomy_02.png/1200px-Blausen_0863_ToothAnatomy_02.png [Accessed 30 Nov. 2017] and B) Morphologic zones of dental pulp Slideshare.net. (2017). Dental pulp. Available at: https://www.slideshare.net/LevakuMaheswarreddy/dental-pulp-54517743 [Accessed 30 Nov. 2017].

DPSCs derived from mesodermal tissues and have been firstly isolated from human dental pulp by (Gronthos et al., 2000), who also named them. These cells exhibit differentiation potential into numerous mesodermal and non-mesodermal tissue cells including osteoblasts, odontoblasts, adipocytes, chondrocytes, endothelial cells, neural cells, and myocytes (Ashri et al., 2015). DPSCs are closely related to mesenchymal stem cells (MSCs), while their gene expression profile is similar to that of bone marrow MSCs. Dental pulp progenitor cells are among the most

attractive cell sources not only for periodontal tissue engineering, but also for bone tissue engineering (BTE), due to their high and multi-lineages differentiation ability. The harvesting procedure of DPSCs from pulp tissue is easier, while produces less pain and discomfort than bone marrow aspiration. Also, DPSCs induce little to no morbidity, and characterized by good interactivity with biomaterials used in bone tissue engineering applications (d'Aquino et al., 2008). Moreover, dental pulp stem cells (DPSCs) are characterized by higher proliferation rate (60-120 PDs) compared to BM-MSCs (30-50 PDs) (Huang et al., 2009), while they display the same immunoreactivity profile with MSCs (Gronthos et al., 2000).

Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (**iPSCs**) are pluripotent stem cells inducted from non-pluripotent (Amini et al., 2012). Human iPSCs show similar properties to those of embryonic stem cells (ESCs), having similar morphology, surface antigen pattern and differentiation potential into all cell types. At the same time they have no ethical and political considerations.

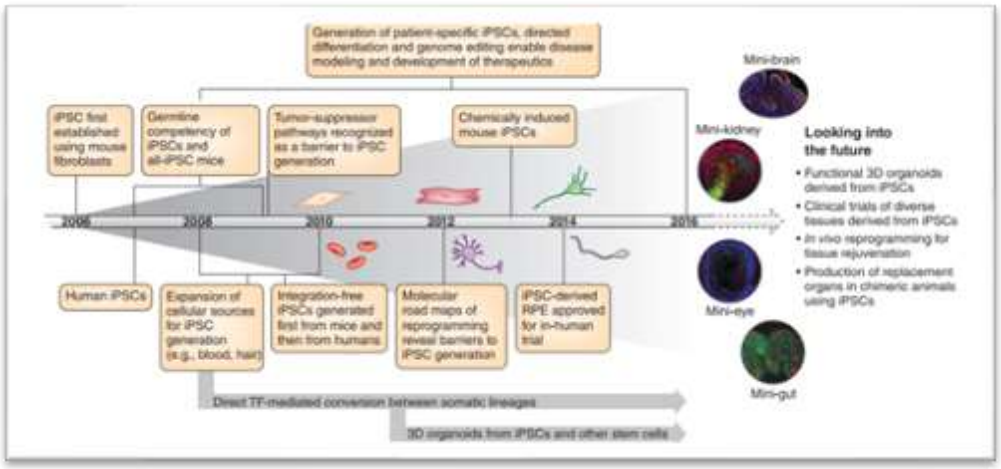


Image 3-8: Milestones in the development of iPSCs. Adapted from: (Li & Belmonte, 2016)

The list of non-pluripotent (somatic) cells capable of generating iPSCs includes cells from all three germ layers. Following the creation of iPS Cells, they should be evaluated for their characteristics (pluripotency, differentiation potential, etc.) using methods like RT-PCR[7] and Western blot. iPS cells can serve as a source for generating on-demand pluripotent stem cells for bone tissue engineering applications, replacing the ESCs considered now as the "gold standard".

[7] RT-PCR: Reverse transcription polymerase chain reaction

On the other hand, induced pluripotent stem cells show an extremely low (approximately 0.01-0.02%) reprogramming efficiency for the most frequently utilized sets of factors (Yamanaka factors and Thomson factors) (Lanza et al., 2013). Thus, extensive population expansion is necessary, making the process both inefficient and costly. In addition, the majority of pluripotent induction techniques (retroviral vector, lentiviral vector, etc.) are not FDA approved and could lead to integration of viral DNA into the chromosome producing tumorigenic mutations creating limitations for further clinical application of iPSCs into tissue engineering (Lanza et al., 2013; Vonk et al., 2015).

Endothelial Progenitor Cells (EPCs)

Vascularization comprises a crucial issue in every tissue engineering application, as much in bone tissue engineering (BTE). Thus, inclusion of proper cells (osteoblasts) capable of migrating and differentiating in response to local cues (e.g., growth factors, ECM) in order to form new blood vessels, a procedure called neovascularization, is of pivotal importance in successful bone regeneration. One approach to achieve improved vascularization of tissue-engineered construct could be the application of **endothelial progenitor cells** (**EPCs**). EPCs were firstly identified in the peripheral blood (PB) by (Asahara et al., 1997) who reported the high neovascularization potential of these cells due to expression of endothelial-associated surface markers (e.g., CD34). These cells represent a small population with high capacity to proliferate, migrate, and differentiate into cells (endothelial cells-ECs) that line the lumen of blood vessels being able to induce neovascularization (Zigdon-Giladi et al., 2014). Endothelial progenitor cells originate from bone marrow and can be isolated from adult peripheral blood (PB), or umbilical cord blood (UCB) using ex vivo techniques, while the most promising are perinatal-derived UCB-EPCs. Moreover, they are classified into two types of cells, depending on their growth characteristics and morphological appearance. The cells of first type called early-outgrowth cells, are spindle-shaped having low proliferative capacity, while the second ones, called late-outgrowth cells, are cobblestone-shaped, having high proliferative potential and considered as “true EPCs” (Fedorovich et al., 2010).

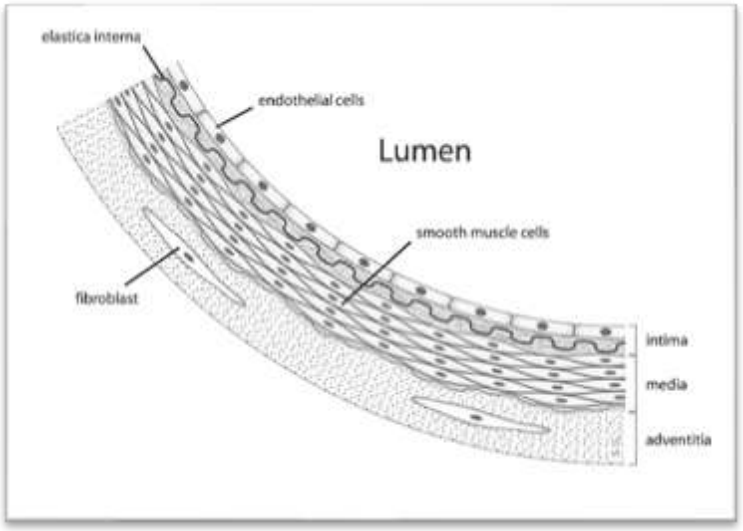


Image 3-9: Endothelium compartment anatomy and EPCs location. Source: https://en.wikipedia.org/wiki/Endothelium#/media/File:Endotelijalna_%C4%87elija.jpg

Endothelial progenitor cells have shown to express endothelial surface markers such as CD34, VEGFR2, and CD133 that used to separate EPCs from hematopoietic stem cells, which also express some of these markers, and thus lead to hematopoietic contamination of harvested EPCs population (Atesok et al., 2012). In addition, EPCs show an advantage over mature endothelial cells (ECs) due to their greater doubling ability (e.g., 10-times more proliferative than HUVECs) and blood vessels forming efficiency (Liu et al., 2012; Murohara, 2010).

Despite their promising properties that make them important candidates for every bone tissue engineering application, endothelial progenitor cells (EPCs) show limitations such as the incapacity to form bone tissue (Hutton & Grayson, 2014). As a result, EPCs should be incorporated in every BTE application in order to induce neovascularization, but the presence of another bone-forming cell type (e.g., MSCs, iPSCs etc.) is necessary.

4. Scaffolds in Bone Tissue Engineering

Biomaterial scaffolds comprise the second pillar of Tissue Engineering. Every tissue engineering application requires a reliable scaffold that offer a three-dimensional matrix for cell attachment and future tissue growth. Scaffold is of crucial importance because survival of most cells in human body is highly affected from proper substrate that support cell proliferation and differentiation, while it shapes fresh-formed tissue.

Moreover, scaffolds can mimic the ECM properties because they provide mechanical support, transfer genetic material and growth factors to the defined site, while they undergo resorption and replacement by new bone during its formation (Hollinger, 2005; Bose et al., 2013).

A scaffold utilized in tissue engineering should meet some requirements:

1. **Pore size and porosity**: a vital feature for tissue engineering scaffolds is high porosity for transport of nutrients in the site of interest. A typical pore size for most TE applications is around 150 μm while bigger pore sizes preferred in applications with high vascularization needs.

2. **Biocompatibility**: the scaffold's ability to support cellular activities (adhesion, proliferation, and differentiation) with non-toxicity effect to the host tissue (Roseti et al., 2017).

3. **Bioactivity**: the capacity of scaffold to interact with neighbor tissue. In this direction the attention has been directed from bioactive, to "smart" biomaterial scaffolds that promote osteoinduction (Roseti et al., 2017).

4. **Bioresorbability**: referred to the ability of scaffold degrade into low molecular weight by-products after time, preferably at a controlled rate, matching those of tissue growth, with no residual side effects and finally promote growth of fresh-formed bone (Hutmacher, 2000).

5. **Mechanical integrity**: scaffold's mechanical properties should meet those of native bone tissue for success of each application. Also, mechanical properties vary widely between cortical and cancellous bone so scaffold material should be selected properly according to which part of bone we want to restore as seen below (Table 3).

Table 3: Mechanical properties of compact (cortical) and spongy (cancellous) bone Source: (Roseti et al., 2017)

	Cortical bone	Cancellous bone
Compressive strength (MPa)	100-230	2-12
Tensile strength (MPa)	50-150	10-20
Strain to failure (%)	1-3	5-7
Fracture toughness (MPam)	2-12	-
Young's modulus (GPa)	7-30	0,5-0,05

6. **Surface characteristics**: surface chemistry of scaffold plays a crucial role in cellular adhesion and proliferation (Hutmacher et al., 2001).

7. **Reproducibility**: one more challenge in tissue engineering is to fabricate reproducible 3-D scaffolds that are able to function for a defined time in the implantation site (Hutmacher, 2000). This necessity has led to the emergence of contemporary scaffold fabrication techniques, which will be thoroughly analyzed below.

8. **Commercialization**: an ideal 3-D scaffold should be fabricated at an acceptable cost enabling commercialization actions (Thavornyutikarn et al., 2014).

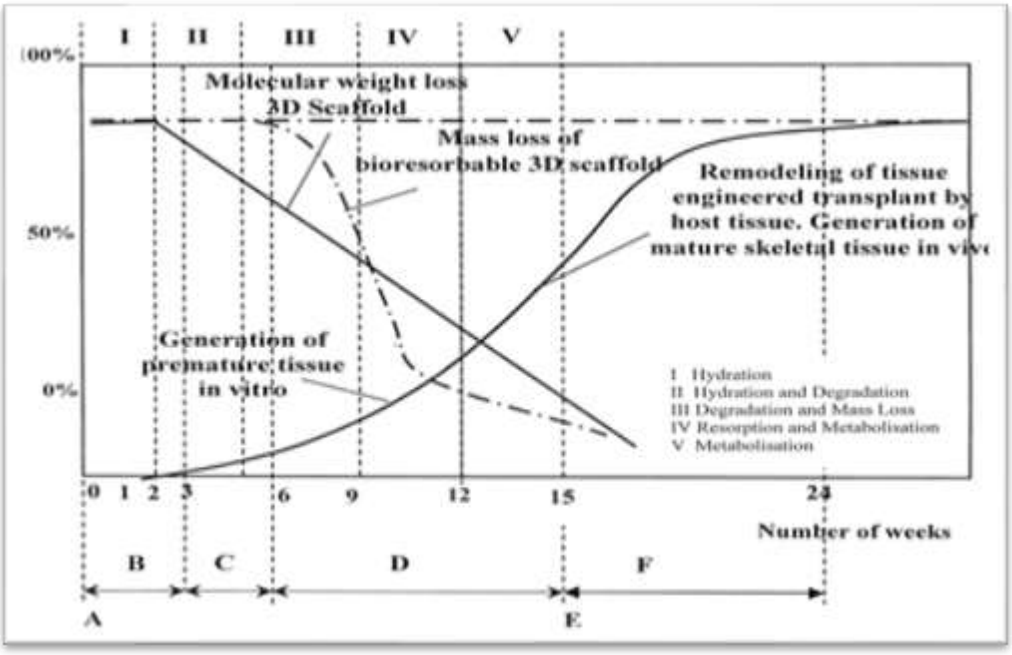


Image 4-1: Graphical illustration of scaffold molecular weight and mass loss against time passage in a typical bone tissue engineering application. (A) scaffold fabrication; (B) cell-seeding into scaffold; (C) initial tissue growth in a spinner flask; (D) growth of mature tissue in a physiologic environment (bioreactor); (E) surgical implantation; (F) tissue-engineered scaffold transplantationand bone tissue remodeling. Adapted from: (Hutmacher, 2000)

The majority of scaffolds currently used in bone tissue engineering (BTE) applications are classified in **polymers** (natural or synthetic), **bioactive ceramics**, and **composites** (hybrids).

4.1. Polymers

Several natural and synthetic materials have been studied for scaffold in bone tissue engineering applications.

Natural Polymers

Natural-derived polymers that have been extensively used in bone tissue engineering applications include **hydrogels** such as **collagen**, **fibrin**, **agarose**, **chitin/chitosan**, and **hyaluronic acid (HA)**. Natural polymers show better interaction with tissue cells due to their high bioactivity, while considered as the first biodegradable biomaterials used in clinical practice (Dhandayuthapani et al., 2011).

Collagen is a natural polymer protein found in native tissue. It is derived from the submucosa of bovine and has been extensively used as suture material for over a century. Back in 1980s, collagen was first used in order to construct tissue-engineered bilayer skin grafts (Yannas & Burke, 1980). Due to its biological origin, collagen displays excellent biocompatibility, biodegradability and low toxicity that made it strong candidate for bone tissue engineering applications (Thavornyutikarn et al., 2014). On the other hand, it suffers from poor mechanical stability, and rapid degradation over time.

Agarose comprises a seaweed-derived polymer exhibiting temperature-sensitive water solubility than can be utilized to entrap mammalian cells (Hutmacher et al., 2001). It was first used for tissue engineering purposes as an experimental material for encapsulating endocrine cells.

Chitin, or poly (b-(1-4)-N-acetyl-D-glucosamine), comprises the second most important polymer in the world. It is a natural polysaccharide that was first identified in 1884. **Chitosan** comprises a derivative of chitin prepared by treating the chitin at 110–120ºC for 2–4 hours in a 40–50% NaOH solution (Park & Lakes, 2007). It is a semi-crystalline polymer showing promising features for scaffold material like porous structure and adequate mechanical properties for bone applications.

Hyaluronic acid or Hyaluronan (HA) is a polysaccharide of the articular cartilage extra cellular matrix (ECM) that composed of N-acetylglucosamine and glucuronic acid. In the last decade, it has been widely utilized in orthopedic surgery (e.g., joint surgery), and treatment of ophthalmologic diseases (e.g., cataract). It is characterized by high water solubility, high angiogenesis, and sufficient biocompatibility.

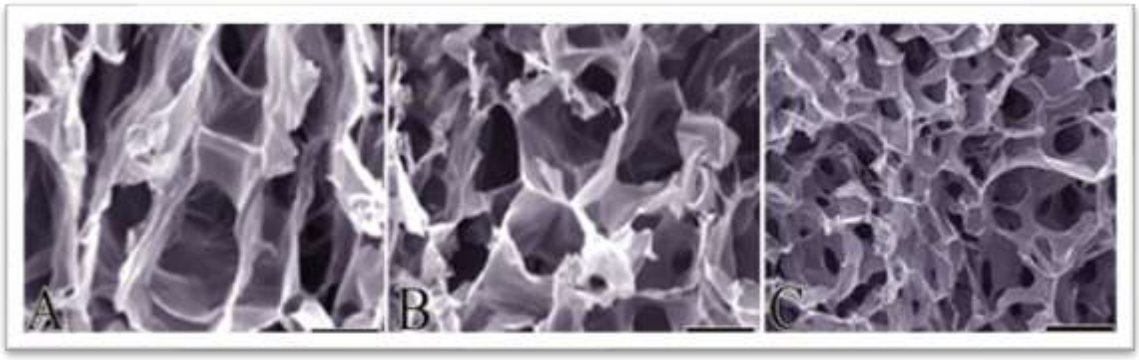


Image 4-2: SEM Images of natural-derived scaffold microstructure. A) Alginate, B) Alginate-chitosan, and C) chitosan. Adapted from: (Oryan et al., 2014)

Synthetic Polymers

Although the natural-derived polymers offer numerous benefits (e.g., excellent biocompatibility), they suffer from extensive degradation time. **Synthetic polymers** not only alleviate the natural-derived polymer limitations, but also offer advantages of fabrication with tailored mechanical and physical properties, shapes, and sizes, and higher sterilizability. The majority of synthetic polymers in BTE applications, are **poly-α-hydroxy esters**.

The most widely studied polymers in tissue engineering field are poly (lactic acid) (**PLA**), poly (glycolic acid) (**PGA**), poly (e-caprolactone) (**PCL**), and their co-polymers.

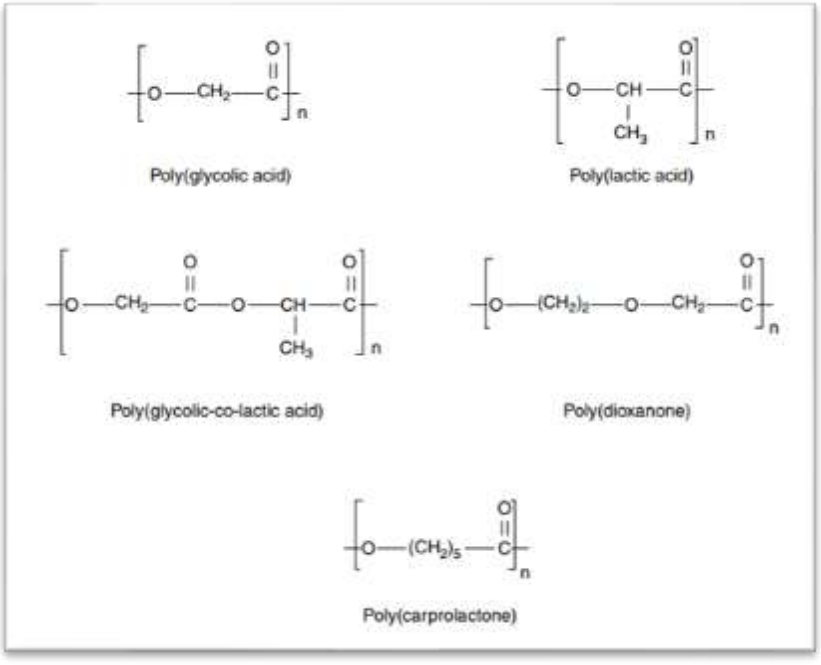


Image 4-3: Chemical formulas of widely used synthetic polymers in Tissue Engineering field. Adapted from: Pallua & Suschek, 2011)

Polyglycolic acid (**PGA**) comprises the simplest aliphatic polyester. It was first synthesized in 1930s by William Carothers, who also considered as the 'father' of nylon. Since 1970, PGA has been widely used in clinical practice as a synthetic absorbable suture under the trade name **Dexon®**. In addition to its excellent biocompatibility, it also characterized by high crystallinity, high melting point (230°C), and low solubility in organic solvents (Atala & Mooney, 1997). However, PGA degrades rapidly over a period of 2-4 weeks because of its hydrophilic nature, leading to complete resorption within 4-6 months and premature mechanical failure of scaffolds. Hence, PGA comprises a controversial selection for tissue engineering applications.

Polylactic acid (**PLA**) was the first polyester utilized for tissue engineering applications. It shows higher solubility in organic solvents, higher hydrophobicity due to an extra methyl group, excellent biocompatibility, and longer degradation time compared to PGA (Atala & Mooney, 1997; Amini et al., 2012). PLA has also three stereoisomers: poly(L-lactic acid) (**PLLA**), poly(D-lactic acid) (**PDLA**), and poly(D,L-lactic acid) (**PDLLA**). Among them, PLLA is mainly utilized in applications required good mechanical properties (e.g., sutures, orthopedic devices), while PDLLA for scaffold fabrication in BTE and drug delivery because of its excellent biocompatibility, and osteoinductivity (Hutmacher et al., 2001). Both PLA and PGA polymers degrade through the mechanism of bulk erosion (Image 4-4). However, release of acidic products can create a local acidic environment that may trigger inflammatory response (Thavornyutikarn et al., 2014).

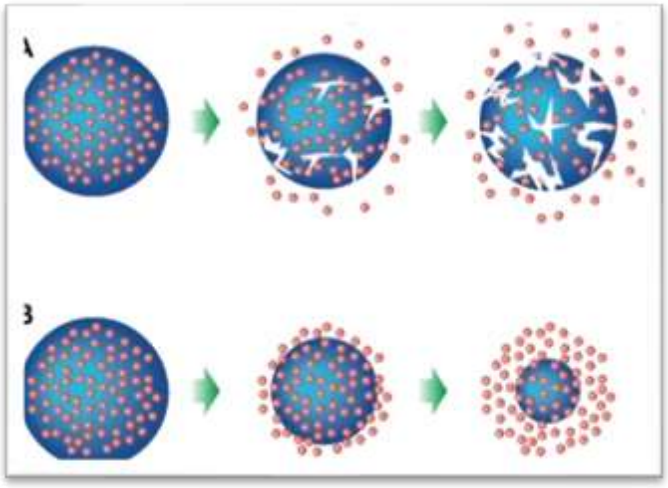


Image 4-4: Degradation mechanisms of A) bulk erosion and B) surface erosion. Adapted from: (Dinarvand et al., 2011)

PCL or **Poly(ε-caprolactone)** has similar structure to PLA and PGA being semicrystalline and rubbery polymer with high solubility, high crystallinity, and slow degradation time. Moreover, it has been utilized to carry antibiotic drugs and thus it is suitable for drug delivery systems and

long-term (1-2 years) implants in bone tissue engineering (Rezwan et al., 2006). As regards degradation times of PLA stereoisomers they can be ranked in the following order **PGA < PDLLA < PLLA < PCL** as seen below (Image 4-5).

Polymers	Biodegradation time (months)	Compressive or tensile strength (MPa)	Modulus (GPa)
PGA	6-12	Fibre: 340–920	Fibre: 7–14
PDLLA	12-16	Pellet: 35–150[a]	Film or disk: 1.9–2.4
PLLA	>24	Pellet: 40-120[a]	Film or disk: 1.2–3.0
PCL	>24	10-15	0.15–0.33
PLGA	Adjustable 1-12	41.4-55.2	1.4–2.8

Image 4-5: Mechanical properties of synthetic polymers used in tissue engineering. Adapted from: (Rezwan et al., 2006)

4.2. Ceramics

Ceramics biomaterials produced from heating of mineral salts. A sub-category of ceramics, called **bioceramics** hold prominent future for hard and soft tissue engineering applications. **Bioactive ceramics**, **hydroxyapatite (HA)**, **tricalcium phosphate-TCP**, and compositions of silicate and phosphate glasses (**bioactive glasses**) are amongst the most-used materials for BTE applications due to their similar properties to bone tissue. Also, they display excellent biocompatibility, low toxicity, high compressive strength, and low ductility (Hollinger, 2005).

Corals

Natural coral graft substitutes, were first used as scaffold materials in the early 1970s while have an pore-rich structure resembling that of native bone. They are characterized as highly biocompatible, biodegradable, and osteoconductive materials. Moreover, coral scaffolds display surface chemistry that promotes cell adhesion and differentiation while can also be used as growth factor carriers (Manassero et al., 2016). Moreover, mechanical properties of corals resembles ones of native bone. The structure of the most used corals for bone regeneration purposes are seen below (Image 4-6).

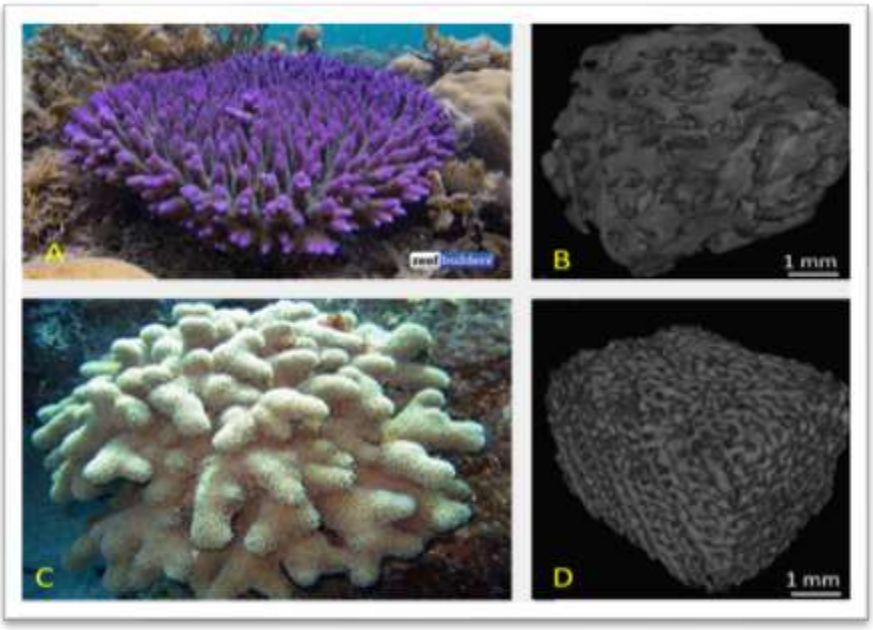


Image 4-6: A) Acropora and C) Porites corals B) Micro-CT reconstruction of fabricated coral scaffolds. Adapted from: (Manassero et al., 2016), https://reefbuilders.com/2017/05/17/acropora-millepora/, http://coralpedia.bio.warwick.ac.uk/en/corals/porites_porites

Table 4: Mechanical properties of corals compared to those of native bone tissue.

	Porites	Acropora	Trabecular bone	Cortical bone
Global porosity (% volume)	47-64	12-60	50-80	5-30
Compressive strength (MPa)	12.1	7.1	1-12	131-283
Young's modulus (MPa)	7620-8360	21300-27900	50-400	17000

Image 4-7: Coral implants of various shapes and sizes for bone tissue engineering applications. Adapted from: (Manassero et al., 2016)

HA and CaP-based bioceramics

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and calcium phosphate (CaP)-based ceramics (e.g., **β-TCP**) are probably the most thoroughly researched bioceramics for biomedical applications. They display excellent biocompatibility, high bioactivity and osteoconductivity. Nevertheless, they show limitations such as: high brittleness, insufficient porosity (mainly TCPs), poor tensile strength despite their high compressive strength, and slow degradation (being virtually inert for years after implantation). In addition, degradation rates of ceramics can be classified as following: **amorphous CaP > amorphous HA > crystalline CaP > crystalline HA** (Thavornyutikarn et al., 2014).

Table 5: Mechanical properties of various ceramics utilized in bone tissue engineering.

	Compressive strength (MPa)	Tensile strength (MPa)	Elastic modulus	Fracture toughness $MPa\sqrt{m}$

Calcium phosphates (CaPs)	20-900	30-200	30-103	<1.0
HA	>400	~40	~100	~1.0
45S5 Bioglass®	~500	42	35	0.5-1
Cortical bone	130-180	50-151	12-18	6-8

Bioactive glasses

The second category of ceramics utilized as scaffold materials includes bioactive glasses such as **bioactive silicate** (**SiO_2**) glasses, **bioactive phosphate** (**P_20_5**) glasses, and **bioactive borate** (**B_2O_3**) glasses. These materials called 'bioactive' because they are able to interact with native tissue with no side effects (Roseti et al., 2017). Bioactive glasses display numerous advantages over ceramics of previous category, showing first of all higher degradability. Moreover, they are more bioactive offering features of osteoconduction and osteoproduction (Class A bioactive materials) compared to HA and CaP (Class B bioactive materials) that exhibit only osteoconduction (Thavornyutikarn et al., 2014).

One frequently used bioactive glass composition, known as **45S5 Bioglass®** containing 45% SiO_2, 24.5% NaO_2, 24.5% CaO, and 6% P_2O_5, was first proposed by Hench and his coworkers in 1969 (Hench, 2006). Since then, 45S5 Bioglass® has been used not only in tissue engineering but also in numerous bone graft commercial products (e.g., NovaBone, NovaMin, and NovaThera) in the fields of bone, cartilage, and teeth repair. Despite their promising properties, bioactive glasses show limitations associated with high brittleness, slow degradation rate and low fracture toughness mainly due to their amorphous structure. As a result, bioactive glasses are not strong candidates for load-bearing applications (Chen et al., 2012). This limitation gave rise to composite (hybrid) scaffolds.

4.3. Hybrid (composites) scaffolds

Composite (hybrid) scaffolds composed of two or more scaffolds selected properly to display the required characteristics. In this way we might use the advantage of the individual material to alleviate the limitations of another material. The combination can be in the form of **co-polymers**, **polymer-ceramic composites,** or **polymer-polymer blends**.

Co-polymers

Co-polymers composed of two or more polymers such as **PLGA** (poly[lactic-co-glycolic acid]), a combination of glycolic acid (GA) and lactic acid (LA) monomers. PLGA comprises the most popular biodegradable co-polymer, displaying advantages such as adjustable degradation rate, and good mechanical properties (Pan & Ding, 2012). Alternating the percentage of LA monomers, displaying long degradation time, and GA monomers that

show shorter degradation time, we will be able to tune the degradability of PLGA co-polymer on demand (Table 6). Except from good ductility, PLGA demonstrates good proliferation rate, and extensive cell-adhesion capability.

Table 6: Degradation time of various PLGA scaffolds. Adapted from: (Middleton & Tipton, 2000)

Polymer	Degradation time (in months)
PGA	6-12
85/15 PLGA	5-6
75/25	4-5
65/35	3-4
50/50	1-2

Since 1974, PLGA has been also used as suture material marketed by Ethicon under the tradename **Vicryl**®. Vicryl® comprises a PLGA co-polymer made of Polyglactin 910, a co-polymer of glycolic and lactic acids in a 90:10 ratio (Hutmacher et al., 2001). Similarly, other co-polymers have been studied, such as PLGA-PCL, PLA-PCL, etc.

Polymer-ceramic composites

By combining polymers and ceramics, we can take advantage of polymers' toughness and plasticity and ceramics' strength at the same time. Human bone composed of inorganic hydroxyapatite crystals (HA) and natural polymer (collagen) fibers, thus composite scaffolds are highly biomimetic (Rezwan et al., 2006). As we have already mentioned, release of acidic by-products during degradation process of polymers like PLA, and PGA may create a local acidic environment triggering inflammatory response, implant failure, or tissue necrosis. The addition of bioactive ceramics eliminates poor bioactivity of synthetic polymers, while creates a non-acidic environment for tissue proliferation.

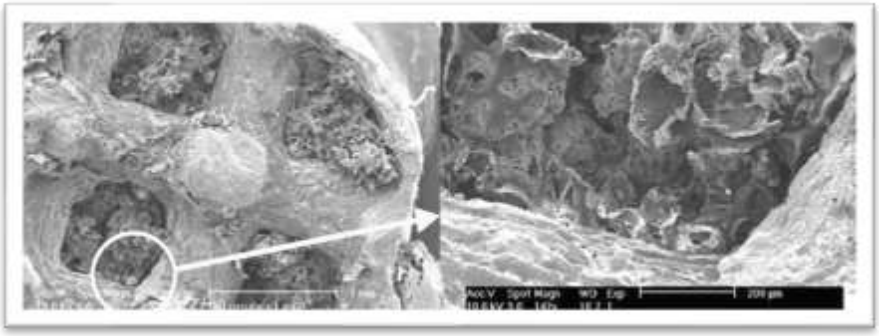


Image 4-8: Global and high magnification SEM images of HA/PLA composite scaffold showing PLA embedded inside HA pores. Adapted from (Hollinger, 2005)

Among several polymer/ceramic scaffolds, polymer/calcium phosphate (CaP) scaffolds such as **PLLA/HA**, **PLGA/HA**, **PGA/β-TCP** composites

show excellent properties. Moreover, **polymer/Bioglass®** composites exhibit enhanced mechanical strength (Thavornyutikarn et al., 2014).

Table 7: Composite scaffolds used for bone tissue engineering and their properties. Adapted from: (Rezwan et al., 2006)

Scaffold composite		Percentage of ceramic (%)	Pore size (μm)	Porosity (%)	Mechanical properties Compressive strength (C), tensile (T), flexural (F) strength (MPa)
Ceramic	Polymer				
1. Dense composites					
HA Fibre	• PDLLA • PLLA	2-10.5 (vol.) 10-70 (wt.)	- -	- -	45 (F) 50–60 (F)
HA	PLGA	40-85 (vol.)	-	-	22 (F)
β-TCP	• PLLA-co-PEH • PPF	75 (wt.) 25 (wt.)	- -	- -	51 (F) 7.5–7.7 (C)
2. Porous ceramics					
Amorphous CaP	• PLGA	28-75 (wt.)	>100	75	
HA	• PLLA • PLGA • PLGA	50 (wt.) 60-75 (wt.)	100-300 800-1800 110-150	85-96 81-91 30-40	0.39 (C) 0.07–0.22 (C)
Bioglass®	• PLGA • PLLA • PLGA • PDLLA	75 (wt.) 20-50 (wt.) 0.1-1 (wt.) 5-29 (wt.)	89 -100 (macro) 50-300 -100 (macro)	43 77-80 - 94	0.42 (C) 1.5–3.9 (T) 0.07–0.08
Cortical bone					50–150(T) 130–180 (C)
Cancellous bone					4–12 (C)

Polymer-polymer blends

Polymer-polymer blends comprise a mixture of two polymers. Polymer-polymer blending differentiate from co-polymerization, being a simpler and more effective scaffold fabrication method for tissue engineering (Li & Mai, 2017). For example, **PLA/PCL** blends are utilized in order to alleviate the high brittleness of PLA with the addition of more rubbery materials such as PCL. Moreover, other widely studied polymer-polymer blends such as **PLA/PEG**, that augment hydrophilicity of PLA, and **PLGA/ polyphosphazenes** blends, that counteract the acidic by-products of PLGA degradation through neutral or basic products released from polyphosphazenes, hold promise future in bone tissue engineering applications (Amini et al., 2012).

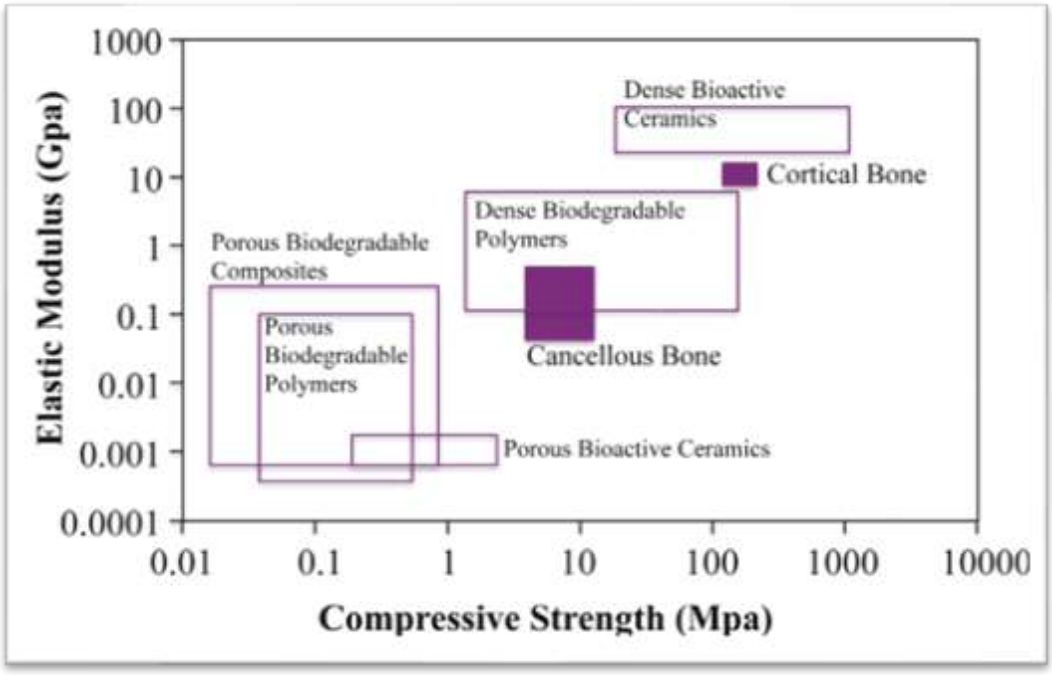


Image 4-9: Mechanical properties of various scaffolds used in tissue engineering field. Adapted from: (Rezwan et al., 2006)

5. Scaffold fabrication techniques

As discussed before, a crucial challenge in the field of tissue engineering is to design and fabricate reproducible 3-D scaffolds enabled to work for a certain time period under load-bearing conditions (Hutmacher, 2000). Three-dimensional design and high porosity play a crucial role in the success of every scaffold-based application because cells and their ECM are organized into three-dimensional structures/tissues. In order to fabricate tissue scaffolds that support cells and guide their growth in three dimensions, several techniques have been developed and they can be classified into categories as: **conventional** and **advanced scaffold fabrication techniques**. Scaffold fabrication technique, among the others, determines features such as **pore size**, **porosity, interconnectivity** and **mechanical properties** of scaffold (Thavornyutikarn et al., 2014). Therefore, the selection of fabrication technique could determine the viability of the whole tissue engineering application.

5.1. Conventional fabrication techniques

Conventional techniques use subtractive (top-down) methods, where part of material is removed from an initial block to reach the desired shape (Roseti et al., 2017). These includes techniques, such as solvent-casting and particle-leaching, freeze-drying, phase separation, gas foaming, electrospinning, and sol-gel technique among others.

Solvent casting/ particle leaching

In this technique, a polymer (usually PLGA or PLLA) dissolved in a solvent (chloroform or methylene chloride) with a water-soluble porogen, salt (NaCl) or sugar that has uniformly distributed particles (crystals) of specific size. After the evaporation of solvent, the polymer/porogen composite is leached in water for a couple of days where salt leaches out producing a porous structure (Image 5-1), with pore size that can be controlled and porosity (Roseti et al., 2017; Mikos & Temenoff, 2000).

The advantages of this technique include ease of fabrication with no need for specialized equipment, sustainable equipment cost and feasibility to tune features such as pore size, and porosity of the fabricated scaffold alternating the size of porogen particles and porogen/polymer ratio. On the contrary, limitations include the long time required for solvent evaporation (days-to-weeks), the extensive use of highly toxic solvents, residual particles in the polymer matrix, ability to fabricate only thin flat sheets and tubes typically up to 3mm thick, and residual solvent that could be harmful to cells and biological tissues (Hutmacher, 2000; Rezwan et al., 2006).

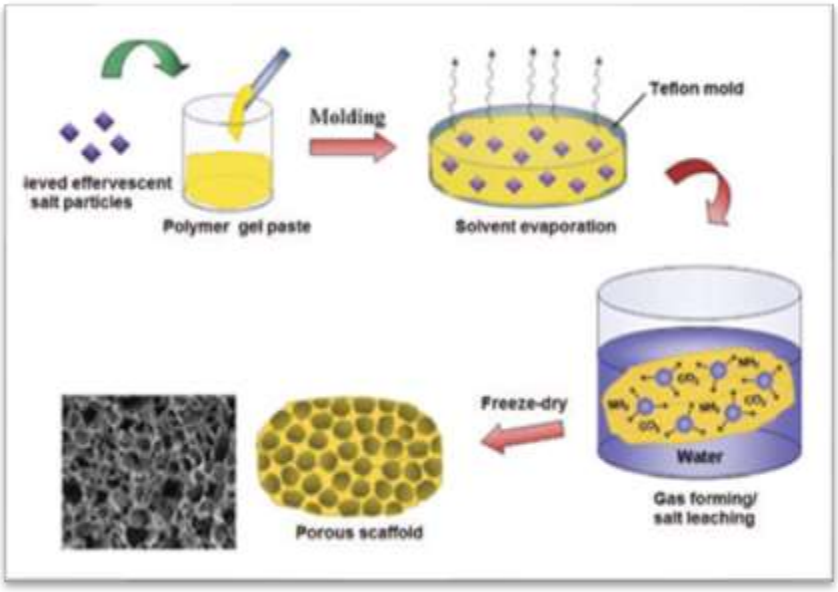


Image 5-1: Schematic representation of solvent-casting/particulate-leaching scaffold fabrication technique. Adapted from: (Loh & Choong, 2013)

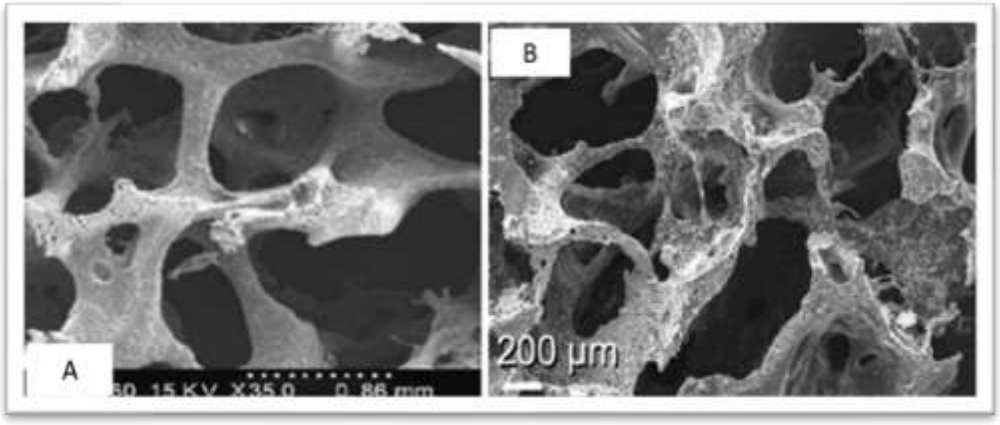


Image 5-2: Typical porous scaffolds produced by solvent casting/particulate leaching technique. A) Adapted from: (Thavornyutikarn et al., 2014), B) Adapted from: (Rezwan et al., 2006)

Freeze-drying (or lyophilization)

First, a synthetic polymer first dissolved into a solvent (typically methylene chloride). Then, distilled water is being put in order to create an emulsion, and polymer/water mixture is being cooled down below its freezing point using liquid nitrogen, so evaporation of the solvent follows (Roseti et al., 2017). After the solvent is completely evaporated, the scaffold is freeze-dried at a pressure of approximately 30 mTorr and temperature at -55^{o}C, that results in the removal of the water and polymer solvent. Subsequently, samples are being placed in a vacuum desiccator at normal temperature for at least 7 days in order to remove any residual solvent (Whang et al., 1995). Finally, a dry polymer scaffold with

large porosity (typically up to 90%) and interconnected porous microstructure remains (Mikos and Temenoff, 2000).

This fabrication technique was first introduced by (Whang et al., 1995) showing several benefits such as: ability to fabricate a scaffold without using high temperatures that could slow down the activity of incorporated biomolecules. Both, pore size and morphology of fabricated scaffold are controllable by tuning several processing parameters (freezing rate, temperature, and polymer concentration). Last but not least, this technique leads to highly interconnected scaffold's pore architecture, while shows no need for washing/leaching step (Thavornyutikarn et al., 2014).

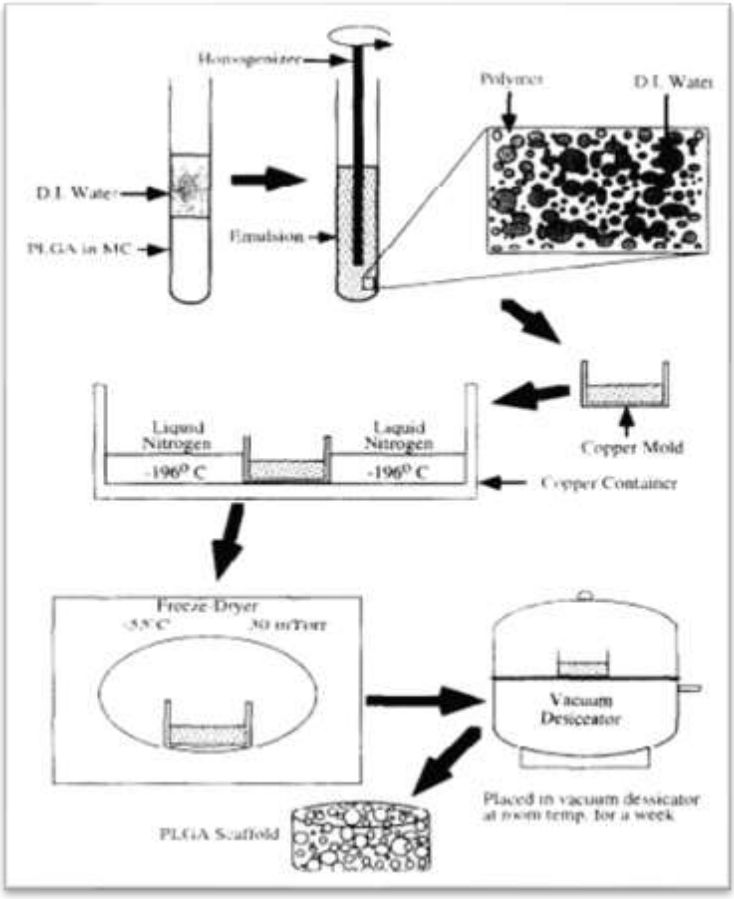


Image 5-3: Schematic representation of the freeze-drying fabrication technique steps. Adapted from: (Whang et al., 1995)

This technique includes numerous limitations like toxic solvents, high energy consumption, and irregular-small (15 - 35 μm) pore size of fabricated scaffold (Thavornyutikarn et al., 2014). Also, freeze-drying technique is a time-consuming fabrication method, requiring days-to-weeks for solvent evaporation (Hutmacher, 2000).

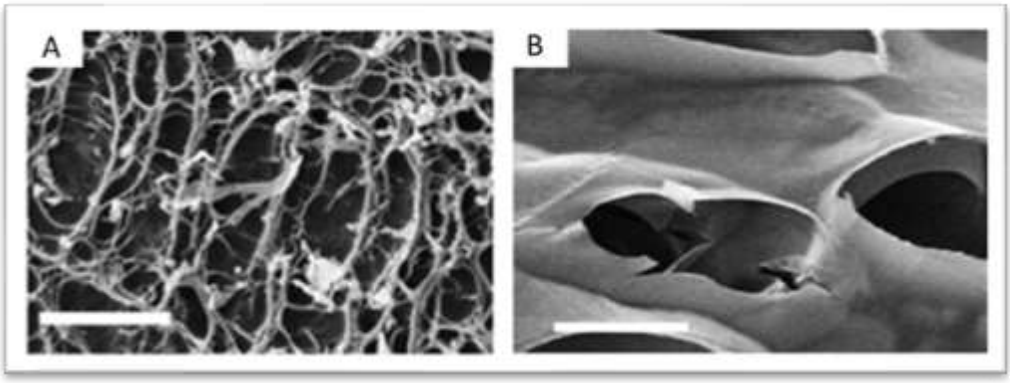


Image 5-4: SEM images of porous scaffolds fabricated by freeze-drying technique using different pressure values A) 6.5 mbar and B) 0.1 mbar. Adapted from: (Lu et al., 2013)

Fiber bonding

Fiber bonding is one of the earliest techniques proposed for scaffold fabrication by (Mikos et al., 1993). In this technique, a polymer (e.g., PLLA) put in methylene chloride and cast over PGA fibers (Image 5-5). Subsequently, the solvent is evaporated, and the construct is being heated above the temperature that melts the above two polymers (McIntire et al., 1998). As a result, the PLLA is being melted first and fills all voids that had been left behind by the PGA fibers. After that, the PGA-PLLA composite is cooled down and the PLLA, that used to normalize the PGA fibers and keep the mesh away from being destroyed, is selectively removed by dissolution in methylene chloride. So, fibers when PGA begins to melt, they become welded together at their cross-points forming a highly porous scaffold, instead of being collapsed (Mikos & Temenoff, 2000).

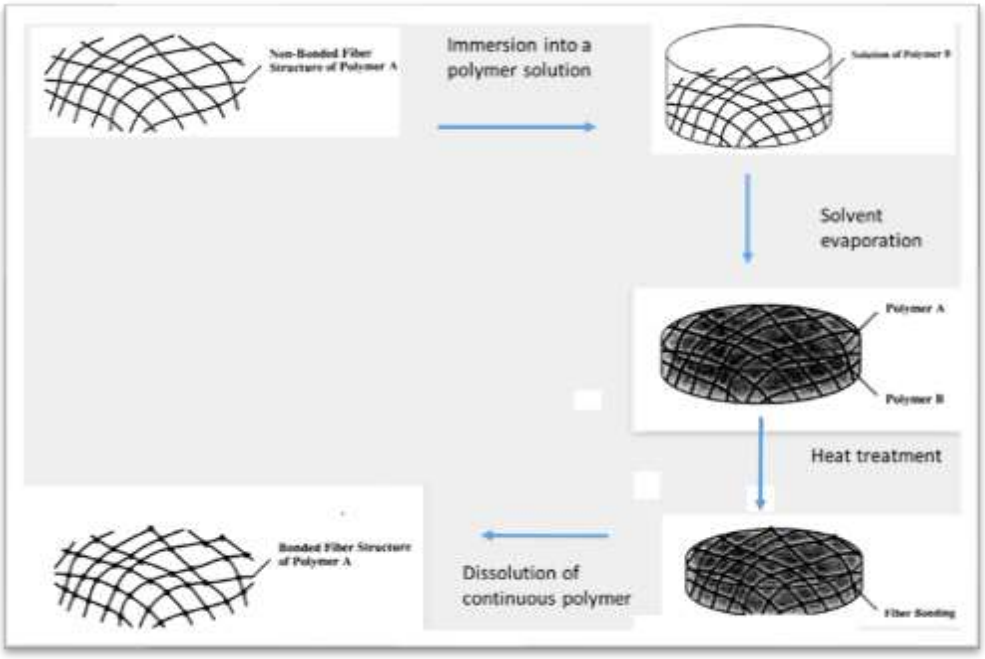


Image 5-5: Schematic representation of fiber-bonding technique steps. Adapted from: (Mikos et al., 1993)

Fiber bonding despite its ease and high porosity of fabricated scaffold shows limitation due to its restricted number of polymer combination and limited ability to control scaffold porosity, and pore size (McIntire et al., 1998). Moreover, the combination of toxic solvents like methylene chloride and high temperatures may raise concerns in applications that incorporate biological agents (e.g., cells or growth factors) (Mikos & Temenoff, 2000).

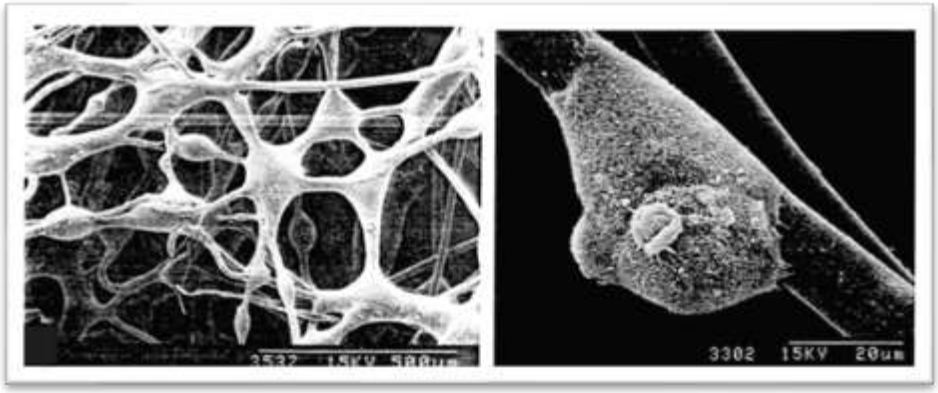


Image 5-6: (L to R) SEM images of PGA nonwoven fiber mesh embedded in PLLA., Hepatocytes attached to a PGA fiber 18h after initial plating. Adapted from: (Mikos et al., 1993)

Powder-forming processes

This technique comprises one of the two techniques used for the fabrication of scaffolds. A mix of ceramic particles in water or ethanol, is being used to shape the pre-sintering ceramic body (called “green” body). In addition,

chemical porogens (e.g., sucrose, camphor, gelatin or PMMA beads) with a surfactant for example are added (Chen, 2011). These fillers are evaporated during heating of ceramic suspension, leaving behind scaffold pores. In order to obtain green bodies of ceramics, several powder-forming methods have been proposed. (Table 8).

Table 8: Methods of obtaining bodies for 3D ceramics. Adapted from: (Ishizaki et al., 1998)

Dry processes • Loose packing • Compaction • Uniaxial pressing • Cold isostatic pressing (CIP)
Wet processes • Slip casting • Injection molding • Phase separation/freeze drying • Polymer replication • Gel casting

Among these techniques, polymer replication (also called polymer-sponge) process, offers the ability to create dispersion of powder in a specific template, that eventually leads in a size of pores that can be controlled in fabricated scaffolds (Thavornyutikarn et al., 2014). This method used firstly by (Chen et al., 2006) to produce a 45S5 Bioglass® scaffold that had porosity >90% and pore size 510-720 μm. Binders (such as polysaccharides, polyvinyl alcohol-PVA, and polyvinyl butyl-PVB) are being also put in to ceramic slurries, in order to strengthen the mechanical ability and maintain the structural integrity of the green body before the sintering of product.

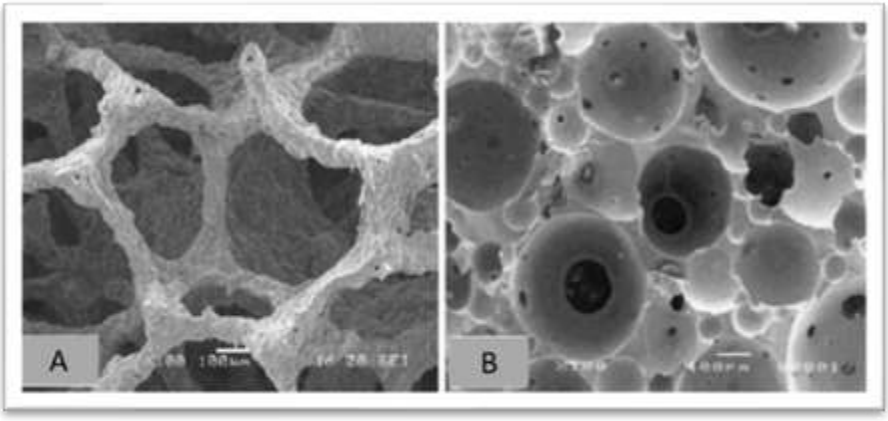


*Image 5-7: SEM images of porous scaffolds fabricated by **A.** polymer replication technique (Chen et al. 2008) and **B.** sol-gel technique (Thavornyutikarn et al., 2014)*

Powder-forming techniques comprise simple methods to use, giving a scaffold similar to bone. On the other side, insufficient mechanical integrity has been

shown, that resulted in making them unsuitable for use in load-bearing applications (Thavornyutikarn et al., 2014).

Sol-gel technique

Sol-gel technique comprises another method for fabrication of bioceramic scaffolds. It is a versatile process, including the formation of a solution by adding a surfactant, after condensation and gelation reactions (Chen, 2011). With this technique, ceramic scaffolds can be fabricated in many shapes (Image 5-7) with high surface area. Nonetheless, the poor mechanical strength of produced scaffold, making this method inappropriate for load-bearing application.

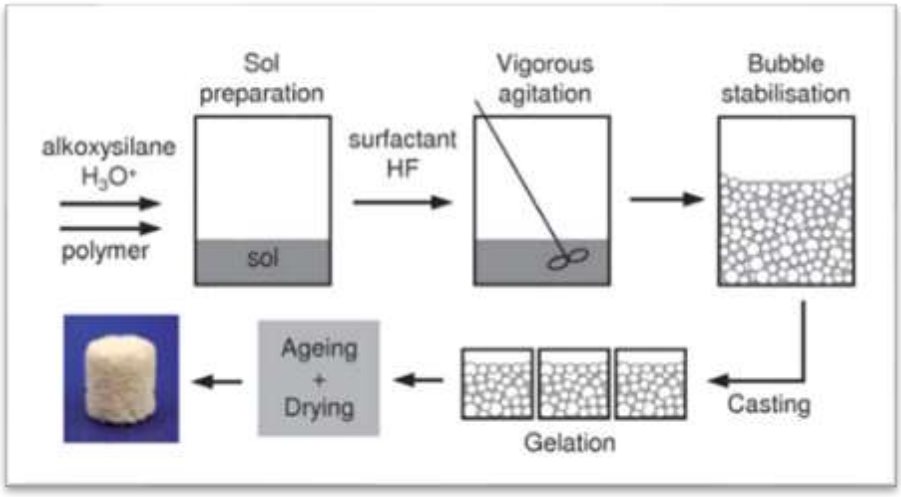


Image 5-8: Schematic representation of sol-gel technique steps. Adapted from: (Valliant & Jones, 2011)

Gas foaming/supercritical fluid technology

First, solid discs of biodegradable polymers (e.g., PGA,PLGA or PLLA) are created by compression molding through a heated mold. Then, polymer discs are put in a place where they are being pressurized at pressures, as 15 MPa for example with CO_2, nitrogen or water until mix is being full of gas bubbles (Zhu & Che, 2013). Subsequently, pressure is being reduced a lot to atmospheric pressure and creation of pores occurs. This technique forms a structure with pore size of 30-700 μm and porosity up to 85% (Thavornyutikarn et al., 2014).

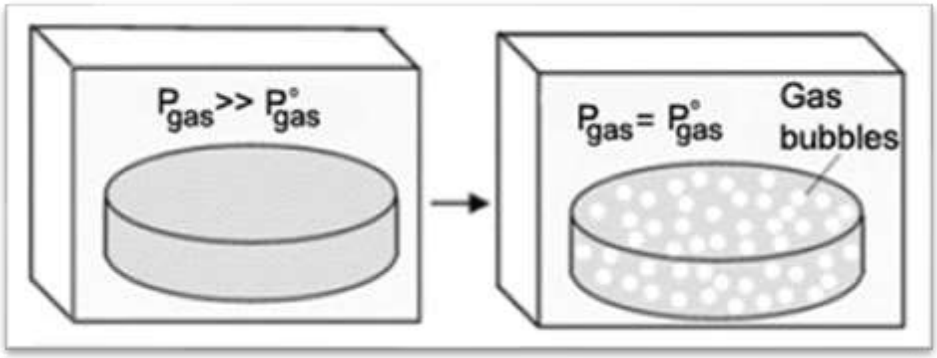


Image 5-9: Gas foaming technique: polymers are exposed to high pressure (e.g., 5.5 MPa) resulting to saturation of the gas and then decrease of pressure leads to the creation of bubbles. Adapted from: (Puppi et al., 2010)

Despite gas-foaming technique does not use organic/cytotoxic solvents and we can tune the size and morphology of the scaffold by changing fabrication parameters (e.g., temperature, pressure), it suffers from several limitations. The limitation of this process are more or less: use of heat during compressive molding that limits the utilization of cells, the closed and non-interconnected pore structures that lead to mechanical integrity, and the creation of a layer without pores at the surface of the scaffold (Roseti et al., 2017). To manage an interconnected pore complex, Harris and his coworkers incorporated gas-foaming with leaching (Image 5-10). Using this technique, they achieved PLGA scaffolds production and levels of porosity up to 97% (Harris et al., 1998).

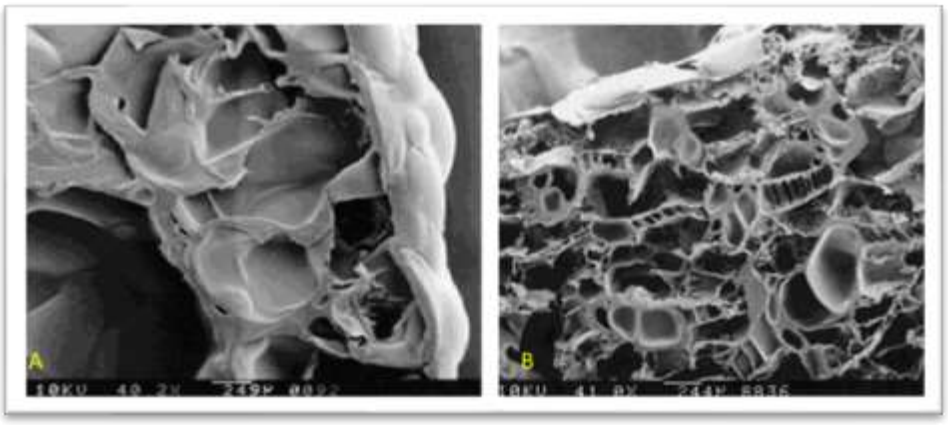


*Image 5-10: SEM images of polymers matrices fabricated by **A)** gas foaming process and **B)** gas foaming/particle leaching technique. By combining these two-fabrication method, we achieve higher porosity. Adapted from: (Harris et al., 1998)*

Thermally induced phase separation (TIPS)

Phase separation method can be performed either thermally or by a nonsolvent, while using a nonsolvent, the fabricated scaffold displays a pore structure that is not likely in tissue engineering (Lu et al., 2013). Thermally induced phase separation (TIPS) is a low temperature method, where a solution of polymers is cooled rapidly while a liquid-liquid phase separation occurs, in order to pass from the following phases: a polymer-rich and a polymer-poor

phase (Roseti et al., 2017). After the sublimation[8] of solvent, the polymer-rich phase solidifies forming a matrix, while the polymer-poor phase is taken away and a porous network if being left behind. This results to obtaining a 3D porous scaffold.

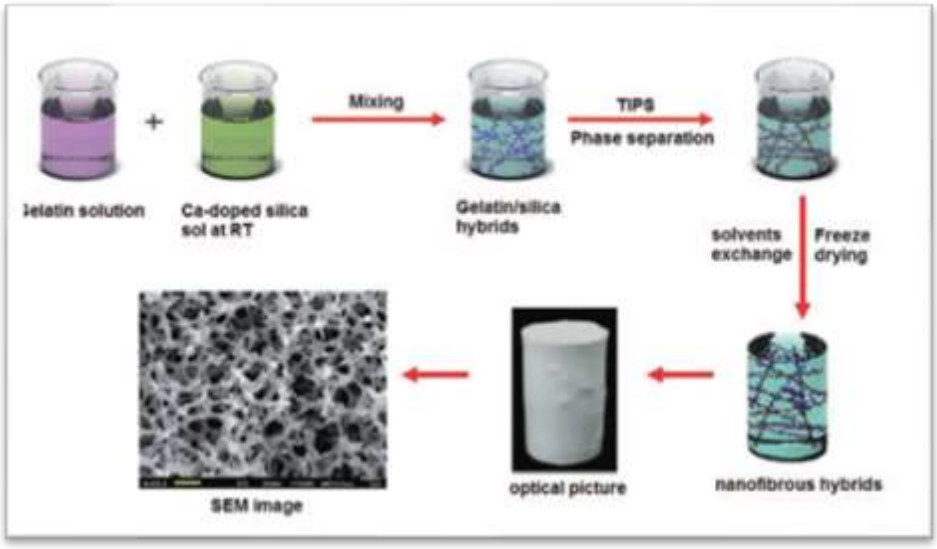


Image 5-11: Thermally Induced Phase Separation (TIPS) fabrication method. Adapted from: (Loh & Choong, 2013)

The most important benefit of this method is the nano-scale fibrous structure of scaffolds that mimics the ECM architecture providing a better environment for cell attachment (Zhu & Che, 2013). One more benefit of TIPS technique is the excellent porosity (>95%) of fabricated scaffolds especially when some of the above techniques are being combined like freeze-drying.

On the other hand, despite the high porosity achieved with this technique, pore size is typically < 200μm limiting its probable applications in bone tissue engineering (Hutmacher, 2000). Moreover, this technique shows further limitations like the use of organic solvents that may cause severe inflammatory responses and long time for solvent sublimation (>48 hours) (Rezwan et al., 2006).

[8] Sublimation is the transition of a substance from the solid phase to the gas phase, but without the intermediate liquid phase.

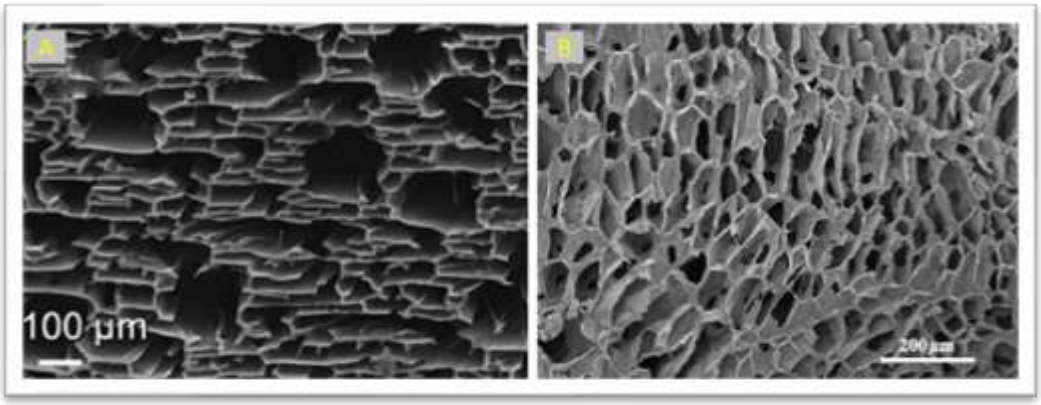


Image 5-12: SEM images of scaffolds fabricated using phase-separation technique. Adapted from a) (Boccaccini and Blaker, 2005), b) (Ma et al., 2000)

Electrospinning (textile technology)

Electrospinning is a fabrication technique that uses electrical charges to create fine fibers of several nanometer and form a nanofibrous architecture having surface areas able to absorb proteins and binding sites to cell membrane receptors (Roseti et al., 2017). An original electrospinning system can carry the following parts (Image 5-13): a **syringe pump with polymers inside**, a **high voltage power supply** to creating an electric field, and a grounded **collector** to collect the fibers that are being produced. The fabrication process starts with a voltage of 10kV value for example and is being applied to a capillary tube. Because of the applied high voltage, a charge repulsion is added in the polymer solution, which prevents the surface tension (Lu et al., 2013). As the electric field is being rising ,the charge repulsion will pass over the surface tension of the droplet and generates a charged liquid jet (Roseti et al., 2017). As this jet travels, the solvent (e.g., chloroform, methanol, etc.) evaporated and jet solidified to create a fibrous membrane.

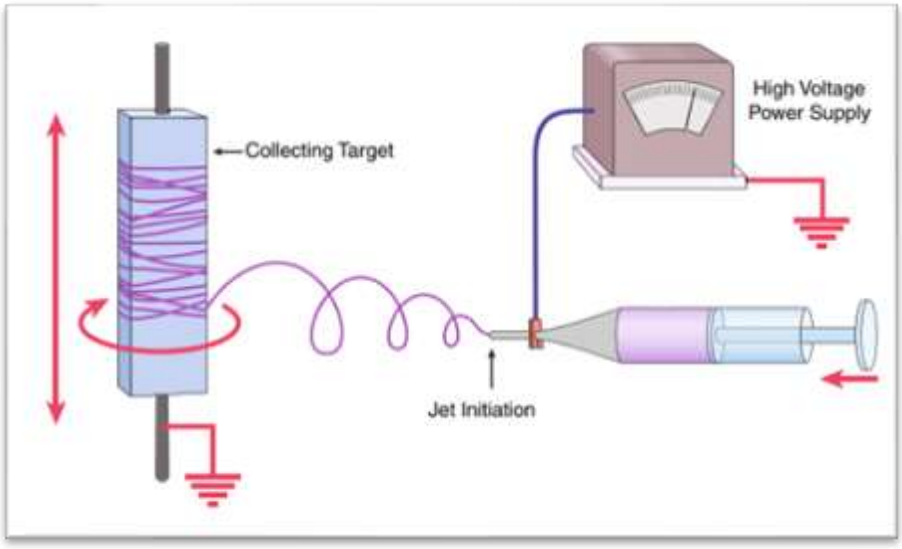


Image 5-13: Schematic representation of electrospinning technique. Adapted from: (Pallua & Suschek, 2011)

Electrospinning technique displays the ability to utilize a variety of polymers such as PGA, PLA, PLGA, PLLA, and PCL and other polymers such as collagen, silk fibroin, and chitosan to produce fibers of several nano-micro meters (Di Martino et al., 2011; Zhu & Che, 2013). Moreover, the produced nanofibers using this technique can be used through incorporation of bioactive molecules. Several variables, including polymer molecular weight and concentration, surface tension, solution viscosity, electric field strength and voltage, polymer flow rate, needle tip design, type of collector used (stationary or rotating), tip to collector distance, and ambient parameters (humidity, temperature etc.) could be tuned so to control the fiber diameter and morphology better (Bhardwaj & Kundu, 2010).

Electrospinning has been extensively researched and gained considerable popularity in the past decade, especially in bone tissue engineering because of the equivalence of the produced fibers to bone ECM (Li & Mai, 2017). Except from nanoscale of produced fibers, one more benefit of electrospinning is excellent scaffold porosity (>90%) with small/interconnected pores. Electrospinning also provides a simpler and inexpensive technique compared to other fabrication methods (e.g., phase separation) (Pham et al., 2006). On the contrary, the biggest limitation of the mentioned method is that it could result in being toxic to cells or other biological agents if not completely removed (Roseti et al., 2017).

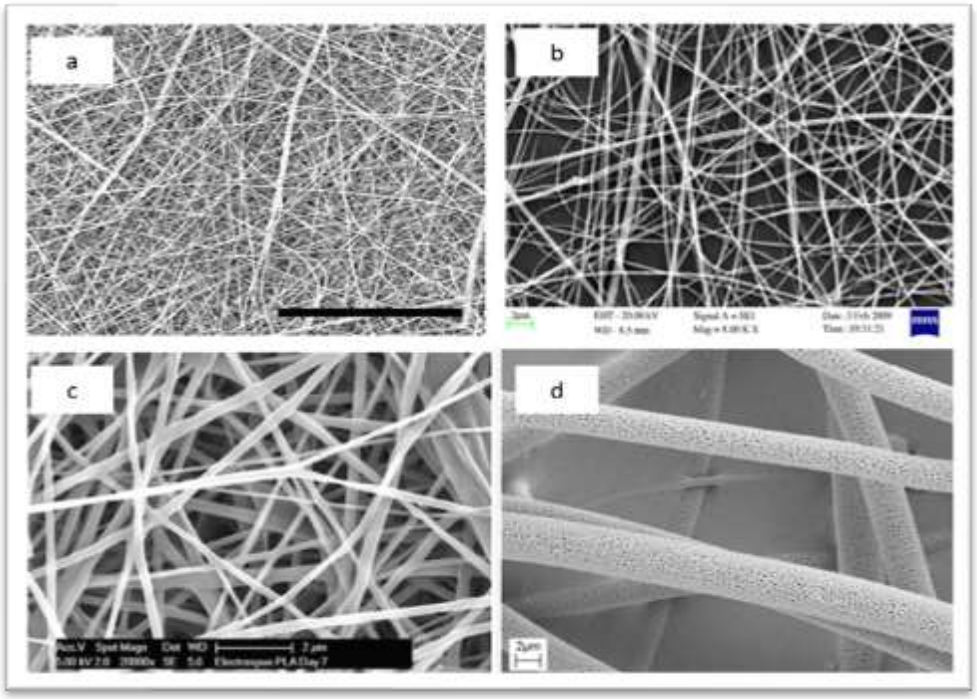


Image 5-14: Various SEM images of scaffolds fabricated with electrospinning technique. a) Electrospun fibers of 5% PCL solution. Adapted from: (Pham et al., 2006). b) Electrospun fibers of 4% PEO. Adapted from: (Bhardwaj and Kundu, 2010). c) Electrospun PLA fibers. Adapted from: (Li and Mai, 2017). D) Electrospun PLLA microfibers with micropores on their surface. Adapted from: (Di Martino et al., 2011)

Table 9: Summary of properties, advantages, and disadvantages of several fabrication techniques.

Technique	Pore size (μm)	Porosity (%)	Advantages	Disadvantages
Solvent casting/ particle leaching	20-50	30-300	Ease of fabrication; low cost; tunable scaffold features	time-consuming process; insufficient mechanical integrity; use of organic solvents; possibility of residual solvent
Freeze-drying	15-35	> 90	good porosity ; pore structure with high interconnectivity	small pore sizes; use of toxic solvents; insufficient mechanical integrity for use in load-bearing application;
Fiber bonding	-	-	Simple method	Limited number of polymer combinations; use of toxic solvents; limited control of pore size and porosity
Powder-forming processes	300-700	> 80	Simple method; high porosity similar to cancellous bone; high surface area to volume ratio	insufficient mechanical integrity;
Sol-gel techniques	> 600	> 70	High surface area	Insufficient mechanical integrity for use in load-bearing application; possibility of solvent residual
Gas foaming	30-700	> 85	Free of organic toxic solvents; controllable pore size and porosity	formation of an nonporous outer layer; Insufficient mechanical integrity for use in load-bearing application; use of excessive heat that may hamper the incorporation of biological agents
TIPS	5-600	< 90	Simple method; excellent porosity; pore structure with high interconnectivity; nano-scale pore sizes	Use of organic solvents; shrinkage issues
electrospinning	1-10	90	Simple method; high interconnected porosity; high surface area to volume ratio; nano-scale pore size; inexpensive process compared to the rest conventional techniques	Use of organic solvents; insufficient mechanical integrity for use in load-bearing application

Disadvantages of conventional scaffold fabrication techniques

Conventional fabrication methods that were described before, can produce porous scaffolds of various types having good porosity, sufficient mechanical integrity for load-bearing applications, good biocompatibility etc. However, these techniques are incapable of producing 3-D scaffolds with precise pore size, geometry, morphology, and pore interconnectivity (Thavornyutikarn et al., 2014). Moreover, the production of scaffolds using most of these techniques require organic solvents to dissolve polymers or porogens to create pores that may have opposite results on the supported cells. Thus, **additive manufacturing (AM) techniques** have emerged, enabling the creation of scaffolds with tailored porosity and pore size/shape.

5.2. Additive manufacturing (AM) techniques

Additive manufacturing (AM) techniques, also named as **Rapid Prototyping (RP)** Technologies or **Solid Freeform Fabrication** (**SFF**) methods, are conjugated as a complex of processes that can produce scaffolds layer-by-layer with complex shapes from a computer aided design (CAD) file (Hutmacher, 2000). The main AM techniques used for the construction of scaffolds in tissue engineering are **Stereolithography** (**SLA**), **Fused Deposition Modeling** (**FDM**), **Selective Laser Sintering** (**SLS**), **Inkjet 3D Printing** (**3DP**) and **Bioprinting** that will be thoroughly discussed below.

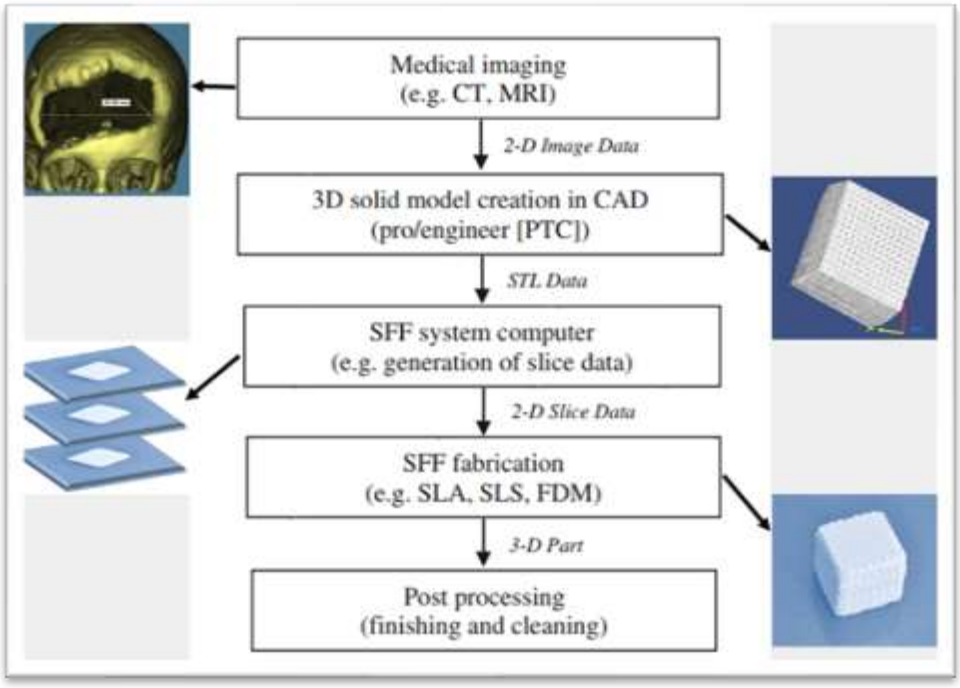


Image 5-15: Steps followed to fabricate 3D scaffolds in every additive manufacturing technique. Adapted from: (Roseti et al., 2017; Leong et al., 2003)

1. Stereolithography (SLA)

Stereolithography (SLA) was introduced by Charles Hull who came up with this technique in his patent created back in 1986. A typical SLA system contains a tank filled with photo-sensitive liquid resin, a platform with ability of moving, a UV laser to irradiate the resin, and a mirror (Thavornyutikarn et al., 2014). The SLA process starts by importing a computer-aided design (CAD) file, created by a medical image giving the power to form patient-specific scaffolds with the use of clinical imaging techniques (e.g., CT, MRI etc.). This CAD file is being switched to a **standard tessellation language** (**STL**) file containing the contributes of triangles which created layer-by-layer the surface of the 3D scaffold-structure. Then, STL file is being cut into thin layers forming a **slice file** (**SLI**) which is being put into SLA apparatus (Mota et al., 2012).

Subsequently, UV laser irradiates and deposits a film of a photo-sensitive liquid resin into the built system in some pattern defined by the CAD file (Skoog et al., 2013). The thickness of fabricated layer on each cycle (cure depth) is crucial for every stereolithography application and determined by the light energy that cures the photosensitive resin (Melchels et al., 2010). Once a film is completely solidified, the system is vertically reduced, and next layers of 3D scaffold are created. Above steps are continued until a full 3D scaffold will be created.

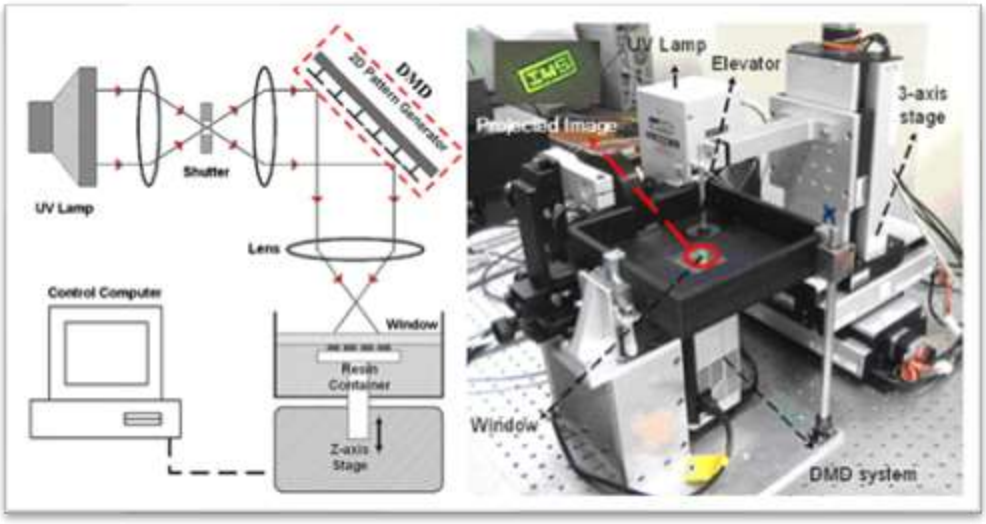


Image 5-16: Schematic representation and photograph of an SLA system. Adapted from: (Kang et al., 2009)

SLA is used in 3D scaffolds within a range of several biomaterials: synthetic polymers, PCL, and PDLLA; bioceramics, such as β-TCP, and suspensions of HA; and composites such as bioactive glass/methacrylated PCL, PDLLA/HA, and PPF/ HA (Bose et al., 2013; Thavornyutikarn et al., 2014). However, the use of bioceramics either alone or in composite scaffolds, can be controversial due to their high viscosity that could mess up the above mentioned process (Melchels et al., 2010). Due to this limitation, many researches have used non direct stereolithography techniques which are beyond of the scope of this study.

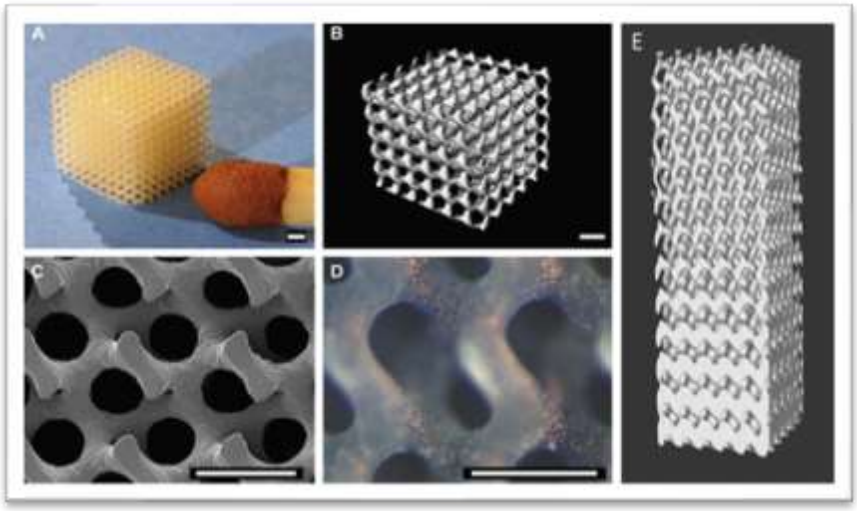


Image 5-17: Images of several PDLLA scaffolds fabricated by SLA. A) Photograph, B) μCT reconstructed image, C) SEM image, D) microscopy of a PLLA scaffold seeded with mouse pre-osteoblasts and E) micro-CT reconstructed image. Adapted from: (Melchels et al., 2009; Melchels et al., 2010b)

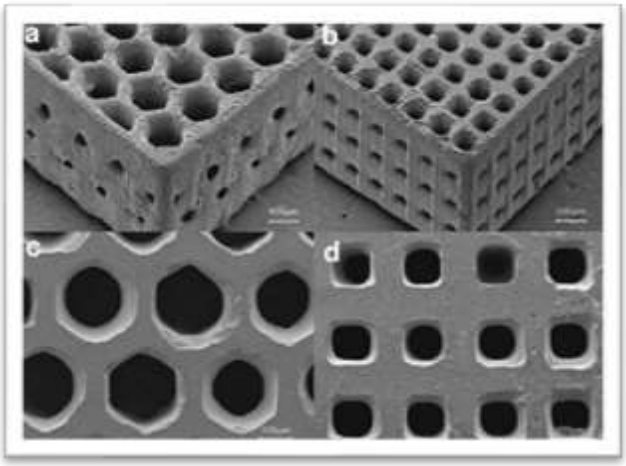


Image 5-18: Various PPF scaffolds constructed using stereolithography (SLA) technique. Adapted from: (Jansen et al., 2009)

Stereolithography (SLA) as a scaffold fabrication technique, can fabricate scaffolds with complicated inside features like well-defined pore size, pore interconnectivity, and pore gradients (Skoog et al., 2013). It shows excellent accuracy (< 50 μm), excellent porosity (up to 90%), and high spatial resolutions (both vertical and lateral) producing scaffold layers usually in the range of 25-150 μm, while scaffold pores can be below 1 μm (Thavornyutikarn et al., 2014). Moreover, SLA fabricated scaffolds are able to incorporate cells or growth factors, a feature that expand their potential uses in tissue engineering field.

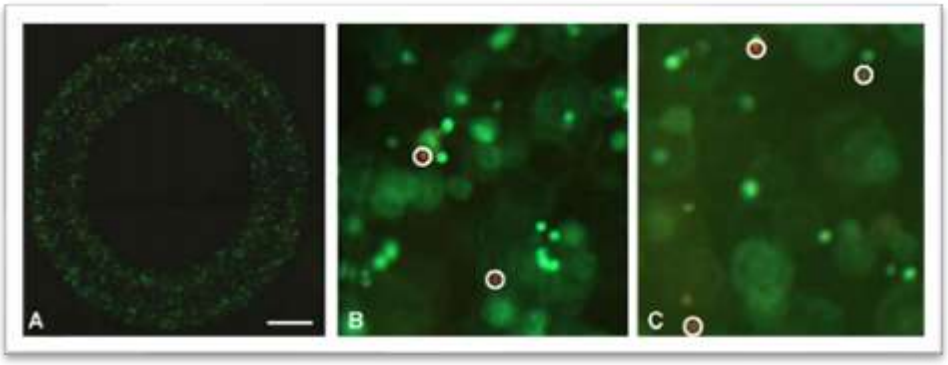


Image 5-19: Human dermal fibroblasts encapsulated in PEG hydrogel scaffolds fabricated using SLA technique. A) Low magnification image, B) 10x fluorescent image 2h after fabrication, C) 10x fluorescent image 24h after fabrication. Red spots in the images identify the dead cells, while fluorescent green identify alive cells. Scale bar represents 1 mm. Adapted from: (Arcaute et al., 2006)

There are several restrictions on SLA technique, such as: requirement for high cost machinery systems, limited number or photopolymer resins, skin soreness created by photo-sensitive resins utilized in this technique, and scaffold shrinkage during polymerization (Roseti et al., 2017; Thavornyutikarn et al., 2014; Melchels et al., 2010).

In order to enhance spatial resolutions and design flexibility of fabricated scaffolds, SLA technique has been modified from early developed system (Skoog et al., 2013). In that way, three-dimensional scaffolds having features less than 5 μm can be created and three advanced stereolithographic methods can be mentioned: **micro-stereolithography** (**μSLA**), **two-photon polymerization** (**2PP**), and **digital light processing** (**DLP**).

Advanced stereolithographic processes

Microstereolithography (**μSLA**) comprises an advanced stereolithographic technique, where laser ray is centered with more accuracy upon photosensitive resin, reducing the point size to the scale of some micrometers. This achieved by projecting the scaffold pattern, that comes from CAD file, onto the surface of the photosensitive resin (Image 5-20) (Bartolo et al., 2008). With this way, each film of scaffold is solidified in only one irradiation, speeding up the whole fabrication process.

Microstereolithography displays higher accuracy (0.2 μm), and better resolution in the range of 0.5-10 μm compared to standard SLA process (Mota et al., 2012). Moreover, the layer thickness achieved using this technique can be reduced to approximately 1 μm (Thavornyutikarn et al., 2014).

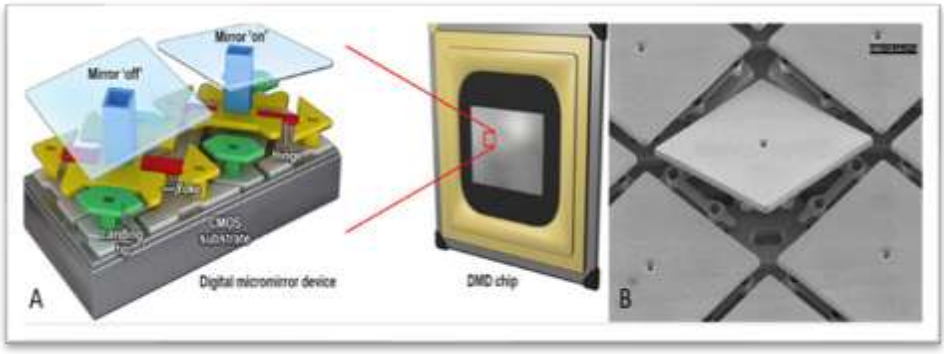


Image 5-20: A) Graphic illustration of DMD layout. Pixels of mirror can be individually turned on (+10°) and off (-10°), B) SEM image of a DMD showing the microstructure. Adapted from: A) https://www.nature.com/articles/nmeth.f.402 B) https://spectrum.ieee.org/tech-history/silicon-revolution/chip-hall-of-fame-texas-instruments-digital-micromirror-device

Two-photon polymerisation (**2PP**) comprises a more advanced stereolithographic approach where photosensitive resin is polymerized upon nearly simultaneously saturation of two photons with low power, which together introduce enough energy to destroy the labile bond and begin the polymerization reaction (Melchels et al., 2010). In this technique, biomaterial molecule simultaneously absorbs two photons, not one, being excited to higher state. To produce these photons, a femtosecond laser, working at almost800 nm wavelength is preferred due to short pulse width and high peak power (Narayan et al., 2010). Laser beam focused into a volumetric pixel (voxel) of photo-curable resin making it possible to fabricate 3D scaffolds in micro/nano scale. Using 2PP technique spatial resolution up to 100 nm can be obtained (Lee et al., 2008). In addition, this method comprises an ultra-fast scaffold fabrication technique (Bartolo et al., 2008).

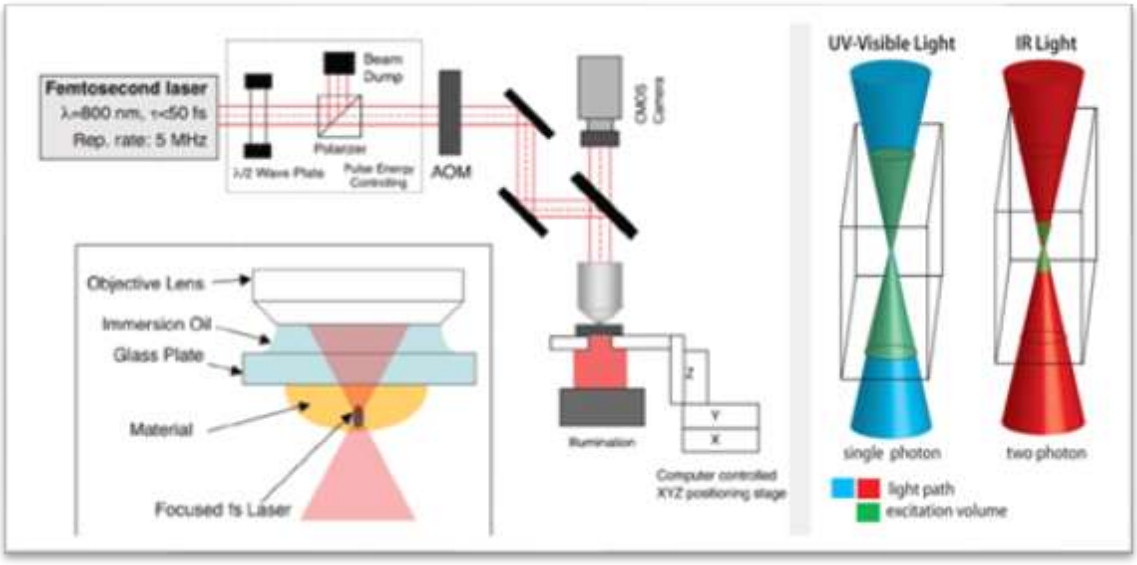


Image 5-21: A) Two-photon photopolymerization (2PP) enables 3D micro/nano scaffold fabrication by curing a resin voxel at once. Adapted from: (Paz et al., 2012)

Digital light processing (**DLP**) is an SLA technique, using digital mirror devices (DMDs) or mask generators (Image 5-22). The image of scaffold layer is created onto the DMD using UV light, so as a complete scaffold layer can be created at once (Melchels et al., 2010). DLP technique displays benefits of reduced machinery cost as it does not require a laser source, and higher fabrication speed because of liability of one scaffold film at time (Bartolo et al., 2008). The DLP-based method is successfully utilized to create scaffolds from bioceramics such as 45S5 Bioglass®, β-TCP or Alumina (Al_2O_3). DLP process has displayed spatial resolution approximately 40 μm, while layer thickness ranges from 15 to 70 μm (Thavornyutikarn et al., 2014).

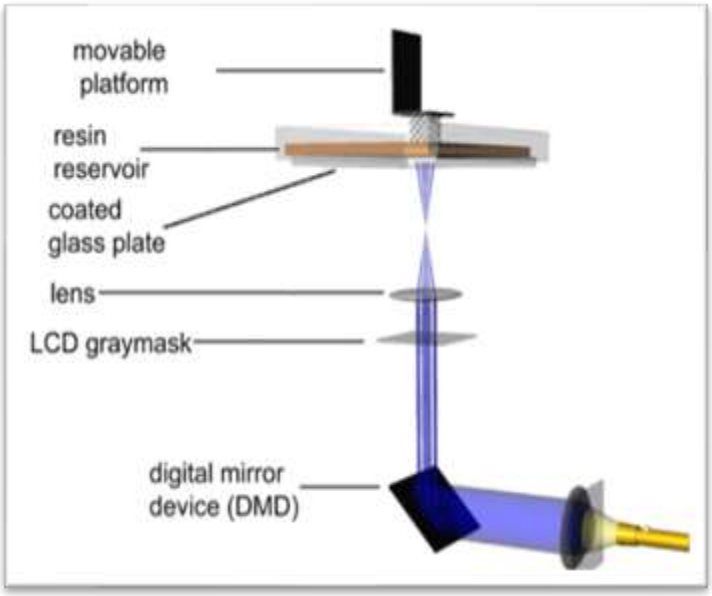


Image 5-22: A typical top-down DLP setup. Adapted from: (Melchels et al., 2010)

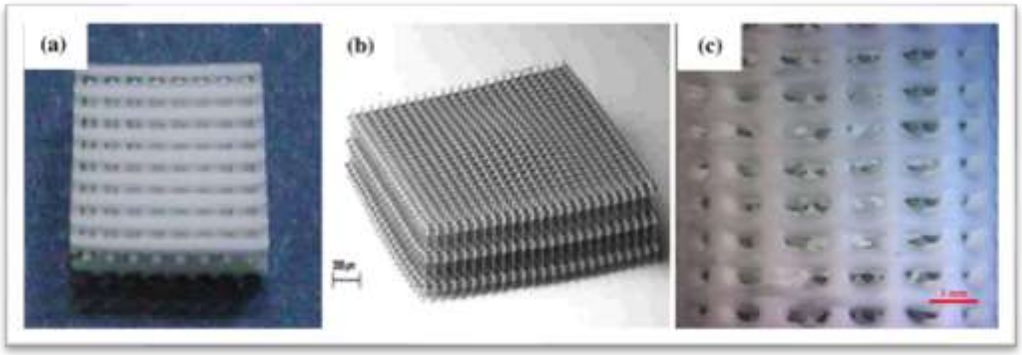


Image 5-23: Various scaffolds fabricated using advanced SLA techniques: A) HA/TCP structures prepared using μSLA, B) methacrylated oligolactones created using a 2PP system, and C) 45S5 Bioglass® using DLP system. Adapted from: (Thavornyutikarn et al., 2014).

2. Fused Deposition Modeling (FDM)

The extrusion-based rapid prototyping (RP) method, also named **Fused Deposition Modelling** (**FDM**), was initiated and patented by Crump in 1989, while it was first marketed by Stratasys Inc. later in 1992 (Crump, 1992). In this process, scaffolds fabricated by liquefying some material, usually thin thermoplastic filaments, through a heated mouthpiece with a small hole. Subsequently, thin material filaments are laid down as strings on a build platform to create a three-dimensional (3D) scaffold, following a pattern predefined by a CAD file.

The hole moves in both x and y planes so that fiber is left on a parallel series of material rods to form a film (Thavornyutikarn et al., 2014). After the complete construction of each layer in the xy plane, build system is reduced (in z axis) and procedure is being repeated, building a new film on top of the previous. Another approach of FDM technique uses two autonomous holes that can deposit two not identical materials at the same time. The first extrusion hole is employed to construct the 3D scaffold, while the second one extrudes a supporting material (Yeong et al., 2004).

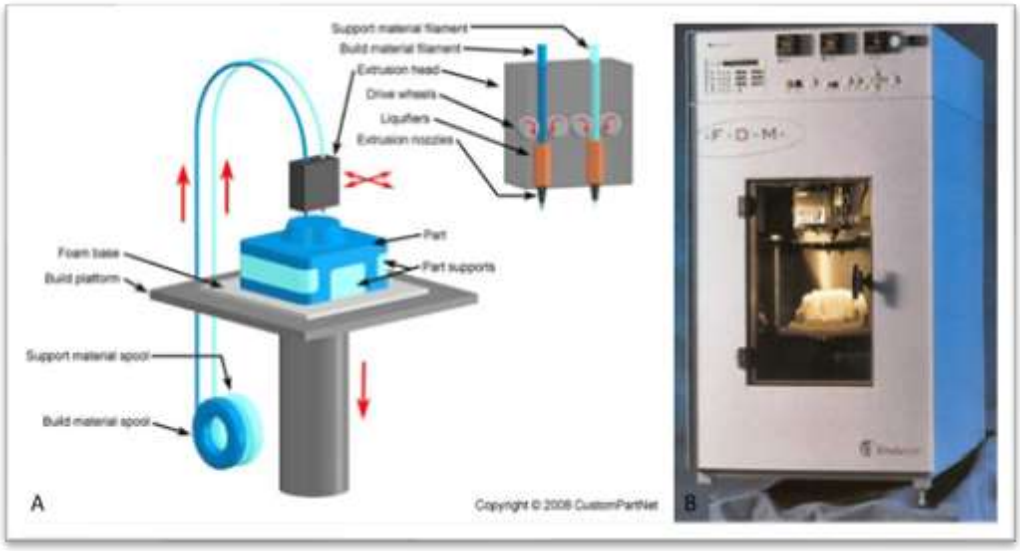


Image 5-24: A) Fused Deposition Modeling (FDM) process schematic diagram B) FDM 1650 system produced by Stratasys Inc.

FDM process is successfully utilized for producing scaffolds using PCL, PLGA, PCL/HA, and PCL/TCP as building materials (Yeong et al., 2004). Among these materials, PCL comprises an ideal material for this technique due to its low temperature (-60°C), increased dissolution temperature, good stability to various environmental conditions (e.g., temperature, moisture etc.) and adequate flexibility (Zein et al., 2002).

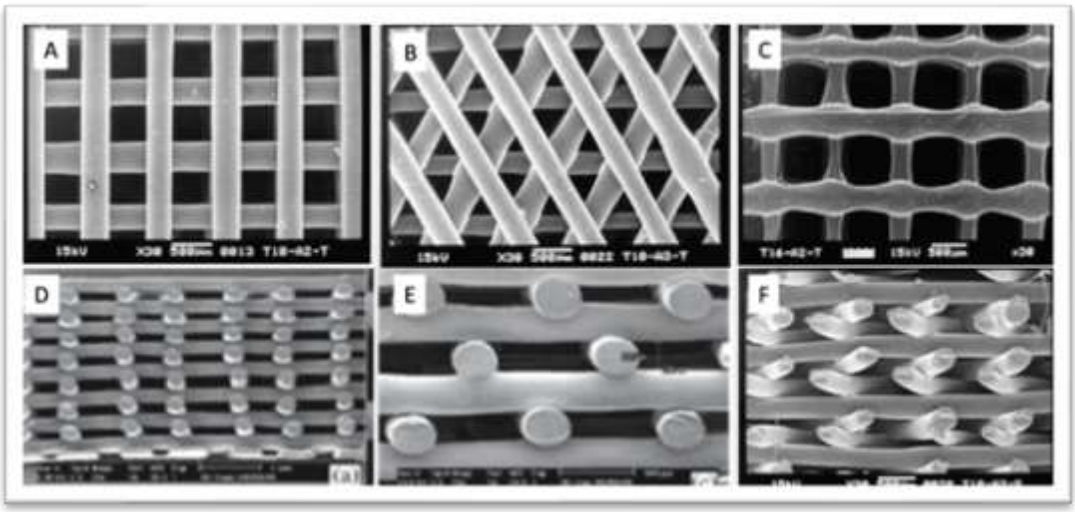


Image 5-25: SEM images of various 3D scaffolds prepared with FDM technique. A, B, C, and F Adapted from: (Zein et al., 2002); D, and E Adapted from: (Woodfield et al., 2004)

The benefits of FDM technique could be the good mechanical strength and high material porosity giving versatility in lay-down pattern design, no material trapped into the fabricated scaffold, low cost, and no toxic solvent requirement (Roseti et al., 2017; Yeong et al., 2004).

Nonetheless, FDM suffers from several limitations such as: material restriction because of the use of filament-based materials only in molten phase, high operating temperatures that may lead to decomposition of building materials and limited spatial resolution to 100-500 μm in x and y planes and diameter of extruded filament in the z direction (Thavornyutikarn et al., 2014). Last but not least, the lack of micropores in fabricated scaffolds discourages the utilization of this technique in tissue engineering applications that incorporates biological factors (Yeong et al., 2004).

To alleviate limitations of conventional FDM process, various modified FDM processes have been developed. **Multi-head deposition system** (**MHDS**), **low-temperature deposition manufacturing** (**LDM**), **precision extruding deposition** (**PED**), **pressure-assisted microsyringe** (**PAM**), and **robocasting**.

Advanced Fused-deposition modelling (FDM) processes

Multi-head deposition system (**MHDS**) comprises an advanced FDM technique that incorporates more than one extrusion heads enabling the creation of scaffolds with complex composition and shape using more than one biomaterial (Thavornyutikarn et al., 2014). In this way, spatial resolution can be enhanced to the range of several of tens of microns, the list of biomaterials is expanded, while the ability to create scaffolds with micropores enables the incorporation of biological agents. On the other hand, MHDS maintains the limitation of high processing temperatures that accompanies conventional FDM process.

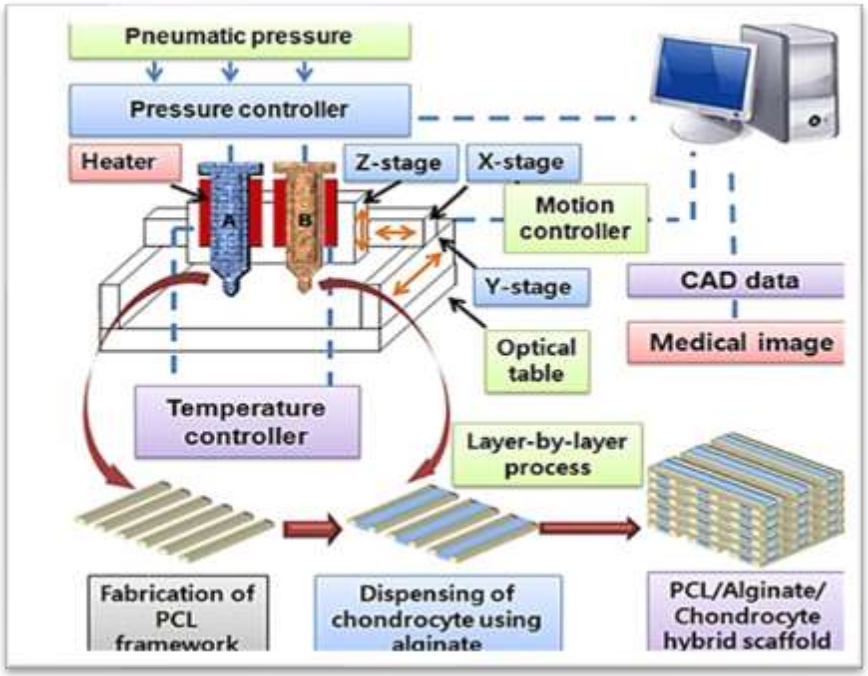


Image 5-26: Schematic representation of MHDS procedure. Adapted from: (Kundu et al., 2013)

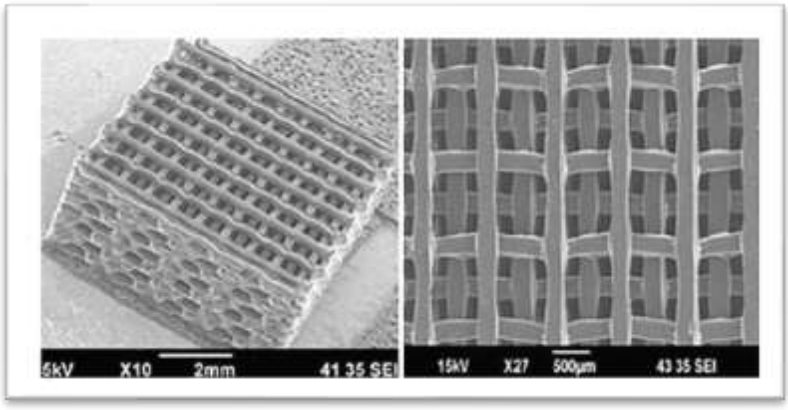


Image 5-27: SEM images of PCL/PLGA scaffolds fabricated via MHDS process. Adapted from: (Kim & Cho, 2009)

Low-temperature Deposition Manufacturing (**LDM**) was developed as an choice method addressing the problem associated with high processing temperatures of conventional FDM process. It was first introduced by (Xiong et al., 2002) and includes the production of 3D scaffolds at a low temperature to compress the material mixture when put on the build platform (Thavornyutikarn et al., 2014). Scaffolds fabricated using this process are able to incorporate several biomolecules, though the need for solvent clearance through freeze-drying process still remains a controversial factor (Mota et al., 2012). LDM is successfully utilized to fabricate PLLA/TCP scaffolds that incorporated BMP growth factors (Xiong et al., 2002).

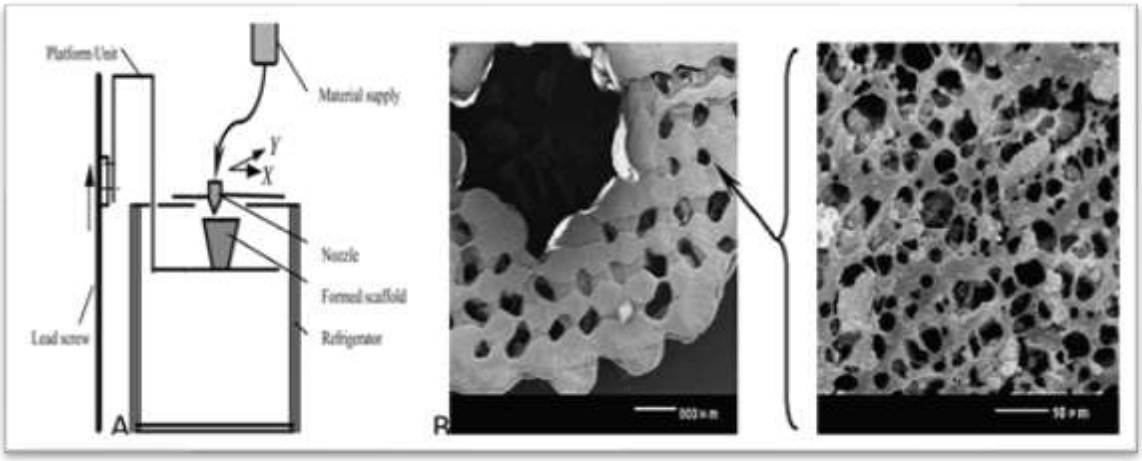


Image 5-28: A. Schematic representation of LDM process. B. Low and high magnified SEM images of PLLA/TCP composite scaffold Adapted from: (Xiong et al., 2002)

To address the problem of requirement for filament preparation in FDM process, **Precision Extruding Deposition** (**PED**) emerged. It uses pellet-formed biomaterials that can be directly deposited into desirable scaffold patterns. This method was introduced by (Wang et al. 2004), though it requires high temperatures making difficult to create 3-D scaffolds able to help with cell growth and proliferation.

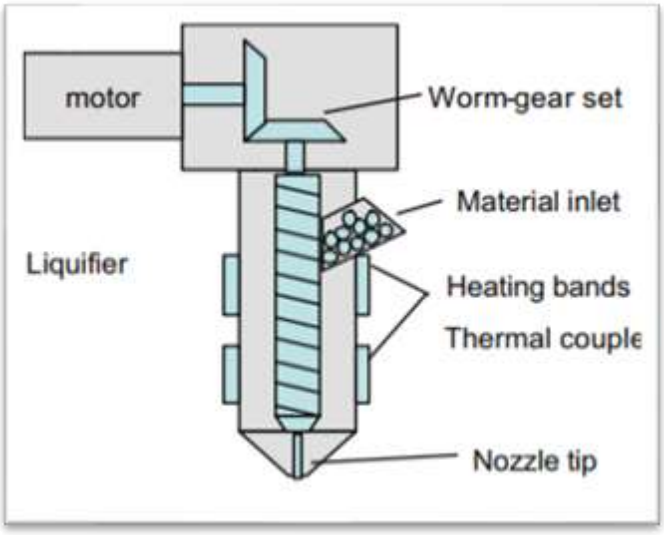


Image 5-29: PED extruder. Adapted from: (Wang et al. 2004)

Pressure-assisted microsyringe (**PAM**) comprises another FDM method initiated by (Vozzi et al., 2002). This method includes a micro-positioning system with a pressure-controlled microsyringe filled with a fine-bore (diameter 10-20 μm) exit needle (Mota et al., 2012). By tuning processing parameters fine resolution, in a value of even 10 μm can be achieved (Yeong et al., 2004). The method displays the capacity to fuse several biomolecules in fabricated scaffolds, though its small nozzle inhibits incorporation of bigger particles (Thavornyutikarn et al., 2014).

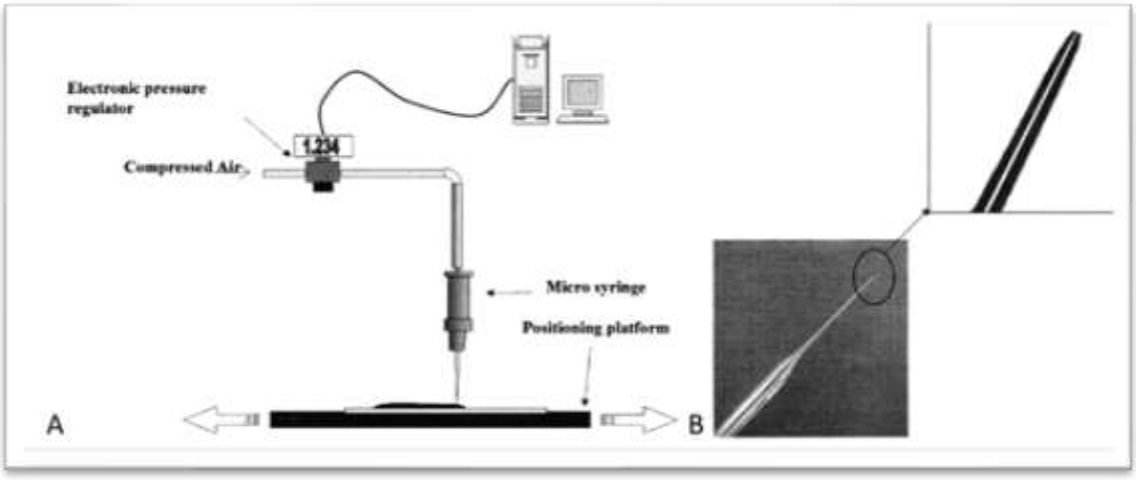


Image 5-30: Schematic representation of PAM system. B) Photograph of capillary needle tip. Adapted from: (Vozzi et al., 2002)

Robocasting also called 'robotic deposition' or '**direct-write assembly**' includes robotic deposition of highly concentrated colloidal suspensions via hole in an oil tube (Bartolo et al., 2008). The method is used to create scaffolds (e.g., β-TCP, HA, PCL/CaP, HA/PCL etc.) with tailored architecture design. This process shows the advantage of building ceramic scaffolds with no need for supporting material as inks are able to support their own weight during scaffold convention (Miranda et al., 2006).

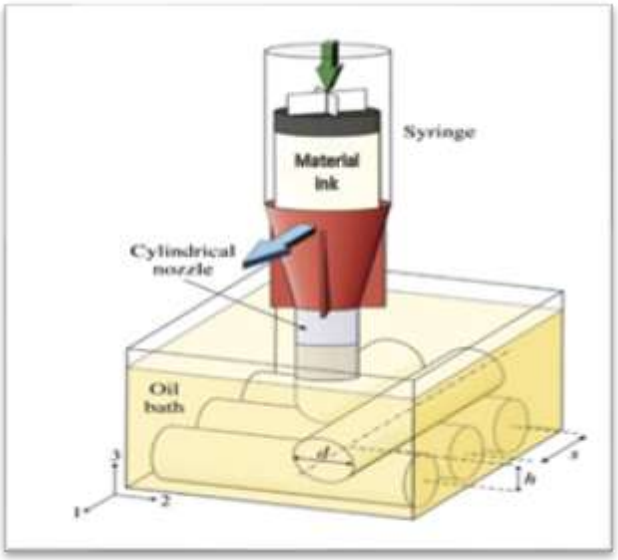


Image 5-31: Schematic representation of robocasting process. Adapted from: (Martínez-Vázquez et al., 2010)

3. Selective Laser Sintering (SLS)

Selective laser sintering (SLS) initiated and patented by (Deckard, 1989) at University of Texas in Austin and was commercialized in 1992 by DTM corporation (later named 3D Systems). It is a family member of rapid prototyping (RP) techniques that uses CO_2 laser ray to merge selected regions of a polymer powder in a powder surface, creating this way, a material film. The material chamber is firstly heated in a temperature just below the glass passage temperature (T_g) of polymer to reduce the energy needed in the combining process (Chu, 2006). Subsequently, the laser ray is scanned over the powder surface, following the cross-sectional data carried by a CAD file, raising the powder temperature just above the glass passage phase temperature and causing the powder particles fuse together to form a scaffold layer at time (Hutmacher et al., 2004). Once the first layer is solidified, the powder bed is lowered by a pre-defined distance (layer thickness), while a roller laid down the next material layer on the top of the bed. This process is being continued until the whole scaffold will be formed, while the unfused material powder around and within the scaffold provides structural support throughout the fabrication process (Thavornyutikarn et al., 2014). Finally, unprocessed powders surrounding the fabricated scaffold have to take off after the fabrication process is completed in order to be used again in next applications.

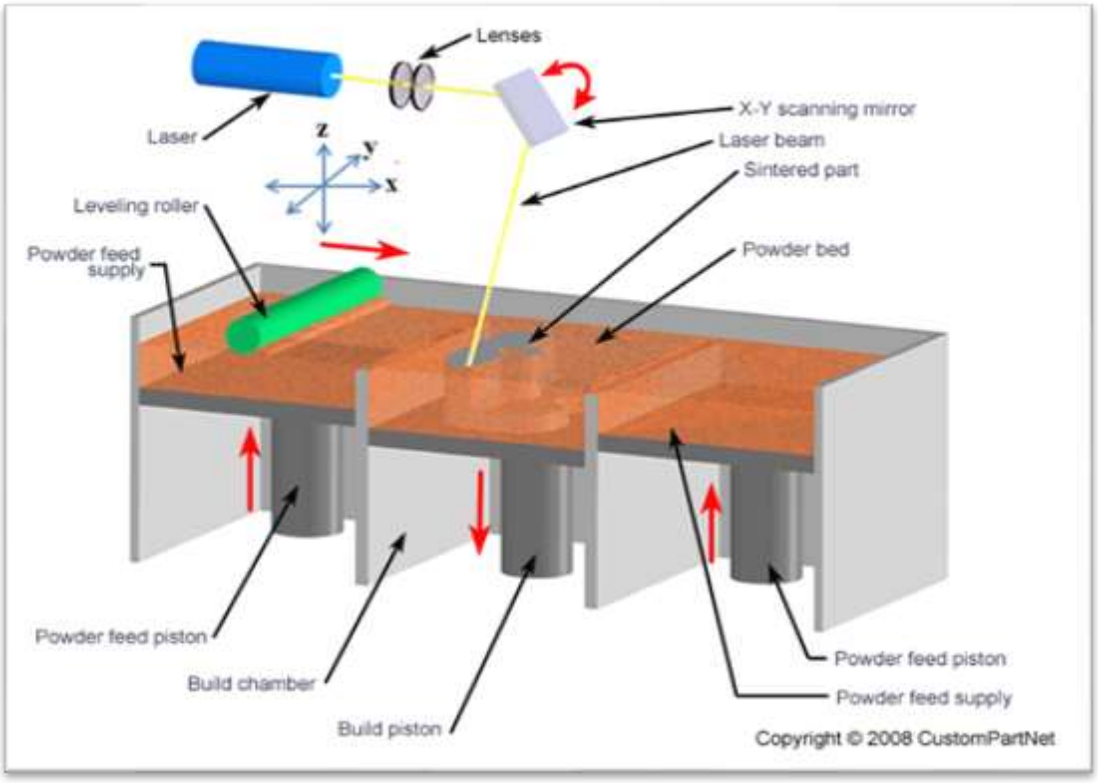


Image 5-32: Schematic representation of SLS process. Source: https://www.custompartnet.com/wu/selective-laser-sintering

A major challenge in every scaffold fabrication procedure using SLS technique, is tracing an optimal mix of various parameters such as laser strength, beam focal spot, scan speed, scan spacing, and powder composition (Thavornyutikarn et al., 2014). SLS method was also used to create tissue-engineering scaffolds from 1) polymers such as PCL, PLLA, and PHBV[9]; 2) ceramics such as β-TCP; and 3) polymer/ceramic composites such as PLLA/HA, PCL/HA, PCL/TCP, and PLGA/HA (Thavornyutikarn et al., 2014; Duan et al., 2010).

[9] poly(hydroxybutyrate–co-hydroxyvalerate)

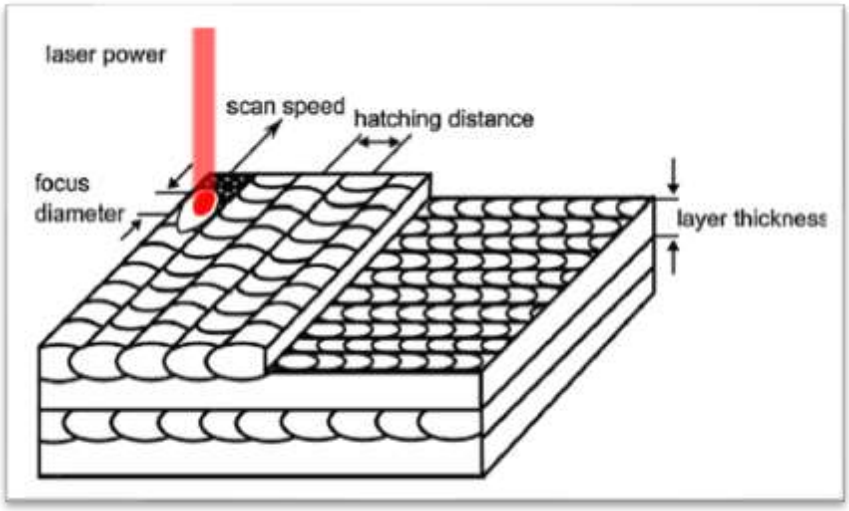


Image 5-33: SLS processing parameters. Adapted from: (Abele et al., 2015)

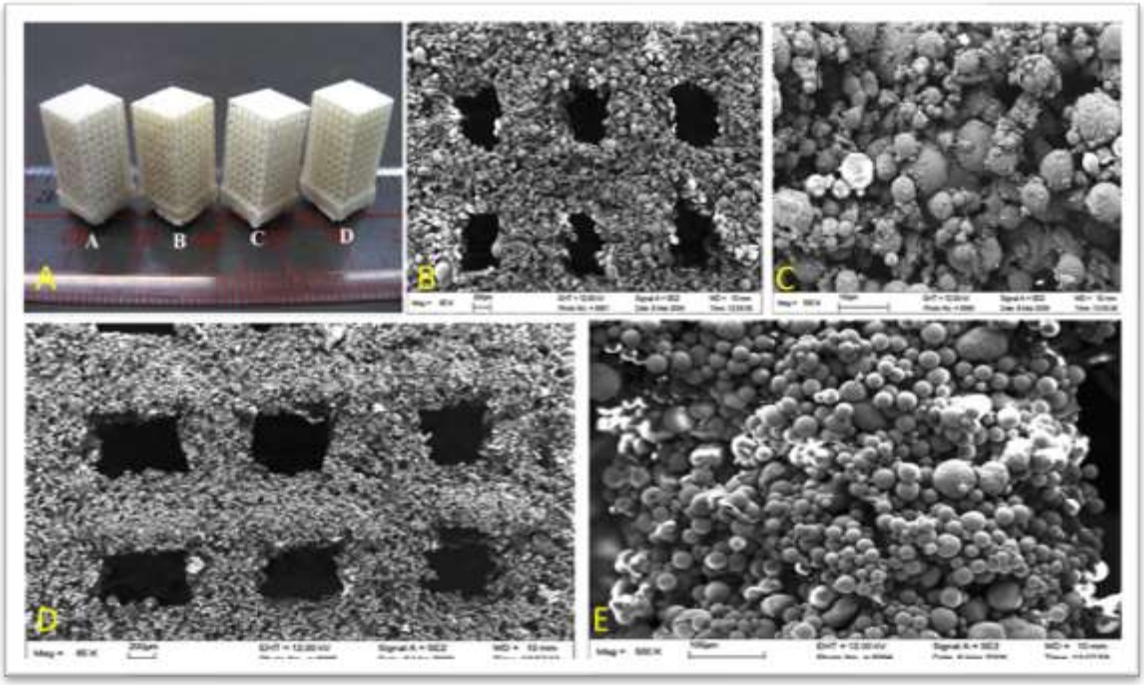


Image 5-34: Various scaffolds fabricated using SLS technique. A) Images of PHBV, CaP/PHBV, PLLA, and HA/PLLA scaffolds. SEM images of sintered scaffolds: (B and C) CaP/PHBV magnified 85x and 500x respectively, (D and E) PLLA magnified 85x and 500x respectively. All adapted from: (Duan et al., 2010)

SLS process is a method well known in tissue engineering applications because of its several advantages. This technique has no need for supporting materials, comprises a solvent-free fabrication technique, while a range of biomaterials can be utilized for scaffold creation (Leong et al., 2003).

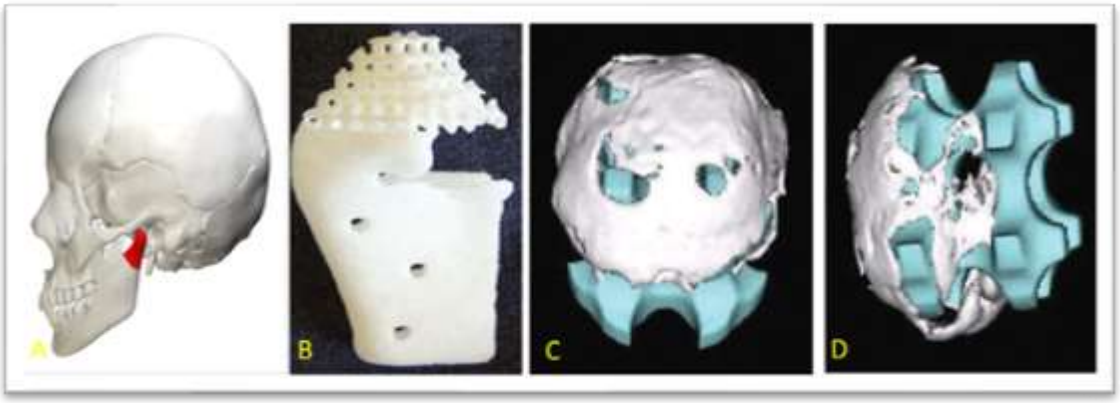


Image 5-35: A) photograph showing mandibular condyle B) PCL scaffold for mandibular condyle fabricated using SLS C&D) scaffold and bone integration after implantation to the defect site. Blue represents scaffold and white represents bone. Adapted from: (Hollister, 2005)

However, limitations of SLS include high machinery cost, use of only thermally stable polymers in the form of fine powder, inadequate porosity (<40%) and pore size (30-2500μm) of fabricated scaffolds, and poor mechanical integrity of fabricated scaffolds that limit the use of this process to non-load bearing applications. Also, spatial resolution is restricted by powder particle parameters and the size of the focal point of laser. Last but not least, SLS displays strong need for post processing phase to remove powders entrapped within the scaffold pores, while bioactive molecules cannot be incorporated in the fabricated scaffolds mainly due to the high processing temperatures (Bartolo et al., 2008; Roseti et al., 2017; Thavornyutikarn et al., 2014; 169; Leong et., 2003; Mota et., 2012).

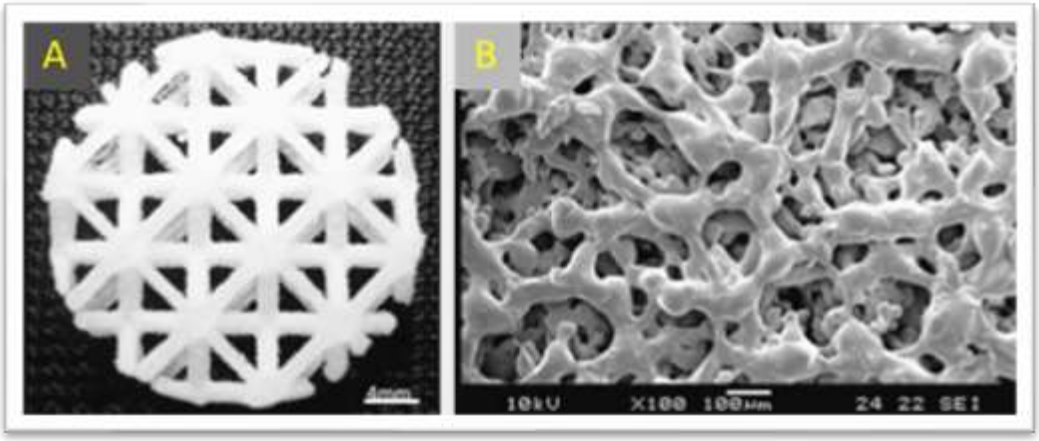


Image 5-36: PCL scaffold fabricated using SLS technique for cardiac tissue engineering application. A) Scaffold macrostructure B) 100x magnified SEM scaffold image. Adapted from: (Yeong et al., 2010)

Surface-selective laser sintering (SSLS)

Surface selective laser sintering (SSLS) comprises an advanced-SLS method first developed by (Popov et al., 2004). In conventional SLS technique, polymer

particles absorb infrared (λ=1060 nm) radiation made them completely melted and fused. Instead, in SSLS, a near-infrared (λ=970 nm) laser is used, so polymer particles do not absorb beam energy. Polymer particles are coated with a small quantity (<0.1 wt. %) of carbon microparticles, limiting the melting process to the surface of each particle. So, bioactive agents that may entrapped within polymer particles can maintain their energy though all fabrication process. As a result, scaffolds fabricated with this technique display both higher biodegradability and bioactivity (Antonov et al., 2005; Thavornyutikarn et al., 2014).

4. 3D Printing (3DP)

Three-dimensional printing (3D-P) is a powder-based freeform fabrication method first initiated at the Massachusetts Institute of Technology (MIT) by (Sachs et al., 1993). 3D printing employs the so-called ink-jet printing technology to create complex 3D objects, including tissue engineering scaffolds, by selective spraying a liquid binder onto thin powder layers (Leong et al., 2003). The layout of a typical 3D printing platform includes a build **platform** that has been put on an up-down elevator system (in Z direction), a **powder spreading roller**, and an **ink-jet print head** mounted on X-Y position rails (Image 5-37).

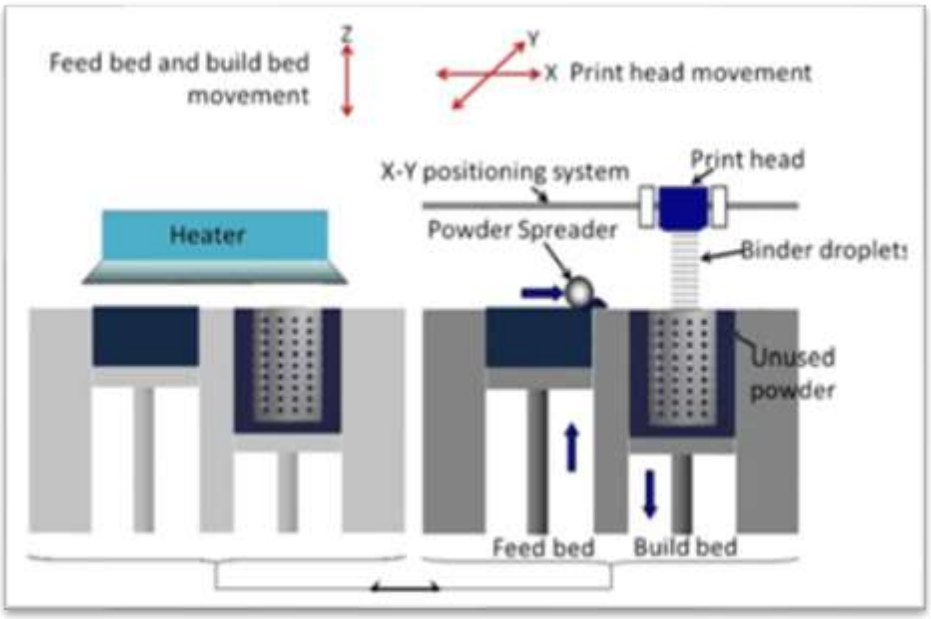


Image 5-37: Schematic representation of the 3DP system. Adapted from: (Fielding et al., 2012)

First, the spreading rollers spread a fine powder layer onto powder bed surface. Subsequently, liquid droplets of combining agent (binder) will selectively put onto the powder layer through print heads forcing the powder parts to merge together so form a solid layer (Thavornyutikarn et al., 2014). Then, platform is lowered to a fixed distance, while a next film of powder is laid on top. The above three steps are replicated iteratively until the final 3D scaffold is created layer-by-layer (Jariwala et al., 2015). After the fabrication is

performed, scaffold is embedded inside unfused powders, thus a post-processing step is required to take away any leftover powder from the created scaffold. In case of ceramic scaffolds, an additional heating procedure is needed so to increase solidity of green part (scaffold after printing) as seen in Image 5-37.

Among various parameters of this technique**, powder packing density**, **powder flowability & stability, binder drop volume, binder saturation**, **binder wettability**, and **powder/binder reactivity** must carefully selected to optimize fabricated scaffold (Bose et al., 2013; Butscher et al., 2011). Powder flowability comprises an essential requirement for both scaffold construction and de-powdering process while it is primarily influenced by size and distribution of material particles, surface roughness and shape.

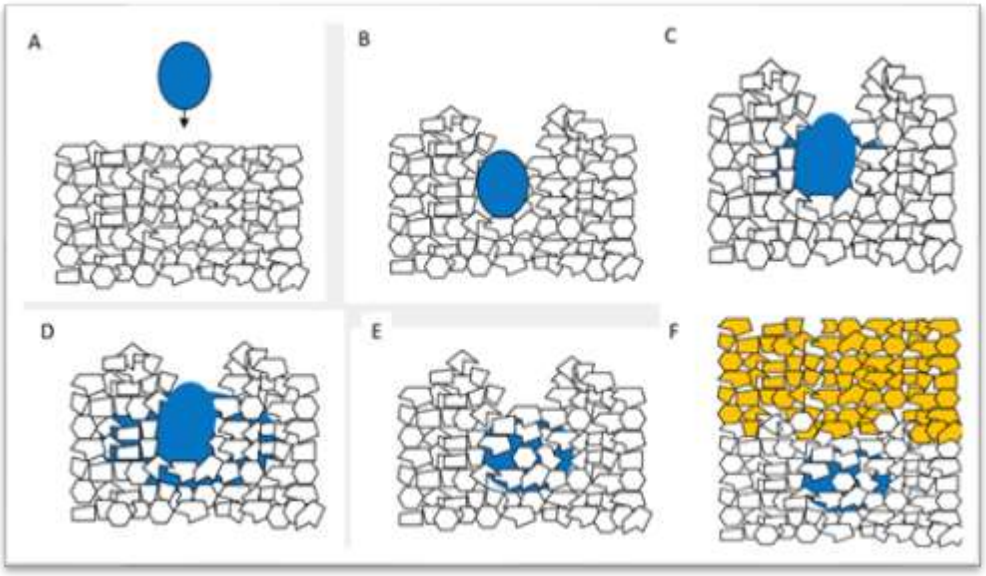


Image 5-38: Binder drop/powder interaction steps during 3D printing. Adapted from: (Butscher et al., 2011)

Three-dimensional (3D) printing has been in the forefront of SFF-related scaffold research. It has been utilized to fabricate scaffolds using a wide variety of materials including polymers (both natural and synthetic), ceramics and composites. The list of biomaterials used in 3D printing process includes synthetic biomaterials such as PLGA, and PLLA; natural-derived polymers such as dextrose, starch, and collagen; ceramics such as TCP, β-TCP, CaP, and HA; and composites such as PLGA/TCP, HA/starch, PCL/PEO, starch/PLLA, starch/cellulose, and PLGA/PLA (; Chu, 2006; Jariwala et al., 2015; Butscher et al., 2011).

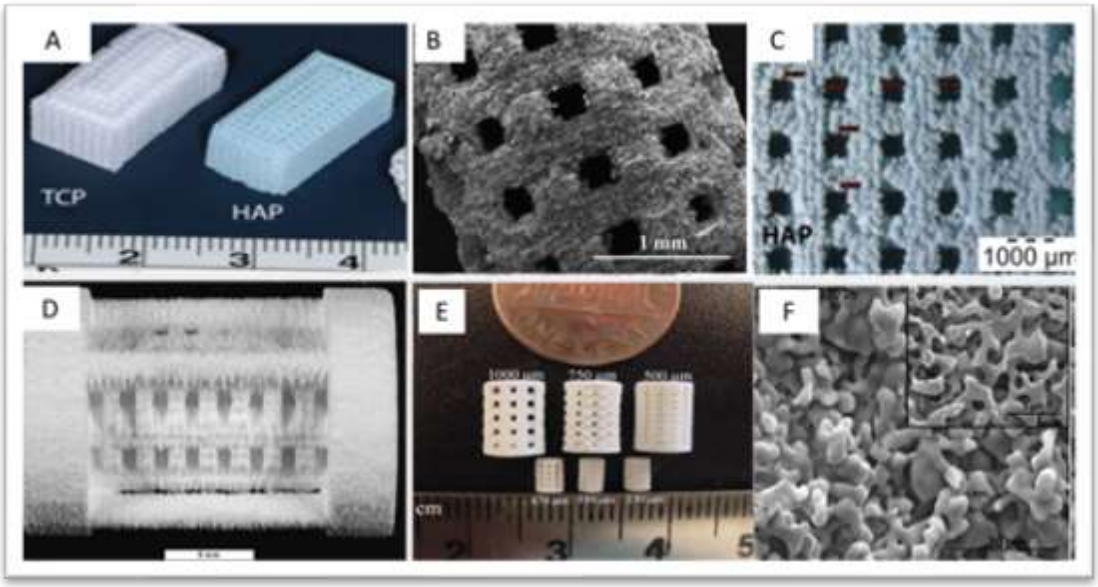


Image 5-39: A) Photograph of TCP and HA scaffolds produced by 3DP. Adapted from: (Warnke et al., 2010), B) SEM image of 3D printed TCP scaffold showing its surface morphology. Adapted from (Tarafder et al., 2013) C) SEM image of HA scaffold fabricated using 3DP technique. Adapted from: (Warnke et al., 2010), D) 3D printed CaP scaffold. Adapted from: (Butscher et al., 2011), E) Photograph and F) Microstructure of 3D printed TCP scaffolds. Adapted from (Tarafder et al., 2012)

Three-dimensional printing (3D-P) offers several benefits, including wide range of biomaterials that can be processed using this technique as long as they are in powder form, high build speed of system that offers high production rate, no need for additional structure support during scaffold processing, no use of toxic solvents, and low cost (Thavornyutikarn et al., 2014; Mota et al., 2012). Moreover, since 3D-printing performed at room temperatures both pharmaceuticals and biological agents (proteins, cells, growth factors, etc.) can be integrated into fabrication process expanding its potential for tissue engineering applications (Hutmacher, 2000).

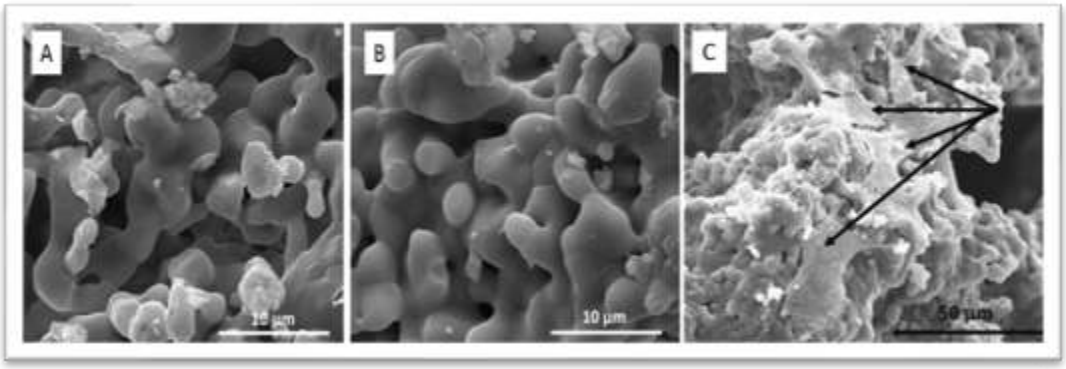


Image 5-40: SEM images of A) pure and B) SiO2/ZnO doped 3D-printed TCP scaffolds C) SiO2/ZnO doped TCP scaffold showing cell adhesion 7 days after cell seeding (black arrows). All Adapted from: (Fielding et al., 2012)

However, 3D Printing suffers from some limitations such as: pore size restricted by powder particle size, and poor mechanical strength due to weak

bonding among powder particles (especially in ceramics), limiting the use of this technique only in non-load bearing applications (Bose et al., 2013; Leong et al., 2003). In order to improve hardness of some scaffolds, a post-processing step of sintering is essential. Nonetheless, this action raises other limitations, such as the shrinkage and distortion of scaffold upon sintering and restriction of biomolecule incorporation due to high temperatures (Shanjani et al., 2010). Also 3DP technique shortcomes on rough and ribbed surface finish raised from the large size of powder. Also, ribbed surface on final scaffold may be challenge the incorporation of cells into the application.

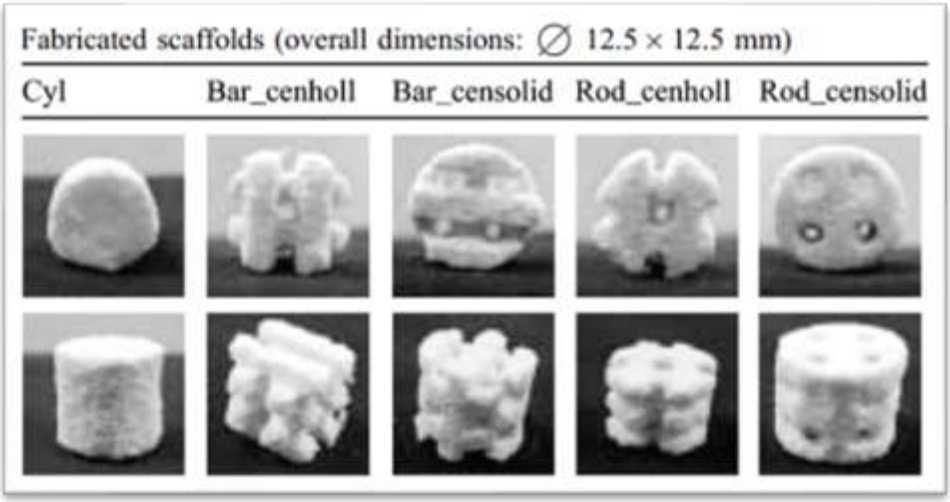


Image 5-41: 3DP-fabricated scaffolds of various shapes and sizes. Adapted from (Lam et al., 2002)

5. Bioprinting

Recently, a new tissue engineering-based strategy has been emerged, referred as **tissue or organ printing**. This strategy focused on developing scaffolds integrating additive manufacturing (AM) techniques, while incorporates cells and tissues at once (Mota et al., 2012). Tissue or organ printing, also called **Bioprinting**, is considered an evolution of tissue engineering field that focus on organ creation. Bioprinting combines cells and biomaterials together in order to create tissue-like structures, while systems can be sorted as: 1) **laser-based**, 2) **inkjet based**, and 3) **extrusion-based**.

Image 5-42: Organ printing concept system: 3D functional tissues and organ are printed on-demand. Adapted from: (Ozbolat & Yu, 2013)

Inkjet-based Bioprinting

Inkjet Bioprinting introduced in the early ‘00s. In this technique, a pre-polymer with included cells, called **bio-ink**, is archived in a cartilage. Subsequently, a printer head, connected to the bio-ink cartilage, is deformed, typically by a **thermal or piezoelectric actuator**, and pushed so to create picolitre bio-ink droplets that then deposited onto a substrate following a CAD file (Mandrycky et al., 2016).

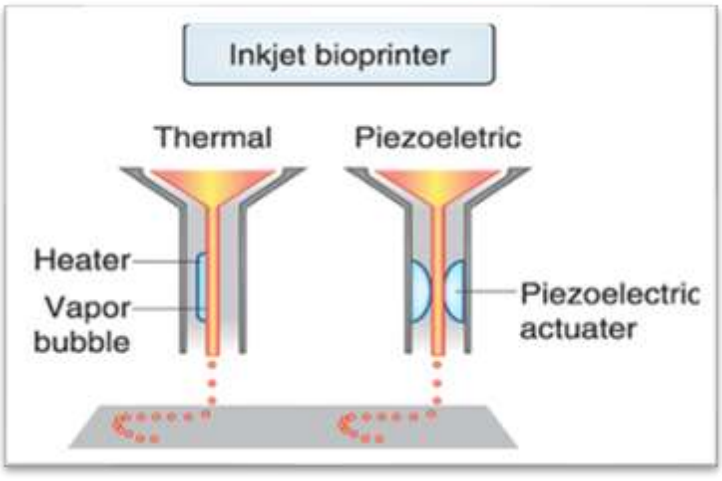


Image 5-43: Schematic representation of inkjet Bioprinting technique. Adapted from: (Murphy & Atala, 2014)

The above method displays many benefits like **low price**, **high printing speed** due to parallel work of multiple print heads, and relative **high cell viability** (80-90%). Also, this technique allows the integration of multiple

print heads to deposit cells of various types (Roseti et al., 2017; Guillemot et al., 2011; Ozbolat & Yu, 2013; Mandrycky et al., 2016).

As regards the limitations of inkjet Bioprinting technique, the most important concerns the restricted cell density of fabricated constructs due to the use of exclusively low cell concentrations (<5x10^6 cells/ml). This restriction rises from the small orifice diameter that results in cell sedimentation or aggregation and finally clogging of the print head (Guillemot et al., 2011).

5.1. Extrusion-based Bioprinting

Extrusion-based bioprinting comprise the well-known type of bioprinting (approximately 30.000 printers sold every year) (Jones, 2012). These platforms assign endless fibers of a mix (biomaterial with embedded cells and hydrogel) through a micro-hole, using plunger, pneumatic pressure, or a mechanical screw plunger (Image 5-44).

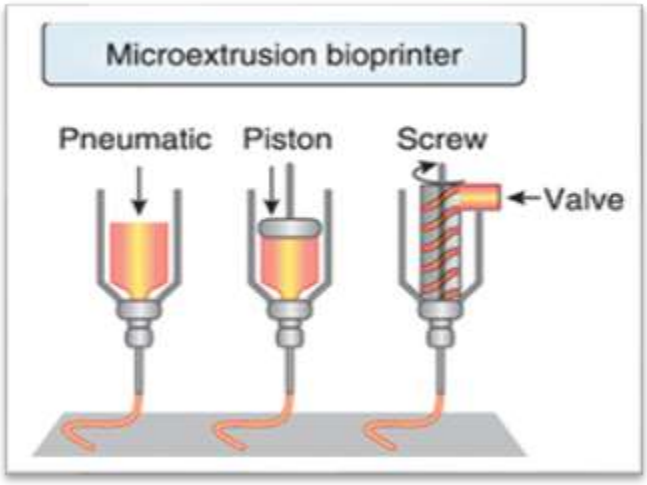


Image 5-44: Schematic representation of extrusion-based Bioprinting. Adapted from: (Murphy and Atala, 2014)

Extrusion-based bioprinting techniques offer a **larger range of biomaterials** because the micro-hole lets to assign high bio-inks (Roseti et al., 2017). Also, they are able to **deposit very high cell densities** having **better structural integrity** compared with inkjet-based techniques (Murphy & Atala, 2014; Ozbolat & Yu, 2013).

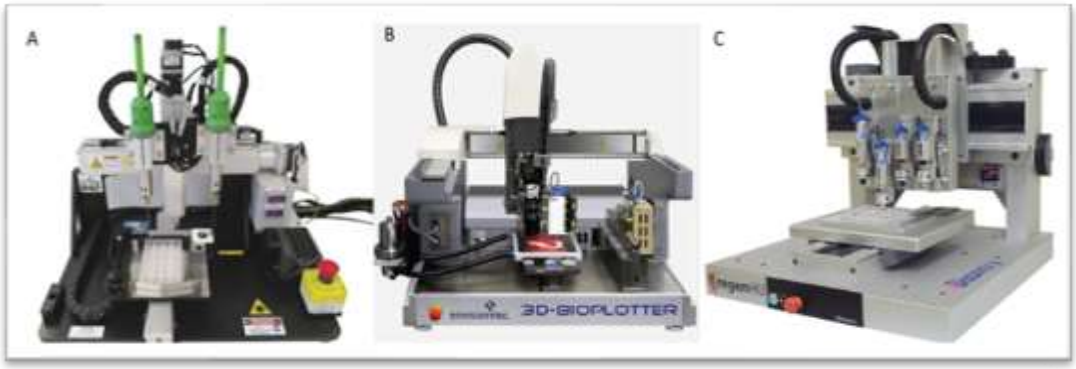


Image 5-45: Commercial bioprinting systems: A) NovoGen MMX Bioprinter (courtesy of Organovo, San Diego, CA), B) 4th generation 3D Bioplotter (courtesy of Envisiontec GmbH, Gladbeck, Germany), and C) 3DDiscovery™ (courtesy of RegenHU Ltd. Villaz, St. Pierre, Switzerland)

As regards its disadvantages, this technique displays the lowest cell viability (40-86%) among all bioprinting techniques mainly due to large mechanical stresses that induce cell deformation (Mandrycky et al., 2016). Concluding, extrusion-based bioprinting offers a balanced choice between cost and accuracy of fabricated constructs.

5.2. Laser-assisted Bioprinting (LAB)

Laser-assisted Bioprinting (LAB) comprises a technique that enables the statement of droplets containing cells and biomaterials. A typical LAB system contains three main parts: a **pulsed laser source**, an **absorbing target-layer filmed with a material**, and **a receiving surface** (Catros et al., 2011).

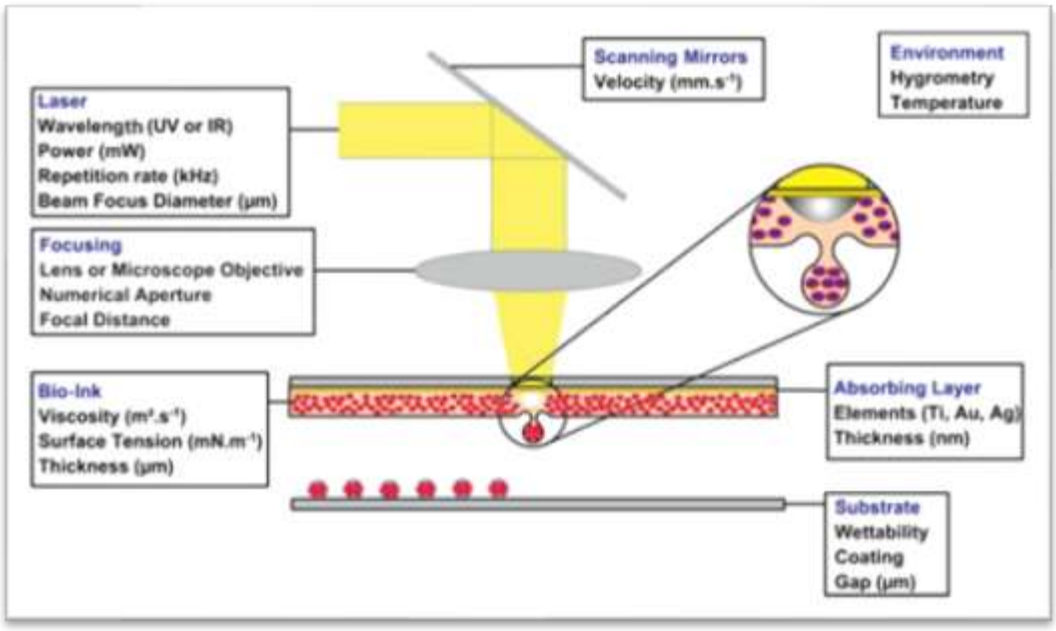


Image 5-46: Schematic representation of laser-based Bioprinting (LAB) process. Adapted from: (Guillemot et al., 2011).

First, a laser beam (approximately in the value of 1 J/cm^2) is focused on the ribbon inducing the creation of a bubble which is lying down till the bio-ink layer is distorted producing a liquid jet with a high value speed. Then, the falling bio-ink droplet is gathered in the surface and is consequently connected (Image 5-46).

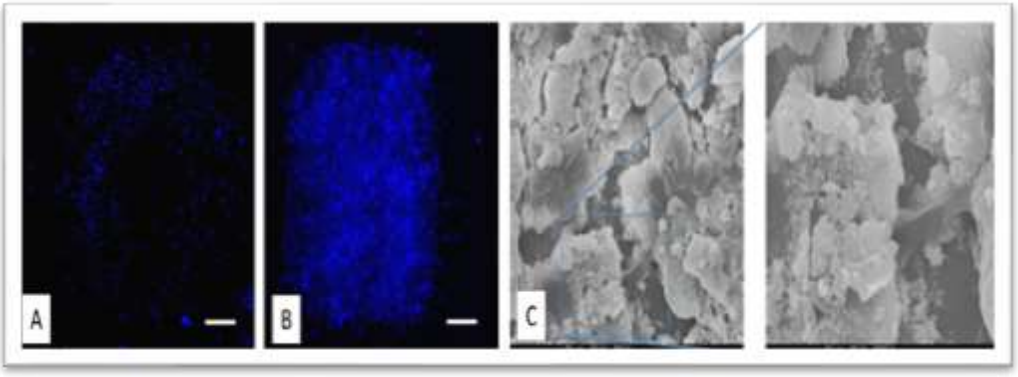


Image 5-47: Fluorescence microscopy image of LAB-created nano-HA with embedded human osteoprogenitor (HOP) cells after Hoechst coloration A) 3 and B) 6 days after fabrication C) Rough and detailed SEM image of nHA surface showing HOP cells lye on the material. Adapted from: (Catros et al., 2011)

Laser-based Bioprinting technique shows several advantages over the rest bioprinting methods. First, it has the ability to place **cells with increased densities and small quantities** of cell suspensionsIt is also capable of **printing many cell types** (e.g., HUVECs, BAECs, etc.), and displays **adaptability within a list of several biomaterial stickiness**. Also, it shows **unbeatable writing resolution** and **shows no clogging of print heads** or nozzles with cells or biomaterials. On the other hand, this method displays the limitations of high equipment cost and poor understanding of unwanted results of laser exposure on the cells (Roseti et al., 2017, 2000; Mota et al., 2012; Guillemot et al., 2011; Ozbolat and Yu, 2013; Mandrycky et al., 2016).

Comparison of Bioprinting techniques				
Parameters	Inkjet-based	Extrusion-based	Laser assisted	References
Material viscosities	3.5–12 mPa/s	30 mPa/s to >6 × 10^7 mPa/s	1–300 mPa/s	(Murphy and Atala, 2014; Guillemot et al., 2010; Chang et al., 2011)
Cost	Low	Medium	High	(Jones, 2012)
Print speed	Fast (1–10,000 droplets per second)	Slow (10–50 µm/s)	Medium-fast (200–1,600 mm/s)	(Guillotin et al., 2010; Murphy and Atala, 2014)
Cell viability	> 85%	40-80 %	> 95%	(Catros et al., 2011; Xu et al., 2005)
Cell density	Low (<106 cells/ml)	High, cell spheroids	Medium (108 cells/ml)	(Murphy and Atala, 2014)
Resolution (droplet size)	High (<1 pl to >300 pl droplets, 50 µm wide)	Medium (5 µm to millimeters wide)	High	(Murphy and Atala, 2014)
Preparation time	Low	Low to medium	Medium to high	(Murphy and Atala, 2014)

6. Growth Factors in Bone Tissue Engineering

Growth factors (GFs) are biomolecules that promote and/or prevent cellular functions like survival, proliferation, migration, adhesion, and differentiation (Lee et al., 2010). Their role is to signal biomolecules on receptor sites on the cell surface, and subsequently secrete factors to them initiating several cellular functions (Ikada, 2006). When GFs influence the same cell that are binded to, the process called **autocrine signaling**, while when they secrete factors on neighboring cells having a different phenotype, the process called **paracrine signaling** (Image 6-1). At last, when GFs are secreted into the blood and then carried by blood and tissue fluids onto a cell located at a distant anatomical site, the process called **endocrine (or hemocrine) signaling**.

Growth factors, unlike hormones, do not typically act in an endocrine manner, they have **short half-lives** (on the order of several minutes), exhibit short-range diffusion and are secreted at low concentration (Andrades et al., 2013). Last but not least, growth factors are usually used as a synonym of **cytokines** (Meyer et al., 2009).

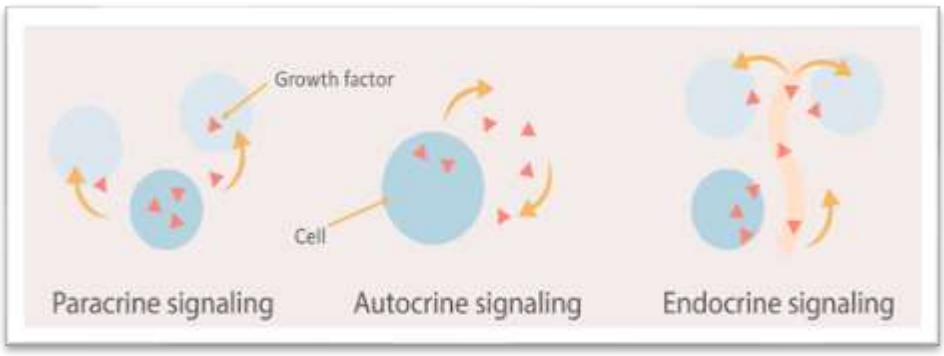


Image 6-1: Growth factor signaling mechanisms

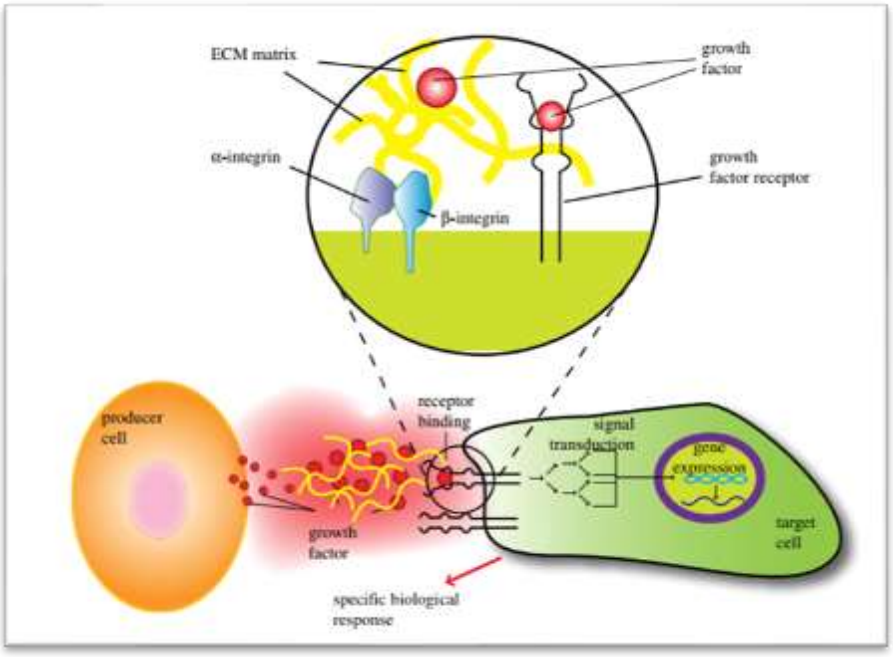


Image 6-2: Growth factor action mechanism. Adapted from: (Lee et al., 2010)

In native bone tissue, growth factors remain encrypted on the extracellular matrix (ECM). Bone matrix contains a great number of GFs including bone morphogenetic proteins (**BMPs**), transforming growth factor-β (**TGF-β**), fibroblast growth factors (**FGFs**), platelet-derived growth factors (**PDGFs**), vascular endothelial growth factors (**VEGFs**) and insulin-like growth factors (**IGFs**) that will be further discussed below. During natural bone healing an orchestrated delivery of GFs takes place. Different types of GFs act either directly on osteoblasts, regulating their growth and function, or inducing angiogenesis (vascularization) in fresh-formed tissue, and osteogenesis through endothelial cell migration and differentiation (Santin, 2009).

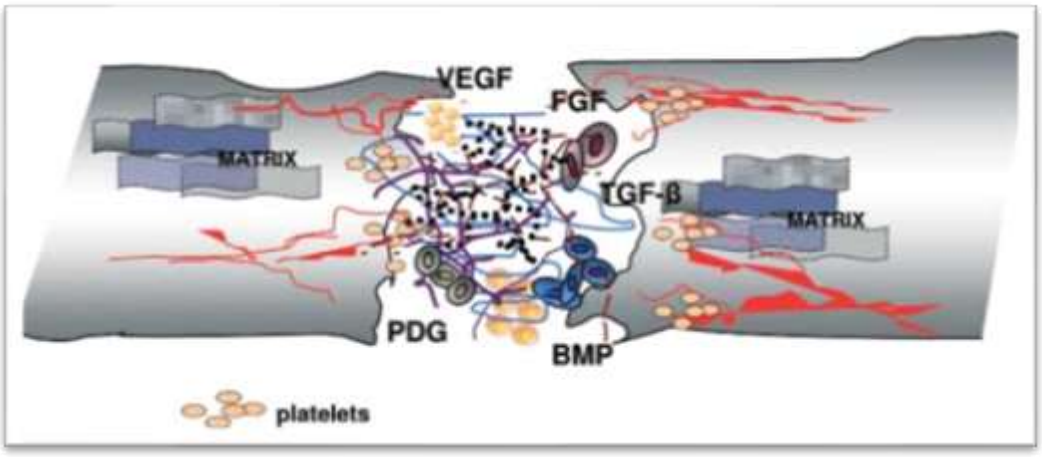


Image 6-3: Growth factor families participating in bone repair process. Adapted from (Devescovi et al., 2008)

6.1. Growth factors in natural bone healing

Several minutes after bone injury a clot is formed (due to vascular disruption), platelets enter the wound in great numbers releasing TGF-β, PDGF, VEGF and IGF chemotactic factors (**inflammation phase**). Subsequently, during **angiogenetic phase**, factors such as VEGFs, FGFs, and PDGFs induce angiogenesis and migration of endothelial cells and formatting tubular blood vessels (Nyberg et al., 2015; De Witte et al., 2018). At the next healing step, called **pre-osteogenic/proliferative phase** FGFs, TGF-β, IGFs stimulate fibroblasts to synthesize key extracellular organic components (e.g., glycoproteins, collagens, and proteoglycans). At final stage, principally BMPs, TGF-βs and IGFs promote the differentiation and proliferation of osteoprogenitor cells to osteoblasts leading to complete tissue remodeling.

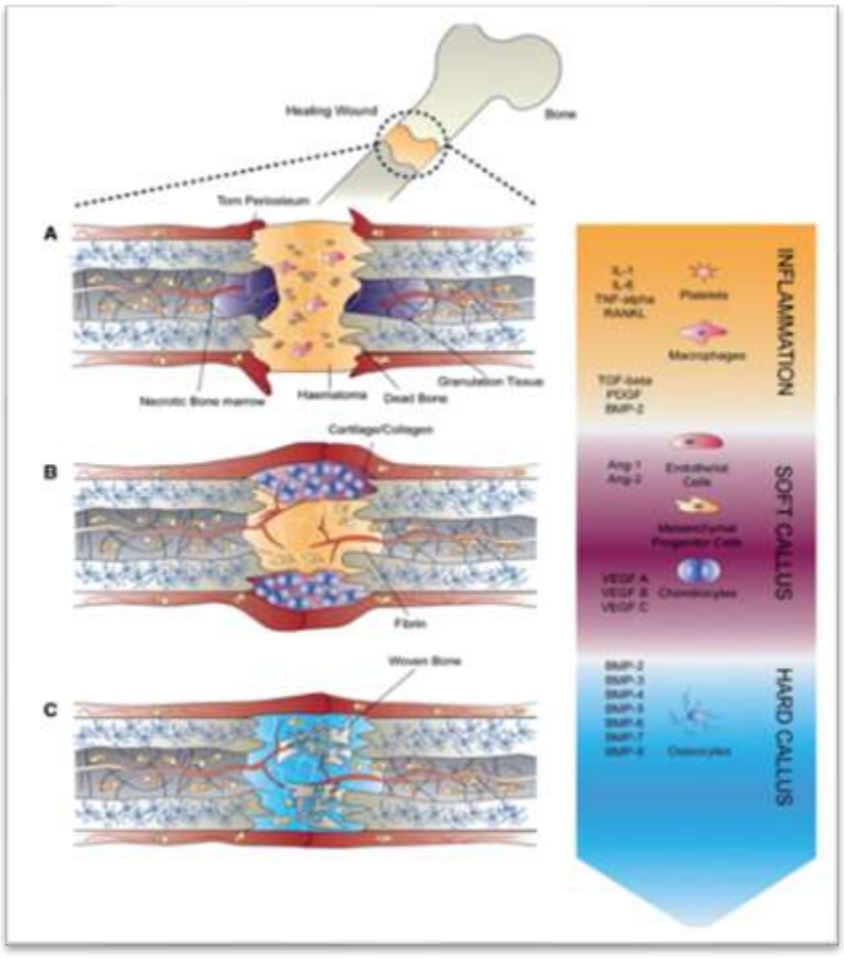


Image 6-4: Bone fracture healing steps. Adapted from: (De Witte et al., 2018)

6.2. Growth factors in Bone Tissue Engineering (BTE)

Except from their critical role in bone modeling and remodeling processes, growth factors are also investigated for utilization in tissue engineering field. Particularly, in Bone Tissue engineering (**BTE**) most common utilized growth factor families are Bone morphogenetic proteins (**BMPs**), Transforming growth factors beta (**TGFs-ß**), Insulin-like growth factors (**IGFs**), and Platelet-derived growth factors (**PDGFs**), Fibroblast growth factors (**FGFs**), and Vascular endothelial growth factors (**VEGFs**).

Bone morphogenetic proteins (BMPs)

BMPs are members of TGF-βs superfamily while have been thoroughly studied for tissue engineering applications. The first BMP was identified by Marshall R. Urist (1965) after his postulations for an element found in native bone with bone regeneration potential.

BMPs act through special receptors that establish a series of phosphorylation events (Fisher et al., 2007). BMPs are among the most potent pre-osteogenic GFs, while having the most widely understood pathway for bone regeneration. So far, more than 30 BMPs have been identified. Among them, BMPs 2, 4, 6, 7, and 9 are considered to be the most osteoinductive stimulating differentiation of pluripotent MSCs to osteoprogenitor and osteoblastic cells (Akter, 2016). BMP-2, BMP-6, and BMP-9, specifically, hold prominent role in MSCs differentiation to osteoblasts (Image 6-5), while BMP-4 and BMP-7 stimulate the maturation of osteoblasts (Andrades et al., 2013). Recombinant BMP-2 and BMP-7 have received FDA approval in 2004 and 2001, respectively, for treating severe bone injures (Image 6-6).

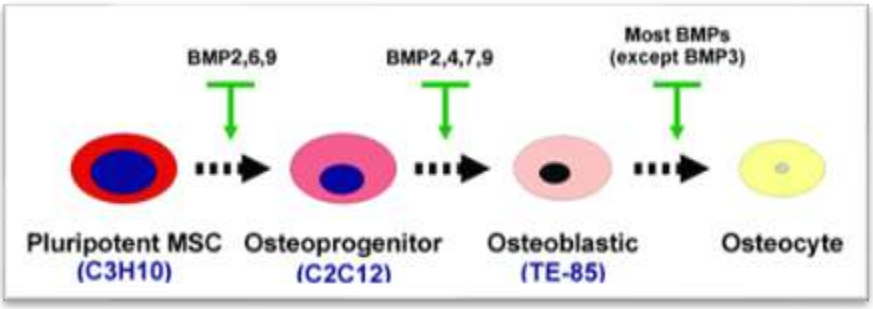


Image 6-5: Osteogenic Hierarchy of Bone Morphogenetic Proteins. Adapted from: (Cheng et al., 2004)

Transforming growth factors beta (TGFs-ß)

Transforming growth factor-ß family consists of **TGF-β1 to TGF-β5**, **bone morphogenetic proteins** (**BMP**), and **growth and differentiation factors** (**GDF**) (Hollinger, 2005). They mainly located in bone, platelets, and cartilage where trigger cell population expansion, proliferation, maintenance, and apoptosis of both osteoblasts and chondrocytes (Fisher et al., 2007). They have

multiple roles as GFs and thus characterized as both **pre-angiogenic and pre-osteogenic factors** (De Witte et al., 2018). Amongst all TGF-βs, TGF-β1 shows high potential for use in BTE as bone formation inducers.

Insulin-like growth factors (**IGFs**)

Insulin-like growth factors-I and -II (IGF) can be found in bone matrix where participate in DNA synthesis (Hollinger, 2005). Moreover, they can prevent collagen degradation by reducing collagenase synthesis. Among the two types of IGFs, **IGF-I** that plays crucial role for longitudinal bone growth, is 4 to 7 times more potent than **IGF-II** but the latter is found in higher concentration in bone matrix (Solheim, 1998). Thus, IGF-I (classified as pre-osteogenic GF) shows higher potential for future tissue engineering application.

Platelet-derived growth factors (**PDGFs**)

Platelet-derived growth factors exists as three similar molecules, PDGF-AA, -BB, and -AB that synthesized by blood platelets. They have been successfully applied with demineralized bone matrix (DBM) inducing osteogenesis in rats (Howes et al., 1988), and combined with IGF-I for periodontal surgery in dogs (Lynch et al., 1991).

Fibroblast growth factors (**FGFs**)

The FGF family consists of 9 polypeptides, but most abundant and best characterized in human tissue are **acidic FGF** (or **FGF-1**) and **basic FGF** (or **FGF-2**). Both FGFs are involved in inflammatory stage of bone healing that trigger angiogenesis. FGF-1 favor chondrocyte proliferation, while FGF-2, that seems to be more potent, is expressed by osteoblasts and play a vital role in bone remodeling process (Hollinger, 2005). Thus, FGF-2 has been used to treat ischemic diseases (Yanagisawa-Miwa et al., 1992) and induce cardiomyocyte differentiation (Chan et al., 2010).

Image 6-6: Commercially available BMPs products. A) Recombinant BMP-7 product marketed as osteogenic protein-1 (OP-1) (Stryker Biotech) B) Recombinant BMP-7 product marketed as Infuse (Medtronic Sofamor Danek).

Vascular endothelial growth factors (**VEGFs**)

VEGFs is another family of GFs that invoke angiogenesis. Currently, there are **six types** of VEGFS**: VEGF-A to E** and **placental growth factor** (**PLGF**) that seem to promote endothelial cell migration/proliferation and tubular blood vessels formation leading to increased angiogenetic network. However, due to the induced vascular permeability, VEGFs delivery can lead to systemic hypotension and edema (De Witte et al., 2018).

Table 10: Clinical studies using different GFs. Adapted from: (Lee et al., 2010)

Growth factor	Administration/ carrier	Clinical target	Result	commercially available
VEGF	infusions (intravenous and intracoronary) cardiovascular disease	cardiovascular diseases	Neutral	-
FGF-2	infusions (intracoronary)	cardiovascular diseases	Neutral	-
FGF-2	alginate microcapsules	cardiovascular diseases	Positive	-
BMP-2	collagen sponge	bone fractures	Positive	Infuse bone graft-Medtronic (https://www.medtronic.com/us-en/d/infuse-bone-graft.html)
BMP-7	collagen matrix	bone defects	Positive	OP-1 Putty-Stryker

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