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ABSTRACT

Surgical treatment of major traumatic pathologies, in which there are critical-size bone defects, includes transplantation of tissue from one site to another in the same patient (autograft) or from one individual to another (transplant or allograft). Problems exist with all these techniques.

The field of tissue engineering and regenerative medicine aims to regenerate damaged tissues, instead of replacing them, by developing biological substitutes, by restoring, by maintaining or improving tissue function.

As our first mundial review on PCL scaffolds in bone tissue, we have reported an experience in repairing that has confirmed its biocompatibility. No signs of local or systemic toxicity were detected in the site of implant and no foreign body reaction was found. Histological examination of samples confirmed the absence of necrosis, inflammation or fibrosis areas.

The radiological and histological evidence of newly formed bone tissue penetrating into the scaffold structure validates its osteoconductivity and osteoinductivity, confirming the possibility to use scaffolds as an alternative to standard bone grafts for the treatment of bone critical size defects.

Key words: PCL scaffolds, critical-size bone defects, tissue engineering.

INTRODUCTION

Disease, injury and trauma lead to damage and degeneration of tissues in the body, which necessitates medical and surgical treatments.

Animals, particularly dogs and cats may be affected by polytrauma. Common situations in which polytrauma occurs include being hit by a car or falling from a height, such as a balcony.

Surgical treatment of major traumatic pathologies, in which there are critical-size bone defects, includes transplantation of tissue from one site to another in the same patient (autograft) or from one individual to another (transplant or allograft). Problems exist with all these techniques.

Autograft technique is expensive, painful, limited by anatomical structure and associated with donor-site morbidity.

In transplants and allografts there are serious problems due to **lack** of available tissue, the risks of rejection by the patient's immune system and the possibility of introducing infection or disease.

The field of tissue engineering and regenerative medicine aims to regenerate damaged tissues, instead of replacing them, by developing biological substitutes that restore, maintain or improve tissue function.

In this study we have performed for the first time a review of the state of art in this field, starting from history until the new experience with biomaterials.

We have described natural and synthetic materials and scaffolds production techniques, focusing in medical fields of application such as bone tissue, blood vessel, neural tissue engineering.

We have also reported an experience in the use of PCL scaffolds in bone tissue repairing.

2. VETERINARY ASPECTS OF POLYTRAUMA

1.1. INTRODUCTION

Animals, particularly dogs and cats may be affected by polytrauma. Common situations in which polytrauma occurs include being hit by a car or falling from a height, such as a balcony.

Polytrauma is the the condition in which the patient is affected by lesions involving two or more different corporeal districts (rachis, head and neck, abdomen, pelvis, limbs) eventually compromising cardiac and/or respiratory function.

In retrospective studies conducted by Kolata and Johnston (1975)[1] and Kolata (1980)[2] it was found that about 13% of admissions for emergencies to veterinary hospitals were for traumatic events. In about 36% of the cases patients had multiple injuries with a mortality rate of 9-12,5%.

A more recent study (Simpson et al. 2009)[3] report a mortality rate of 12%, in his casistic vehicular trauma accounted for 91.1% of cases of trauma, but also in other casistics vehicular trauma is by far the most common cause. The

[1] Kolata RJ, Johnston DE, 1975, Motor vehicle accidents in urban dogs: a study og 600 cases, *J Am Vet Med Assoc*, 167, 938-41

[2] Kolata RJ, 1980, Trauma in dogs and cats: an overview, *Vet Clin North Am Small Anim Pract*, 10, 515-22

[3] Simpson SA et al. 2009, Severe blunt trauma in dogs: 235 cases (1997-2003), *J Vet Emerg Crit Care*, 19, 588-602

incidence of polytrauma is similar in cats and dogs, but for the cats there are more injures of unknown cause because more frequently go outside without owner supervision and the typical high-rise syndrome (the phenomenon of cats falling from higher than two stories). Majority of studies in scientific literature regard dogs.

Dogs in which a trauma occurs, are generally young or middle aged, specially males. a smaller percentage are geriatric patients often with important co-morbilities (heart, renal, endocrine diseases).

1.2. TYPES OF TRAUMA

The most common types of trauma are :

- **thoracic injuries**.

The thorax is the site of the vital cardiopulmonary physiology and consequent delivering of oxygenated blood to tissues; therefore thoracic injury, either alone or in combination, are responsible for inadequate oxygenation. We can have penetrating injuries like stab or gunshot wounds and impalement by a foreign body, or blunt trauma, generally from a vehicular trauma.

Thoracic injuries can produce pulmonary or cardiac contusions, cardiac rupture, rib fractures, flail chest, pneumothorax, hemothorax, pneumomediastinum, aortic tear, diaphragmatic herniation or rupture.

- **abdominal injuries**

are common in vehicular blunt trauma, but we have also trauma from fights, weapons and falls. We can have more frequently hemoperitoneum, but also urinary rupture , abdominal hernia, bile duct rupture, body wall hernia.

- **orthopedic injuries**

Pelvic, femur, spinal, distal limbs, scapular,sacral, radius fracture.
Hip, sacral, elbow luxations

- **head injuries** with significant morbidity and mortality.

- **Soft tissue injury**

  - Abrasions, lacerations, subcutaneous emphysema, major degloving.

1.3. CLINICAL APPROACH TO POLYTRAUMATIZED ANIMAL

Overall outcome of polytraumatized animal depends highly on the organization and function of the trauma reception team and on the timing and type of procedures carried out during the first phase.

1.3.1. Triage

Triage is the process of prioritizing patient on the base of the severity of his clinical status and his consequent need of care. It begins when the owner calls the hospital and the informations he gives, although often unreliable, may be useful to be prepared to the patient's arrival and to suggest the owner an initial emergency management.

At the arrival in hospital the first examination follows the ABCDE rule

A airway

B brething

C circulation

D dysfunction of the central nervous system

E examination

Airway and breathing

For the best outcome of the patient is very important a quick assessment of respiratory system and correction of abnormalities. We have first to assess the patency of airways and adequacy of ventilation.

It is done by :

- visualization (posture, chest wall motion, increased breathing effort, open mouth and paradoxical breathing, flaring of the nares, cyanosis).

- auscultation (noisy breathing such wheezes or stertor, absent or diminished breath soundsand).

- palpation to assess integrity of the chest wall and eventually identify crepitation for subcutaneous emphysema.

Life-threathening problems concerning airway and breathing are apnea, airway obstruction, open chest wounds, pneumothorax, pleural effusion.

Circulation

Also circulation assessment is based on visualization, palpation and auscultation to investigate some important parameters:

- Mucous membrane color

(gums, conjunctiva, penis, vulva) that can lose their normal pink color and assume a pale or white color (anemia or vasoconstriction) , brick red or injected

(vasodilatation due to hyperthermia or sepsis), cyanotic ore blue(severe hypoxemia), yellow (liver disease or hemolysis), brown (methemoglobinemia).

- Capillary refill time

normal 1-2 seconds

shortened < 1/2 second suggestive of vasodilatation

prolonged > 2 seconds suggestive of vasoconstriction

- Heart rate

tachycardia (>160 bpm in dogs, >200 bpm in cats) may be due to hypovolemia, hypoxemia, hypotension, drugs, fever, pain, exercise, excitement.

bradycardia (< 6o bpm in dogs, <140 bpm in cats) may be due to increased vagal tone, electrolyte alterations , cardiac conduction defects

- Pulse quality

The pulse should be palpated to assess rate, rhythm and quality. Pulses can usually be easily palpated from the femoral or dorsal pedal arteries. The strength of the pulse depends on the difference between the systolic and diastolic pressures,rather than the absolute or mean arterial pressure.

If the difference is wide, the pulse feels strong, if abnormally strong it is termed **hyperkinetic.**

If it is small, the pulse feels weak and it is termed **hypokinetic**.

Bilateral pulses should be compared; absence of pulse or a weaker pulse on one side may be caused by thromboembolism.

- Extremity temperature

in case of vasoconstriction extremities are cool

- Mentation

It is important an evaluation of mentation. An altered mental state may reflect a perfusion deficit or a brain problem

Dysfunction of the central nervous system

First, as said before, we have to assess the level of consciousness; an altered mental status (dullness, disorientation, stupor and coma) may be a result of a primary brain problem. We have after to observe gait and body posture. Abnormalities can be attributed to neurological or orthopedic disease. An examination of cranial nerves and of spinal reflexes may determine whether the patient has a neurological disease and the most likely location within the nervous system.

Examination

A complete phisical examination is performed looking for wounds, lacerations, punctures, bruises, fractures, abdominal pain/distension and any other signs of debilitation.

2. TISSUE ENGINEERING and REGENERATIVE MEDICINE

In the last twenty years there has been an upsurge of interests towards the development a new field of research called tissue engineering and regenerative medicine, in wich scientists, engineers, and physicians apply tools from different fields to obtain biological substitutes that can replace ,or help regenerate, diseased and injured tissues and can mimic tissues for diagnostic and research purposes.

2.1 DEFINITION

Tissue engineering **(TE)**

TE stemmed from the field of biomaterials science and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues.

The goal is to assemble functional constructs that restore, maintain, or improve damaged tissues or whole organs.

Examples of engineered tissues approved by the FDA are artificial skin and cartilage ; currently they have limited use in human patients.

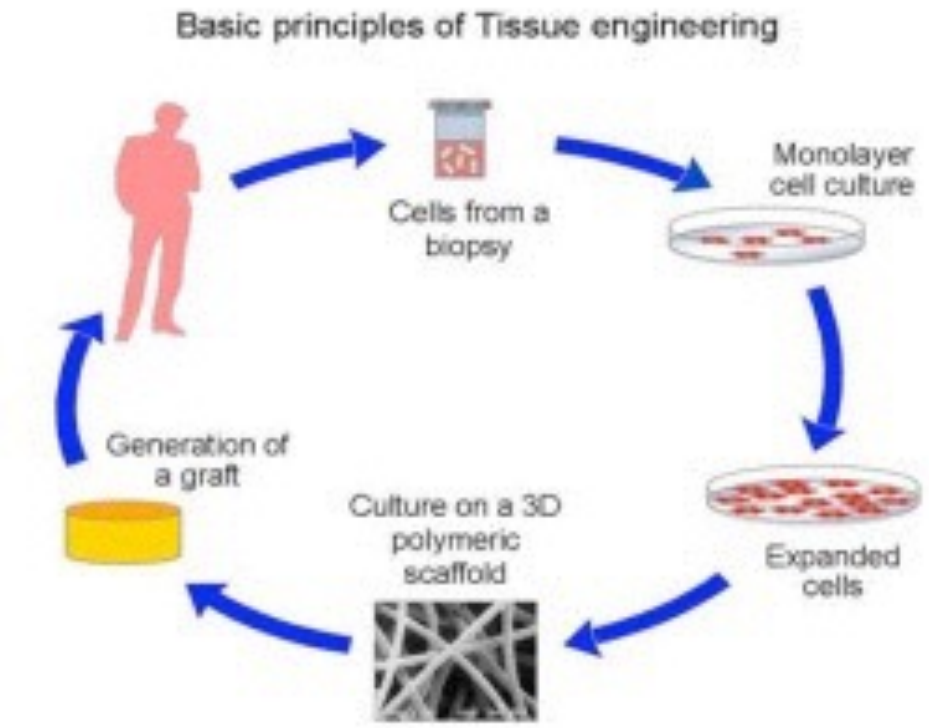

fig.1: from: Energia Ambiente e Innovazione / Anno 2011 / n. 4-5/2011 Luglio-Ottobre 2011

Regenerative medicine (RM)

RM is a broad field that includes tissue engineering and research on self-healing .

Self[4]-healing is the process of recovery where the body uses its own systems, sometimes with help foreign biological material to recreate cells and rebuild tissues and organs. The terms “tissue engineering” and “regenerative medicine” have become largely interchangeable, as the goal is to focus on cures instead of treatments for complex, often chronic, diseases.

This field is continuously evolving. In addition to medical applications, non-therapeutic applications include using tissues as biosensors to detect

[4] Langer, R., & Vacanti, J. P. (1993). Tissue engineering. *Science*, 260(5110), 920–926.

biological or chemical threat agents, and tissue chips that can be used to test the toxicity of an experimental medication.

2.2 HISTORY

2.2.1. Tissue engineering and regenerative medicine in myth and religion

Although developed in recent years, the idea of tissue engineering is as old as man himself.

The Bible describes Eve's creation from Adam's rib.

In the Hindu mythology we find Rakhtbeej , an incredibly powerful demon who has the special ability that every drop of his blood which falls on the ground creates a clone of himself.

Hesiod tells the story of Tityus that was punished by Zeus who had him bound in Hades, the ancient kingdom of the Dead, where two vultures were fed on his liver which, regenerated perpetuating the torture eternally .

He also tells the more famous similar story of Prometheus, who had the liver pecked out by an eagle and continuously regenerated to prolong his suffering.

Another example is the story of the birth of the Kaurava brothers told in the Adi Parva of the Mahabharata. Their mother Gandhari, after two years of pregnancy, produced a hard mass of flesh like unto an iron ball. The sage Rishi divided into a hundred parts that were let in a hundred pots full of clarified butter and herbs for two years. After this time from

each pot was born a Kaurava brother[5]. This is the first example of cell grow in vitro!

We have three Aristotle's works on natural history: *History of Animals, Generation of Animals and Parts of Animals*. He stated that animals in the early developmental stages have a higher potential for regeneration and made detailed descriptions about regeneration on the limbs of salamanders and deer antlers . He sustained the idea that biological form originates from undifferentiated matter .

On Goethe's Faust , Wagner create the homunculus , an artificial human being produced through the alchemical art. Unlike a naturally produced baby, he is instantly knowledgeable and wise. Although he looks like a perfect little man, his body is insubstantial and he stays inside the alchemical flask in which he was made[6].

First mention of the use of biomaterial is in the history of Vishpala, an Indian Queen , in the ancient texts called the Rig-Veda. (compiled between 3500-1800 BC) .According to the text she lost her leg in battle and was fitted with an iron leg so that she could return to fight.

A Celtic Irish god, Nuada, lost his left hand in battle and his brother, Dian Chect, the god of healing, made him a hand of silver.

Herodotus wrote of a prisoner of war, Hegesistratus, who escaped from his bonds by cutting off part of his foot. He fashioned a wooden prosthesis and walked 30 miles, unfortunately only to be captured and executed.

The Roman scholar, Pliny the Elder, wrote of a general who lost a hand in battle between 218 and 210 BC. An iron hand was fashioned for him so he could hold his shield and returne to battle.

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[5] Mishra, P. How India Reconciles Hindu Values and Bio- tech [*Online Newspaper Article*]. New York City: The New York Times, 2005.

[6] Goethe, J.W.V., and Greenberg, M.F. Faust. Part Two. New Haven: Yale University Press, 1998.

2.2.2. Ancient application of tissue engineering and regenerative medicine

The use of sutures for wound closure exists since Neolithic period. The early Egyptians used linen sutures, in India and South Africa were used the heads of large biting ants, in Europe catgut[7].

The Ebers Papyrus, written around 1500 BC details the use of honey as a topical application for wounds, abscesses, sores, burns and skin conditions. The honey was used as an antibiotic barrier to prevent infection. They use also lint and grease. Lint acted as scaffold to promote wound regeneration[8].

Skin grafts were employed in India since 2500 B.C[9].

One of the earliest instances of plastic surgery can be found in the Sushruta Samhita, an important medical text from India, commonly dated to the 6th century B.C., and attributed to the physician Sushruta .

Sushrutha was the first recorded physician to perform rhinoplasty, and is often heralded as the father of both skin grafting and plastic surgery. He repaired noses removed as punishment for crimes such as theft and adultery, ear and lips.

For Egyptians losing a limb was worse than death and they believed that it would affect the amputee in the afterlife. The theology of Osiris, the god of the dead, stated that the body, in order to be effective during the afterlife, should be

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[7] Ratner, B.D. History of biomaterials. In: Ratner, B.D., Hoffman, A.S., Schoen, F.J., and Lemons, J.E., eds. *Biomaterials Science—An Introduction to Materials in Medicine*. 3rd ed. London: Academic Press, 2013, pp. xli– liii.

[8] Jaklenec, A., Stamp, A., Deweerd, E., Sherwin, A., and Langer, R. Progress in the tissue engineering and stem cell industry ‘‘Are we there yet?’’. *Tissue Eng* Part B Rev 18, 155, 2012.

[9] Chick, L.R. Brief history and biology of skin grafting. *Ann Plast Surg* 21, 358, 1988.

complete. Therefore they made false body parts out of fiber for burial purposes to “aid” the deceased. Two extant Egyptian prosthetic toes document their practical use: the “Greville Chester” toe dated back to 600 B.C. ,made of a mixture of linen, animal glue and tinted plaster and the “Cairo” toe dated between 950 and 710 B.C.,made of leather and wood. Researchers believe that these prosthetic toes were used also in life[10].

In Capua, in 1858 was discovered an artificial leg made of bronze and iron with a wooden core that dates to about 300 B.C.

Dental implants were performed by the ancient Mayans. They made out of pieces of shell, shaped in the form of the teeth.

Aurelius Cornelius Celsus (25 BCE–50 CE), described the use of braided suture. Celsus wrote about controlling haemostasis by making ligatures in many places’which would twist around the vessels.

Galen of Pergamon (131–211) was the first to describe the use of gut string as a suture material to sew severed tendons in gladiators and also recommended using silk suture when available.

2.2.3. More recent history

Medieval period was a dark period for medicine. Medieval medicine was mainly based on the ancient works, of Greek physicians Galen and Hippocrates . One of the prevailing theories about disease in medieval medicine was that of the human body relating to the four elements (earth, air, fire and water) and to four bodily humours (blood, phlegm, yellow bile and black bile). It was believed that health could be maintained or restored by balancing

[10] Nerlich, A.G., Zink, A., Szeimies, U., and Hagedorn, H.G. Ancient Egyptian prosthesis of the big toe. *Lancet* 356, 2176, 2000.

the humours, and by regulating air, diet, exercise, sleep, evacuation and emotion.

An history of tissue engineering start again in 1665, when Hooke (1635-1703) viewing by microscope thin cutting of cork discovered empty spaces contained by walls, and termed them pores, or cells.

Abraham Trembley (1710-1784) discovered the remarkable regenerative capacity of the hydra. He decided to cut the polyp in half. He predicted that if the two parts regenerated, the organism must be a plant since animals were not known to regenerate complete individuals. He saw that each half of the polyp regenerated into a complete new polyp, like a plant, but he was notwithstanding this sure that it was an animal. These results, published in 1774, were a first step in the study of regenerative potential of cells[11].

In 1805, Oken stated that "All life is based on individual cells."

In 1838-1839, Schleiden and Schwann on their microscopic findings formulated their "Cell Theory" This theory summarized their findings as:

- Cells are organisms and all organisms consist of one or more cells.

- The cell is the basic unit of structure for all organisms and that plants and animals consist of combinations of these organisms which are arranged in accordance with definite rules.

Virchow[12](1821-1902) with his famous words, "Omnis cellula e cellula... " said that cells arise from pre-existing cells, confuting Schleiden and Schwann's idea of spontaneous cell generation and presented his ideas about regeneration stating that tissue regeneration is dependent on cell proliferation.

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[11] Lenhoff, S.G., Lenhoff, H.M., and Trembley, A. Hydra and the Birth of Experimental Biology, 1744: Abraham Trembley's Me´moires Concerning the Polyps. Pacific Grove, CA: Boxwood Press, 1986.

[12] Virchow, R., and Chance, F. Cellular Pathology, as Based upon Physiological and Pathological Histology. Twenty Lectures Delivered in the Pathological Institute of Berlin During the Months of February, March and April, 1858. New York: R. M. De Witt, 1860.

He also observed that a whole organism does not get sick, but only certain cells or groups of cells. With this theory Virchow launched the field of cellular pathology. He stated that all diseases involve changes in normal cells, and therefore all pathology is cellular pathology.

Levert in 1829 tested various metallic sutures on dogs. He observed that when arteries had been tied with silk leaguers, on killing animals, he found abscesses at the site of leaguers, on the contrary using threads of lead, gold, silver or platinum he found leaguers encysted, without inflammation or suppuration. He reported also better performance of platinum sutures[13].

In 1924 Arthur Zierold, reported on the defects of metallic sutures: for iron and steel rapid corrosion, for copper, aluminum, and zinc tissue discoloration and for gold, silver, lead, and aluminum lack of mechanical robustness[14].

Thiersch in 1874 grew skin cells into granulating wounds and discovered the important influence of granulation tissue on wound healing .

Loeb in 1897 took the first step to maintain blood cells, connective and other tissue outside the human body in plasma or serum[15].

Harrison was the first to grow frog ectodermal cells *in vitro* in 1907, thus developing the first neuronal tissue culture line[16].

Carrel in 1912 was able to grow pieces of chick embryo in various media, initially maintained for 85 days, and subsequently for years[17].

Rous and Jones in 1916 introduced the use of **trypsin** to degrade matrix proteins and separate cells.

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[13] Levert H.S. Experiments on the use of metallic ligatures, as applied to arteries.*Am J Med Sci* 4**,**17, 1829

[14] Zierold A.A. Reaction of bone to various metals. *Arch Surg* 9**,**365, 1924

[15] Schultheiss D., Bloom D.A., Wefer J., and Jonas U. Tissue engineering from Adam to the zygote: historical reflections. *World J Urol* 18**,**84, 2000

[16] Harrison, R.G., Greenman, M.J., Mall, F.P., and Jackson, C.M. Observations of the living developing nerve fiber. *Anat Rec* 1, 116, 1907.

[17] Fell, H.B. Tissue culture and its contribution to biology and medicine. *J Exp Biol* 57, 1, 1972.

In the following years much research was performed, which led to the ability to grow tissue-specific cell lines *in vitro*.

Enders in 1952 contributed greatly to the use of human embryonic cells.

Sir Harold Ridley observed aviators who during Second World War had retained in their eyes splinters of plastic from shattered canopies of their planes. He found that these accidentally implanted shards were perfectly healed without inflammatory reaction. This observation destroyed the paradigm that human body could not tolerate foreign objects. He asked John Pike of Rayners to fashion a lens of polymethylmethacrylate. First implantation was performed by sir Ridley in 1949 in a 42-years-old women after an extra capsular cataract extraction[18].

A fortuitous observation led Per Ingvar Branemark to observe osteocompatibility of titanium. He was studyng blood flow, with an optical device in titanium attached to a rabbit's leg to study microcirculation in the bone tissue of rabbits through specially modified light microscopes. On removing the device from the bone, he found that the bone and the titanium had become inseparable.

In a subsequent study of microcirculation in volunteers with titanium instruments inserted into their arms for several months he found no signs of rejecting the titanium-enclosed optics.

At that point, Brånemark changing field of study began to investigate the body's ability to tolerate titanium[19].

In 1952 started the use of textiles as vascular implants with the innovative work of Voorhees and colleagues who replaced aortic vessels of dogs with

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[18] Ratner, B.D. History of biomaterials. In: Ratner, B.D., Hoffman, A.S., Schoen, F.J., and Lemons, J.E., eds. Biomaterials Science—An Introduction to Materials in Medicine. 3rd ed. London: Academic Press, 2013, pp. xli– liii.

[19] Branemark, P.I. Osseointegration and its experimental background. *J Prosthet Dent* 50, 399, 1983.

woven Vinyon-N (a polyvinylchloride) tubes[20]. Within few years of Voorhees's work, many studies reported clinical trials with different materials (Nylon, Teflon®, Dacron®, Orlon®), and constructions (woven, knitted, braided) in various diameters (6–20 mm).

Alan Turing in his article of 1952 presented the first computational model of a biological phenomenon. He described how, in circular arrays of identical biological cells, diffusion can interact with chemical reactions to generate up to six periodic spatiotemporal chemical structures and one of these structures, a stationary pattern with a chemically determined wavelength, is responsible for differentiation[21].

2.2.4. Modern history

2.2.4.1- Stem cells

A significant event in medical history, particularly of tissue engineering and regenerative medicine is the discovery of stem cells. The concept of stem cells emerged in 19th century, but their existence was confirmed on 1960s

[20] Voorhees A.B., Jaretzki A., and Blakemore A.H. The use of tubes constructed from vinyon N cloth in bridging arterial defects. *Ann Surg* 135,332, 1952

[21] Turing, A.M. The chemical basis of morphogenesis. *Philos Trans R Soc Lond B Biol Sci* 237, 37, 1952.

1868 The term “stem cell” first appears in scientific literature. Ernst Haeckel a german biologist uses the phrase “stem cell” to describe the fertilized egg that becomes an organism, and also to describe the single-celled organism that acted as the ancestor cell to all living things[22].

1886 William Sedgwick uses the term “stem cells” to describe the parts of a plant that grow and regenerate.

1909 Alexander Maximow , a russian academic explains at the Berlin Hematological Society his theory that all blood cells come from the same ancestor cell[23].

1953 Leroy Stevens, a Maine scientist found in teratomas mixtures of differentiated and undifferentiated cells, including hair, bone, intestinal and blood tissue. He concluded that these the cells were pluripotent, able to differentiate into any cell of a fully grown animal[24].

1957 —E. Donnall Thomas (Nobel prize in 1990), a scientist of Seattle, attempted the first human bone marrow transplantation.

1963 Canadian scientists Ernest McCulloch and James Till performing experiments on the bone marrow of mice and observed that different blood cells come from a special class of cells.[25]

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[22] E. Haeckel, Natürliche Schöpfungsgeschichte, Georg Reimer, Berlin (1868)

[23] Maximow A. The Lymphocyte as a stem cell common to different blood elements in embryonic development and during the post-fetal life of mammals (1909). Originally in German: *Folia Haematologica* 8.1909, 125-134. English translation: *Cell Ther Transplant*. 2009,1:e.000032.01. doi:10.3205/ctt-2009-en-000032.01

[24] L.C. Stevens, "Studies on transplantable testicular teratomas of strain 129 mice," *Journal of the National Cancer Institute*, 20:1257-70, June 1958.

[25] E.A. McCulloch, J.E. Till, The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice,*Radiat*. *Res*., 13 (1960), pp. 115–125

1968 Robert A. Good of the University of Minnesota performs the first successful bone marrow transplant on a child suffering from an immune deficiency .The donor was the sister of the patient[26].

1981 Martin Evans of the University of Cambridge and Gail Martin of the University of San Francisco, conducted separate studies and derived pluripotent stem cells from the embryos of mice[27] [28].

1986 Andrew Lassar and Harold Weintraub of Seattle converted rodent fibroblasts directly into myoblasts , using a single gene (MyoD)[29].

1989 Mario Capecchi, Martin Evans and Oliver Smithies created the first "knockout mice," mice specially bred in the laboratory to be missing specific genes. These mice are created using embryonic stem cells and homologous recombination, in a process in which similar strands of DNA switch genes. These mice are important in understanding various diseases[30].

1997 Dominique Bonnet and John Dick of Canada discovered that leukemia comes from the same stem cells of normal blood cells, supporting the thesis that cancer grows out of stem cells gone off course[31].

1998 — A team at the University of Wisconsin, Madison, led by James Thomson and Jeffrey Jones, reported the creation of the first batch of human embryonic stem cells, which they derived from early embryos. After finding the

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[26] Good RA, Gatti RA, Hong R, Meuwissen HJ. Successful marrow transplantation for correction I deficit in lymphopenic agamma- globulinemia and treatment of immunologically induced pancy- topenia. *Exp Hematol*. 1969;19:4-10.

[27] Evans MJ, et al. *Nature*. 1981;292:154–156.

[28] Martin GR. Proc Natl Acad Sci USA. 1981;78:7634–7638.

[29] Sassoon D. et al., Expression of two myogenic regulatory factors myogenin and MyoDI during mouse embryogenesis, *Nature* 341, 303 - 307 (28 September 1989);

[30] Capecchi M.R.,Generating mice with targeted mutations, *Nat. Med.* **7**, 1086–1090 (October 2001).

[31] Bonnet D., Dick J.E., Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell, *Nature Medicine* **3**, 730 - 737 (1997)

cells were pluripotent, the team sees the potential the cells have for drug discovery and transplantation medicine[32].

2007 stem cells researchers were able to produce stem cells from human somatic cells not derived from embryos[33] .

2.2.4.2 cell growth

Following the discover of stem cells studies were focused to investigate cell growth.

Folkman during 1970s demonstrated the dependence of histogenesis on mass transport requirements of the growing tissue, the importance of cell shape on growth and differentiation and the capacity of isolated cells in presence of cues from their native environment to create original structures. He also proposed a hypothesis that tumor growth is angiogenesis-dependent . He sustained that tumors could not enlarge beyond millimeter diameters without recruiting new capillary blood vessels by a tumors secreted diffusible substance that could stimulate endothelial cell proliferation in host capillary blood vessels[34].

William T.Green in 1975 used rabbit articular chondrocytes to produce a chondroid matrix in subculture. This tissue was compared to articular cartilage by histochemical and electron microscopic techniques. Although both tissues

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[32] Thomson J.A.et al.,Embryonic Stem Cell Lines Derived from Human Blastocysts, *Science* 282, 1145 (1998)

[33] Takahashi K. el al., Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131,861, 2007

[34] Folkman J., and Hochberg M. Self-regulation of growth in three dimensions. *J Exp Med* 138,745, 1973

were similar, the cytology of chondrocytes and the matrix structure of chondroid tissue appeared less mature[35].

Paul S.Russel in 1985 wrote a review on selective cell transplantation, referred to the selection of only a part of an organ or tissue fot transplantation. isolated cells when are placed as a cell suspension into tissue lack any intrinsic beginning structure and lack a template to guide restructuring. An additional problem is the metabolic needs of the cells, because they must be no more than a few hundred microns from a capillary.

J.Vacanti and Robert Langer in 1980s designed appropriate scaffolds for cells growth[36].

Balkrishan G.Matapurkar in 1990s employed mesodermal stem cells obtained from peritoneum to aid regeneration of other organs of mesodermal origin[37].

2006, Shinya Yamanaka and Kazutoshi Takahashi at Kyoto University identified four genes, Oct3/4, Sox2, c-Myc and Klf4, that caused cultured mouse skin cells to become undifferentiated, pluripotent stem cells[38].

On 2 December 2007, Science published a report on creating human induced pluripotent stem cells from human somatic cells; researchers induced pluripotency in adult stem cells. They were able to reverse the biological process whereby embryonic stem cells differentiate into somatic (adult) cells.

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[35] Green W.T. Articular-cartilage repair. Behavior of rabbit chondrocytes during tissue culture and subsequent allografting. *Clin Orthop Relat* Res 124**,**237, 1977.

[36] Langer R., and Vacanti J.P. Tissue engineering. *Science* 260**,**920, 1993

[37] Matapurkar B.G., Bhargave A., Dawson L., and Sonal B. Organogenesis by desired metaplasia of autogenous stem cells. *Ann N Y Acad Sci* 857**,**263, 1998

[38] Takahashi K1, Yamanaka S.,Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell*. 2006 Aug 25;126(4): 663-76. Epub 2006 Aug 10.

The use of somatic cells resolved the ethical problems of the use of embryonic stem cells[39].

2.2.5. The state of art of bioengineered tissues

We have actually availability of autologous or allogenic epidermal (Epicel®, Epidex™, Myskin™, ReCell®), dermal (AlloDerm®, Integra®, Matriderm®), and dermoepidermal (Apligraf®, Orcel®) substitutes We can have regeneration of dentine-pulp complex using cells encapsulated within scaffolds; treatment of periodontal defects through guided bone regeneration membrane, growth factors and cytokines, replacement of lost teeth by transplantation of the bioengineered tooth germ[40].

Transplantation of bone marrow stem cells (encapsulated within a suitable scaffold)[41], to repair articular cartilage (patellar, patellofemoral, and femoral), osteochondral defects[42] and local bone defects[43]have been realized.

Technology has made possible development and availability of tissues and, very recently, organs for clinical applications.

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A bioengineered vessel has been implanted to replace the right intermediate pulmonary artery in a child suffering from single right ventricle and pulmonary atresia[44].

Bioengineered bladders and urethras have been realized. Postimplantation follow-ups showed improved functionality and compliance[45].

A deceased donor trachea was decellularized and served as the scaffold where were seeded autologous epithelial cells and chondrocytes .

Before implantation, the construct was maintained for 4 days in a bioreactor designed to address seeding requirements, provide biomechanical cues, and achieve good laboratory practice[46].

This technology of employing decellularized organ scaffolds is advantageous as the native three-dimensional tissue architecture, vascular tree, and ECM-related cues seem to be well preserved in the organ scaffold[47].

A variety of organs have been bioengineered, some of which, such as the larynx and vagina, have found clinical use[48] [49].

The bioengineered tissue constructs recapitulate the native architecture, physiology, dynamic conditions, intercellular, and cell–matrix interactions more accurately than two-dimensional culture monolayers[50].

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These ex vivo constructs can be also employed as preclinical models for drug screening,pharmacokinetic and pharmacodynamics analyses of drugs,and device testing, with the advantage of decreased costs, increased reproducibility, precise control over culture conditions[51], incorporation of human cells, and systematic evaluation of the product being tested. Specific examples are blood vessels for device (stents)[52] and drug testing; bioengineered blood–brain barriers to test drug permeability and disease modelling[53]; musculoskeletal tissue to optimize drugs for improving muscular growth and function and to evaluate the impact of prosthetic devices on muscular tissues[54]; corneal tissue to conduct tests for toxicology and eye irritancy[55]; skin to test for cytotoxicity[56] phototoxicity[57] and wound healing[58]; reproductive tissues to study sexually

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transmitted infections[59], product efficacy,and, as models of the blood–testis barrier[60], to predict toxicity and permeability in vivo.

A significant advantage is their availability as a superior and more accurate alternative to animal testing.

An important evolution is the fact that the manufacturing processes for these constructs has undergone transition from being manual to automated, consequently dynamic, reproducible, and increasingly compliant with current good manufacturing practices[61].

We have now robotic systems, such as the CompacT SelecT, to conduct automated cell culture[62]; computer-assisted bioprinting to manufacture two- and three-dimensional biological patterns[63]; bioreactors that can perform multiple roles such as nutrient and waste transport, mechanical conditioning, cell seeding, supporting cell viability, and promoting their 3D organization[64]. These automated procedures and manufacturing processes are being continuously scrutinized and optimized, using a variety of quality control tools[65].

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3. SCAFFOLDS

Apart from blood cells, normal cells in human tissues are anchorage-dependent placed in a solid matrix called extracellular matrix (ECM).

In human tissues there are numerous types of ECM, which have multiple components and tissue-specific composition. The main functions of ECM are[66]:

1. provides structural support and physical environment for cells to attach, grow, migrate and respond to signals.
2. gives the tissue its structural and mechanical properties,
3. provides bioactive cues to the residing cells for regulation of their activities.
4. act as reservoir of growth factors and potentiate their bioactivities.
5. provides a degradable physical environment allowing neovascularization and remodeling in response to developmental, physiological and pathological challenges during tissue dynamic processes as morphogenesis, homeostasis and wound healing.

In tissue engineering scaffolds are used to mimic ECM functions with two approaches[67] [68]:

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- **scaffold-guided regeneration** by the use of biodegradable scaffold implanted in the damaged area to direct the growth of cells migrating from surrounding tissue.
- **cell-loaded scaffold implantation**. The scaffold guide cells to grow, synthesize extracellular matrix and other biological molecules, and facilitate the formation of functional tissues and organs.

The best scaffold for an engineered tissue should be similar to ECM of the target tissue, but the multiple functions, the complex composition and the dynamic nature of ECM in native tissues make it difficult to mimic exactly. Therefore, the goal of scaffolding in tissue engineering is to mimic the functions of native ECM, at least partially.

The fundamental features of a scaffold are:

- **architecture**: Scaffolds should provide void volume for vascularization, new tissue formation and remodeling so as to facilitate host tissue integration upon implantation. The biomaterials should have a higly porous structure with an interconnected pore network for nutrient and metabolite transport without compromising the mechanical stability of the scaffold. The biomaterials should also be degradable at a rate matching that of the new matrix production by the developing tissue.

- **biocompatibility**: the biomaterials used to fabricate the scaffolds need to be compatible with the cellular components of the engineered tissues and endogenous cells in host tissue. Cells should be able to adhere, proliferate, migrate and differentiate. When implanted should not induce a severe host immune response.

- **surface properties**: scaffolds surface properties, such as morphology, hydrophilicity, energy and charge, are important in vitro for cell adhesion, migration, phenotype maintenance, intracellular signaling, and in vivo for cell recruitment and healing at the tissue interface.

- **anatomical shape:** external geometry and size should match with those of tissue defect for a good integration and distribution of mechanical loadings.

- **sterilization** : is necessary an adequate sterilization technique to avoid physicochemical changes that can occur to the material employed for the scaffold preparation, particularly when polymeric materials are used..

3.1. POLYMERIC MATERIALS FOR SCAFFOLD DEVELOPMENT

3.1.1. POLYMERS OF NATURAL ORIGIN

The ECM is the best milieu that nature has developed to maintain homeostasis and to direct tissue development. Therefore, in the intention to mimic the ECM to guide morphogenesis in tissue engineering, a strategy has been proposed to isolate the main constituents of the ECM and directly use them after purification, with or without further modifications, following the hypothesis that such biomolecules would maintain the biological information and other physico-chemical features, which would preserve a potential space for new tissue development after cell seeding. This would help to overcome one of the main drawbacks of synthetic materials, which lack cell recognition signals[69]. The main classes of polymeric materials of natural origin employed for scaffold preparation are reported by following.

3.1.1.1. PROTEINS

Collagen is the most abundant protein in the body, present in the extracellular matrix of many tissues (skin, cartilage, bone, tendons, blood vessels, teeth) More than 20 genetically distinct forms have been identified.

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Important characteristics are high mechanical strength, good biocompatibility, low antigenicity and and cell-binding properties[70].

Fibroblasts are responsible for the majority of the collagen production in connective tissue.

Collagen molecules are composed of three α chains. Every α chain is composed of more than a thousand amino acids based on the sequence -Gly-X-Y-. The presence of glycine is essential at every third amino acid position to allow a tight packaging of the three α chains in the tropocollagen molecule and the X and Y positions are mostly filled by proline and 4-hydroxyproline . There are about twenty-five different α chain conformations.The combination of these chains forms the twenty-nine different types of collagen currently known. Only a few types are used to produce collagen- based biomaterials. Type I collagen is the gold standard in the field of tissue-engineering[71].

Collagen sponges have been reported to promote cell and tissue attachment and growth and differentiation of osteoblasts. The disadvantage of collagen is its high degradation rate, with a rapid loss of mechanical properties. To resolve this problem many attempts have been made, as adding mineral crystals, or combining collagen with natural or synthetic polymers or applying various cross linking methods[72].

The principle of a cross-linking reaction relies on the modification of amine and carboxyl groups within the collagen molecules, to allow the formation of covalent bonds. Several methods have been developed to cross-link collagen

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scaffolds[73]. There are three types of techniques : physical, chemical and enzymatic crosslinking.

Physical crosslinking rely on irradiation by ultra-violet wavelengths (UV) or thermal sources to induce the collagen scaffold polymerization. These techniques induce an increase in tensile strength and some fragmentation in the collagen molecular structure . UV crosslinked collagen scaffolds also have an enhanced enzymatic resistance . Besides, UV irradiation has been recently optimized to reduced collagen fragmentation by using glucose in the crosslinking process . However, UV irradiation is only effective for thin and/or transparent scaffolds[74].

A chemical technique consists in the use of aldehydes such as formaldehyde and glutaraldehyde[75]. .Another class of chemicals used is the carbodiimide family[76] . These chemicals can also be used to crosslink collagen to some marginal substances like gold nanostructure or utilized in combination with epoxy . The isocyanate chemical family, especially hexamethylene diisocyanate[77], is also used. However, all these chemical stabilisation techniques leave potentially toxic residues in the collagen-based biomaterial .

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An alternative to covalent bond crosslinking is to promote the formation of ionic bonds between collagen molecules. For this purpose are used polycationic molecules such as chitosan, which create ionic bonds between its amine groups and the carboxyl groups of collagen[78].

Enzymatic crosslinking agents like transglutaminase can be used to enhance tensile strength and enzymatic resistance. Using a biologic polymerization technique there is the advantage that no chemical residues remain in the scaffold structure, eliminating the risk of inducing cytotoxic effects[79].

Many biomolecules can also be added to collagen solution. These biomolecules, typically GAG, elastin and chitosan are added to the compound to enhance the mechanical strength and to modulate cellular functions such as migration, proliferation and differentiation[80] .

Fibronectin is component of the ECM, that induces cell attachment and spreading. This glycoprotein (a disulphide-bonded dimer of 220–250 kDa subunits) serve as a substrate for cell adhesion through the biological activity of several modules: the RGD tripeptide, arginine-glycine-aspartic acid, in the tenth Fn3 module plays an important role and has been incorporated onto the surface of numerous biomaterials; One suggested strategy is to deposit layers of oriented fibronectin to enhance the availability of its cell binding site . Human umbilical vein endothelial cells spread significantly faster in a more spherical way on an oriented fibronectin layer, than on an isotropic layer[81].

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Glycosaminoglycans consist of linear chains of the repeating unit of a disaccharide, generically a hexosamine (glucosamine or galactosamine) and a uronic acid component . With the exception of hyaluronic acid, such chains are attached to a central protein to form the proteoglycans. Due to their ionic character, they can absorb large quantities of water, and this osmotic swelling provides compressive strength[82].

Fibrin is important for haemostasis and spontaneous tissue repair. It is formed by polymerization of fibrinogen in the presence of the enzyme thrombin. Fibrinogen can be isolated from the blood plasma of the patient, to avoid disease transmission and immunogenic reactions. Fibrin is a useful cell delivery matrix for cartilage tissue engineering, especially in combination with other biodegradable substances, such as alginate or hyaluronic acid and It has also been used in the regeneration of the skin, and in the loading and posterior release of growth factors[83].

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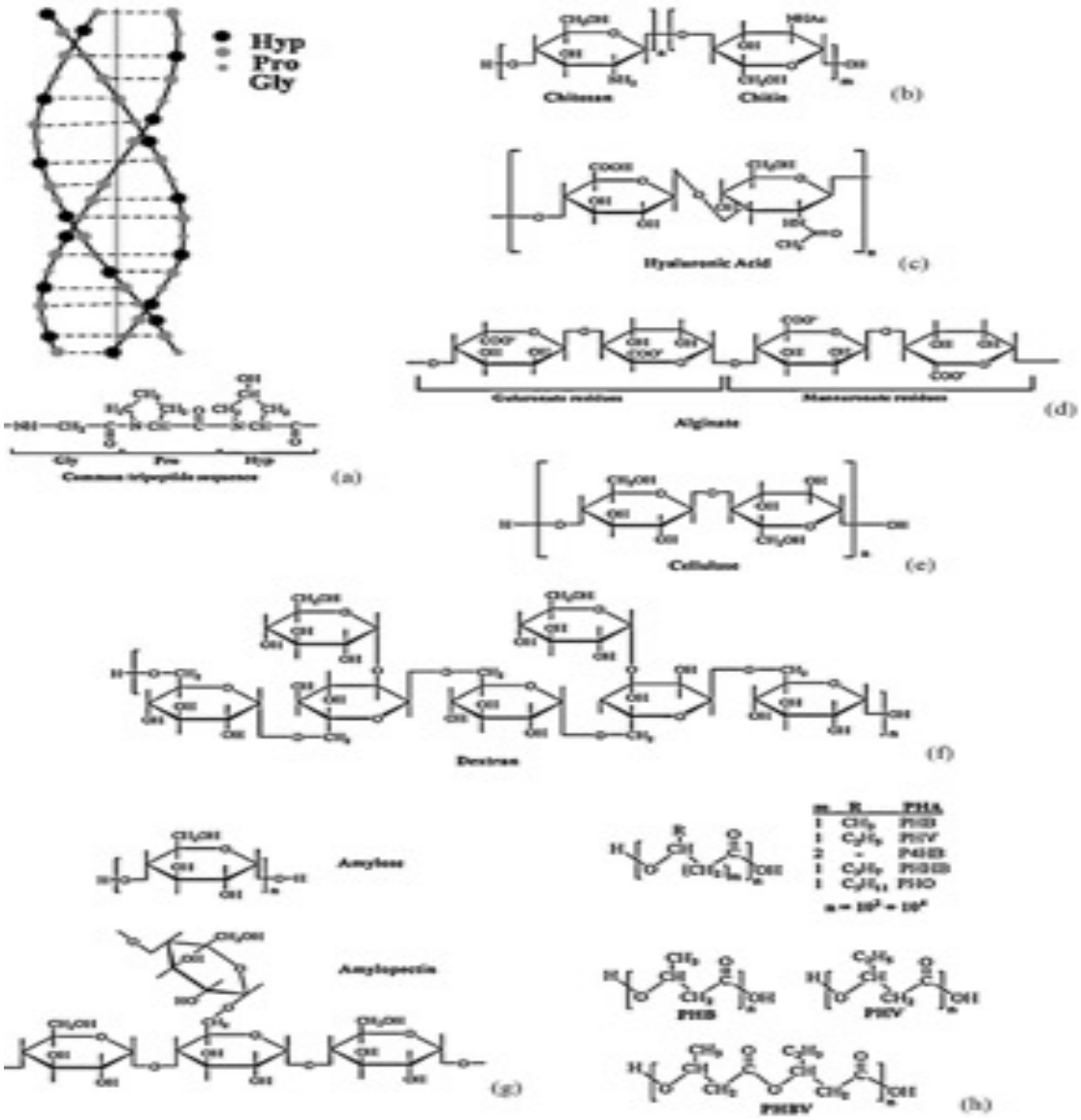

fig.2:. (a) Triple chain structure of collagen fibrils and chemical structure of the most common tripeptide sequence found in collagen composed of glycine (Gly), proline (Pro) and hydroxyproline (Hyp) sequencess; (b) representative chemical structure of chitosan. Chitosan is produced commercially by deacetylation of chitin that yields a heteropolymer; (c) chemical structure of hyaluronic acid (HA); (d) representative chain portion of alginate. Alginates are linear copolymers consisting of blocks of continuous mannuronate residues, guluronate residues or alternating guluronate/mannuronate residues; (e) chemical structure of cellulose; (f) representative chemical structure of dextran. Dextrans are homopolmers with -1,6 linkages in the main chain and -1,2, -1,3 or -1,4 branch linkages as a function of the specific microbial strain; (g) chemical structure of amylose and amylopectin. The ratio of the two components in starch granules varies according to the botanical origin; (h) chemical structure of polyhydroxyalkanoates (PHAs) commonly investigated for applications in tissue regeneration.

from D. Puppi et al. *Progress in Polymer Science* 35 (2010) 403–440 411

Silk fibroin, fibrous protein obtained from silk-worms, has great biocompatibility and important physical and chemical properties[84].

In tissue engineering, silk fibroin has been used for multiple types of scaffolds fabricated with a wide range of chemical, structural and biomechanical modifications . Silk sponges have been used for cartilage and fat , silk tubes for blood vessels and silk fibers for ligaments[85]. Porous sponge scaffolds are suitable for bone tissue formation, by enhancing cell attachment, proliferation and migration. In addition, the high porosity facilitates nutrient and waste transport[86] .

3.1.1.2. POLYSACCARIDES

Polysaccharides consist of monosaccharides linked together by *O*-glycosidic linkages. Their physical properties, including solubility, flow behaviour, gelling potential and/or surface and interfacial properties, depend on differences in the monosaccharide components , linkage types and patterns, chain shapes and molecular weight[87]. They have some excellent properties which make them the polymer group with the longest and widest medical applications experience. They are synthetized in plants, algae and marine crustaceans and also produced by bacteria and fungi[88]. They have excellent properties like non-toxicity, water solubility or high swelling ability by simple

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chemical modification, stability to pH variations, and a broad variety of chemical structures[89]. Some disadvantages of these materials are low mechanical, temperature and chemical stability, and proneness to microbial and enzymatic degradation[90].

Chitin and Chitosan Chitin is a copolymer of N-acetylglucosamine and glucosamine residues linked by β-1,4-glycosidic bonds. Chitosan is the deacetylated form of chitin. The names chitin and chitosan are not precisely defined. Chitin usually refers to a copolymer with a degree of acetylation (DA) of more than 40% and insoluble in dilute acids. The name chitosan is used for a copolymer with less than 40% DA that, generally is soluble in dilute acid[91].

Pure chitosan is non-toxic, free of antigenic effects, biocompatible, biodegradable and polar. It has been used to prepare a variety of forms such as powders, hydrogels, fibers, membranes, beads and porous scaffolds for many medical and biological applications . For tissue engineering applications, chitosan scaffolds have been prepared by means of different tecniques, such as freeze drying and freeze gelation methods, rapid prototyping and internal bubbling process[92].

Chitosan has been combined with a variety of materials, such as alginate, hydroxyapatite, hyaluronic acid , calcium phosphate, poly(methyl methacrylate),

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poly(L-lactic acid) , growing factors, for potential application in orthopedics and cell-based Tissue Engineering[93].

Chitosan (CTS) is able to enhance bone formation in vitro and in vivo but, for its mechanical weakness and instability and its incapacity to maintain a defined shape , chitosan scaffolds alone cannot imitate all the properties of natural bone. On the contrary the development of composite materials with chitosan mimics all the properties of bone. Several studies have been conducted with CTS and Hydroxyapatite (HAp) composite materials for bone tissue engineering. Hydroxyapatite is one of the most stable forms of calcium phosphate and is a major component of the bone (60 to 65%)[94]. It is used in many fields, including orthopedic, dental and maxillofacial applications. Therefore, HAp has recently emerged as an important compound for artificial bone preparation. It stimulates osteoconduction being gradually replaced by the host bone after implantation. It is being used for orthopedic replacements, especially in bone regeneration and dental implant treatment. The mechanical properties of the **chitosan/hydroxyapatite** composites play a significant role in bone tissue engineering. The intermolecular hydrogen bond and chelate interaction between the CTS and HAp contribute to good mechanical properties. There is a possible interaction between the NH_2 group and primary and secondary –OH group of CTS with Ca^{2+} (metal coordination interaction) of HAp. This interaction might be responsible for the higher mechanical strength of the composite scaffolds as compared to CTS and HAp alone[95]. **Chitosan and alginate** hybrid scaffold have been realized with significantly improved mechanical and biological properties as compared chitosan alone. Enhanced

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mechanical properties were attributable to the formation of a complex structure of chitosan and alginate[96]. Incorporation of collagen into chitosan improves cell attachment ability.

Combination with antibiotics or growth factor , like imidazole or PDGF has shown to induce bone formation[97].

In periodontal tissue engineering chitosan/collagen scaffolds have been loaded with plasmid and adenoviral vector encoding human transforming growth factor-beta1 (TGF-beta1) and human periodontal ligament cells (HPLCs) have been seeded in this scaffold, The scaffold containing Ad-TGF-beta1 exhibited the highest proliferation rate, and the expression of type I and type III collagen up-regulated After implanted in vivo,transfected HPLCs not only proliferated but also recruited surrounding tissue to grow in the scaffold.

The biocompatibility and similarity of chitosan to glycosaminoglycans (GAG) natural present in the ECM of cartilage make it particularly attractive as a candidate for the repair of cartilage defects[98]. Limited adhesion of chondrocytes and MSCs on chitosan limits its use. Therefore chitosan has been combined with various materials like poly(l-lactic acid)[99] and hyaluronic acid[100] to improve chondrocyte attachment and consequent cell adhesion, proliferation and biosynthetic activity. Other studies have been performed with a thermosensitive

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chitosan-pluronic hydrogel and chitosan scaffolds loaded with epidermal growth factor with positive results[101].

Hyaluronic acid Hyaluronic acid (HA) is used extensively in tissue engineering scaffolds. It is a nonsulfated glysocaminoglycan (GAG) composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine. HA is present in all vertebrates. It is a major constituent of the ECM, in various connective tissue not only as structural element, but also interacting with binding proteins, proteoglycans and other bioactive molecules, contributing to the regulation of water balance, behaving like a lubricant for the articular cartilage surface[102].

It has a good biocompatibility and viscoelastic properties, non immunogenic properties, widespread availability. It interacts with cell-surface receptors, promotes cell migration, facilitates ECM remodelling, stimulates production of collagen II, aggrecation and cell proliferation[103]. Popular natural hydrogels, such as alginates and fibrin, are useful to contain or immobilize cell suspensions[104]. Their properties have been enhanced with additives like HA . Surfaces of HA biomaterials have been coated with ECM proteins, such as type I collagen and fibronectin, to favor cell

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attachment and tissue formation[105].To provide long term stability and increase mechanical strength covalent crosslinking and photocrosslinking have been used[106].

Through the esterification of carboxyl groups of hyaluronic acid with various therapeutically inactive and active alcohols, it has been possible to synthesize biopolymers with medically desirable properties that are significantly different from those of hyaluronic acid itself. In particular, the esterification prevents fast enzymatic degradation of hyaluronic acid in vivo and allows prolonged scaffold integrity in concert with new tissue formation. The ethyl ester HYAFF7 and the benzyl ester HYAFF 11 are two of the most studied polymers[107].

Alginates Alginate is a naturally occurring anionic and hydrophilic polysaccharide. It is one of the most abundant biosynthesized materials , and is derived primarily from brown seaweed and bacteria. Alginate contains blocks of (1–4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers . Typically, the blocks are composed of three different forms of polymer segments: consecutive G residues, consecutive M residues and alternating MG residues. Physical properties of alginates depend on molecular weight, composition and extent of the sequences. The molecular weight (MW) of alginate influences the degradation rate and mechanical properties of alginate-based biomaterials. Higher MW decreases the number of reactive positions

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[105]Shu XZ et al., Liu Y, Palumbo F, Prestwich GD. Disulfide-crosslinked hyaluronan-gelatin hydrogel films: a covalent mimic of the extracellular matrix for in vitro cell growth. *Biomaterials* 2003;24:3825–34.

[106] Allison DD, Grande-Allen KJ. Review. Hyaluronan: a powerful tissue engineering tool. *Tissue Eng* 2006;12:2131–40.

[107] Grigolo B, De Franceschi L, Roseti L, Cattini L, Facchini A. Down regulation of degenerative cartilage molecules in chondrocytes grown on a hyaluronan-based scaffold. *Biomaterials* 2005;26: 5668–76.

available for hydrolysis degradation, which further facilitates a slower degradation rate[108].

It is used in many applications as a biomaterial and especially as the supporting matrix or delivery system for tissue repair and regeneration. Due to its properties in terms of biocompatibility, biodegradability, non-antigenicity and chelating ability, alginate has been widely used in a variety of biomedical applications including tissue engineering, drug delivery and in some formulations preventing gastric reflux . Commercial alginates are extracted primarily from three species of brown algae (Laminaria hyperborea, Ascophyllum nodosum, and Macrocystis pyrifera)[109]. Alginate purification is necessary to achieve control of material degradation, because the stability of alginate in aqueous solutions is affected by impurities, such as transition metal ions, as well as by the presence of oxygen and solution pH[110].

Alginate hydrogels have been studied for cartilage and bone regeneration applications as scaffolds and matrix for entrapment and delivery of biologically active molecules or cells[111]. They have been combined with chondrocytes and directly injected into the site of interest or moulded and then implanted . After four weeks of implantation the chondrocytes were viable and had produced ECM proteins consistent with cartilage[112].

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[108]Izydorczyk M, Cui S.W, Wang Q Polysaccharide gums: structures. Functional properties, and applications. In *Food carbohydrates: chemistry, physical properties, and applications* Cui S.W 2005pp. 263–307. Eds. Boca Raton, FL:CRC Press; Taylor & Francis Group.

[109] Smidsrød O, Skjåk-Braek G. Alginate as immobilization matrix for cells. *Trends Biotechnol* 1990;8:71–8.

[110] Holme HK, Lindmo K, Kristiansen A, Smidsrød O. Thermal depoly- merization of alginate in the solid state. *Carbohydr Polym* 2003;54:431–8.

[111] Eiselt P, Yeh J, Latvala RK, Shea LD, Mooney DJ. Porous carriers for biomedical applications based on alginate hydrogels. *Biomaterials* 2000;21:1921–7.

[112]Chang SCN, Rowley JA, Tobias G, Genes NG, Roy AK, Mooney DJ, et al. Injection molding of chondrocyte/alginate constructs in the shape of facial implants. *J Biomed Mater Res* 2001;55:503–11.

Alginates have been also studied in non-load-bearing applications: alginate gels with a high guluronic acid content were able to support proliferation of murine marrow cells and their differentiation along the osteoblastic lineage[113]. The presence of the adhesion peptide RGD and of a BMP- 2 derived oligopeptide has been shown to increase the amount of bone formation. Chitosan–alginate gels have also been investigated for bone regeneration. The in vivo studies evidenced rapid vascularization, deposited connective tissue and calcified matrix within the entire scaffold structure[114].

Starch, which is the major dietary source of carbohydrates, is the most abundant storage polysaccharide in plants, and occurs as granules in the chloroplast of green leaves and the amyloplast of seeds, pulses, and tubers (Sajilata, et al., 2006). Chemically, starches are polysaccharides, composed of a number of monosaccharides or sugar (glucose) molecules linked together with -D-(1-4) and/or -D-(1-6) linkages. The starch consists of 2 main structural components, the amylose, which is essentially a linear polymer in which glucose residues are -D-(1-4) linked typically constituting 15% to 20% of starch, and amylopectin, which is a larger branched molecule with -D-(1-4) and -D-(1-6) linkages and is a major component of starch. Amylose is linear or slightly branched, has a degree of polymerization up to 6000, and has a molecular mass of 105 to 106 g/mol. The chains can easily form single or double helices. Amylopectin on the other hand has a molecular mass of 107 to 109 g/mol. It is highly branched and has an average degree of polymerization of 2 million, making it one of the largest molecules in nature. Chain lengths of 20 to 25 glucose units between branch points are typical. About 70% of the mass of starch granule is regarded as amorphous and about 30% as crystalline. The amorphous regions contain the main amount of amylose but also a

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[113] Wang L et al., Evaluation of sodium alginate for bone marrow cell tissue engineering. *Biomaterials* 2003;24:3475–81.

[114] Li Z, Ramay HR et al. Chitosan- alginate hybrid scaffolds for bone tissue engineering. *Biomaterials* 2005;26:3919–28.

considerable part of the amylopectin.[115] The crystalline region consists primarily of the amylopectin (Sajilata, et al., 2006)[116].Starches are totally biodegradable and inexpensive, and can be processed by diverse techniques into diverse shaped items (3D porous scaffolds, microparticles, bone cements) .

To better resist thermomechanical degradation, and make them stronger and more easily processed starch-based polymeric systems have been blended with ethylene vinyl alcohol (SEVA-C)[117], cellulose acetate (SCA), polycapro- lactone (SPCL)[118] and poly(lactic acid) (SPLA)[119] for a wide range of biomedical applications, including bone cements, hydrogels for drugs controlled delivery, bone substitutes.

Cellulose is a polysaccharide consisting of a linear chain of several hundred to over ten thousand b(1 - 4) linked D-glucose units. It was discovered and isolated from green plants by Payen in 1838. It is the most abundant biopolymer on Earth, synthesized by grasses, woody plants, many forms of algae, fungi and some species of bacteria[120].

Cellulose synthesized by *Acetobacter xylinum* is identical to plant cellulose in chemical structure, but it can be produced without contaminant

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[115] Galliard T, Bowler P. Morphology and composition of starch. *Crit Rep Appl Chem* 1987;13:55–78.

[116] Sajilata MG et al., Resistant DOI:dx.doi.org starch—a review. *Comprehensive Rev in Food Sci and Food Safety*. 2006;5(1):1–17.

[117]Neves NM et al.,The morphology, mechanical properties and ageing behavior of porous injection molded starch- based blends for tissue engineering scaffolding. *Mater Sci Eng* C 2005;25:195–200.

[118] Gomes ME et al., Cytocompatibility and response of osteoblastic-like cells to starch-based polymers: effect of several additives and processing conditions. *Biomaterials* 2001;22:1911–7.

[119] Duarte ARC et al., Preparation of starch-based scaffolds for tissue engineering by supercritical immersion precipitation. J Sup Fluids 2009;49:279–85.

[120] Klemm D. et al., Cellulose. In: Steinbüchel A, editor. *Biopolymers*, vol. 6. Weinheim: Wiley-VCH; 2002. p. 275–319.

molecules, such as lignin and hemicelluloses, and does not require intensive purification processes and it is remarkable for its mechanical strength and good biocompatibility[121], thus it has been applied in tissue engineering[122].

Oxidized cellulose has relatively high stability during manipulation and exposure to the cell culture environment, and biocompatibility, which can be further improved by modification with biomolecules, particularly chitosan. It induces the most appropriate spreading and phenotypic maturation of cells, but not considerable cell proliferation. It was proved useful in the construction of bioartificial tissues or organs, where high proliferation activity of cells is not desired. Therefore a possible application of this material is for developing bioartificial vascular prostheses, where excessive growth activity of cells on the material may cause stenosis and failure of these replacements[123].

Bacterial cellulose has been also proposed as potential scaffold material for cartilage TE, showing in *in vitro* studies to support proliferation and ingrowth of human chondrocytes[124].

A highly porous cellulose matrix was created by lyophilisation of a cellulose gel. The behavior of human osteoblastic cells on the investigated cellulose matrix confirmed that it was not cytotoxic as cells were able to adhere and proliferate on it. It was found that the cellulose matrix containing silanol, carboxyl, or/and hydroxyl groups combined with calcium ions induced hydroxyapatite formation on its surface during the biomimetic mineralization.

Comparison of different matrix precalcification methods showed that the highest mineralization rate and larger deposited mass of hydroxyapatite were

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[121] Bae S, Shoda M. Bacterial cellulose production by fed-batch fermentation in molasses medium. *Biotechnol Prog* 2004;20: 1366–71.

[122]Petersen N1, Gatenholm P., Bacterial cellulose-based materials and medical devices: current state and perspectives, *Appl Microbiol Biotechnol*. 2011 Sep;91(5): 1277-86.

[123] Klemm D. et al., Bacterial synthesized cellulose—artificial blood vessels for microsurgery. *Prog Polym Sci* 2001;26:1561–603.

[124] Svensson A. et al., Bacterial cellulose as a potential scaffold for tissue engineering of cartilage. *Biomaterials* 2005;26:419–31.

achieved on the matrix of carboxymethylated cellulose activated with a calcium hydroxide solution[125].

Dextrans are polysaccharides with molecular weights ≥1000 Dalton, which have a linear backbone of α-linked D-glucopyranosyl repeating units. Three classes of dextrans can be differentiated by their structural features:

•Class 1 dextrans contain the α(1→6)-linked D-glucopyranosyl backbone modified with small side chains of D-glucose branches with α(1→2), α(1→3), and α(1→4)-linkage. The class 1 dextrans vary in their molecular weight, spatial arrangement, type and degree of branching, and length of branch chains, depending on the microbial producing strains and cultivation conditions.

•Class 2 dextrans (alternans) contain a backbone structure of alternating α(1→3) and α(1→6)-linked D-glucopyranosyl units with α(1→3)-linked branches.

•Class 3 dextrans (mutans) have a backbone structure of consecutive α(1→3)-linked D-glucopyranosyl units with α(1→6)-linked branches.

The physical and chemical properties of purified dextrans depend on the microbial strains from which they are produced and by the production method. For their high molecular weight they have good mechanical properties, and are highly hydrophilic, but they are resistant to protein absorption and cell adhesion. However the active hydroxyl groups of dextran can be chemically modified to incorporate various functional groups developing specific characteristics[126]. Dextran has been chemically engineered to form various scaffolds, including

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[125] GrandeCJ,TorresFG,GomezCM,CarmenBan˜óM.Nanocomposites of bacterial cellulose/hydroxyapatite for biomedical applications. *Acta Biomater* 2009;5:1605–15.

[126] Leathers TD. Dextran. In: Steinbüchel A, editor. *Biopolymers*, vol. 5. Weinheim: Wiley-VCH; 2002. p. 300–21.

spheres, tubules] and hydrogels][127]. These nano- and microstructured biological scaffolds are highly efficient drug delivery systems, tissue regeneration devices and cell therapy vectors[128].

Microbial Polyesters. Polyhydroxyalkanoates (PHAs)[129], are a family of microbial polyesters widely investigated also for biomedical applications. In particular the homopolymer polyhydroxybutyrate (PHB) and its copolymer with hydroxyvalerate (PHBV), are the most promising materials for such field of applications.

PHB[130] was discovered by Maurice Lemoigne of the Pasteur Institute, Paris in 1925 while studying *Bacillus megaterium.* PHB is associated with the growth and cell division of bacteria, it is stored as energy source and it directly relates with the physiological active state of cell. Author has observed that PHB accumulation in *Alcaligenes* sp. and *Pseudomonas* sp. increases at the exponential phase of growth. This observation led to the conclusion that bacteria make and store PHB when nutrients get exhausted and when cell is at its higher stage of growth[131].

PHAs and their composites have been used in medical field for many devices including sutures, suture fasteners, meniscus repair devices, rivets, tacks, staples, screws, bone plates and bone plating systems, surgical mesh, repair patches, slings, cardiovascular patches, orthopedic pins,adhesion

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[127]Lévesque SG, Shoichet MS. Synthesis of cell-adhesive dex- tran hydrogels and macroporous scaffolds. Biomaterials 2006;27:5277–85.

[128]HovgaardL,BrøndstedH.Dextranhydrogelsforcolon-specificdrug delivery. *J Controlled Release* 1995;36:159–66.

[129] Freier T. Biopolyesters in tissue engineering applications. *Adv Polym Sci* 2006;203:1–61.

[130] Miller ND, Williams DF. On the biodegradation of poly--hydroxybutyrate (PHB) homopolymer and poly-[beta]- hydroxybutyrate-hydroxyvalerate copolymers. *Biomaterials* 1987;8:129–37.

[131] Gangurde NS et al., Development of eco-friendly bioplastic like PHB by distillery effluent microorganisms, *Environ Sci Pollut Res Int*. 2013 Jan;20(1):488-97.

barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, atrial septal defect repair devices, pericardial patches, bulking and filling agents, vein valves, bone marrow scaffolds, meniscus regeneration devices, ligament and tendon grafts, ocular cell implants, spinal fusion cages, skin substitutes, dural substitutes, bone graft substitutes, bone dowels, wound dressings, and hemostats[132].

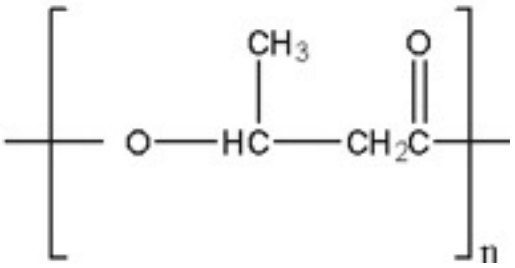
PHB is biocompatible to various cell lines, including osteblastic, epithelial cell and ovine chondrocytes, but its low mechanical strength limits its use in load-bearing applications. Using plasticizers, such as triethylcitrate, or blending with other polymers the properties of PHB can be changed. Mechanical properties can be improved blending PHB with PHBV, while flexibility increase blending with PHBHHx[133].

[132] Chen G-Q, Wu Q. The application of polyhydroxyalkanoates as tis- sue engineering materials. *Biomaterials* 2005;26:6565–78.

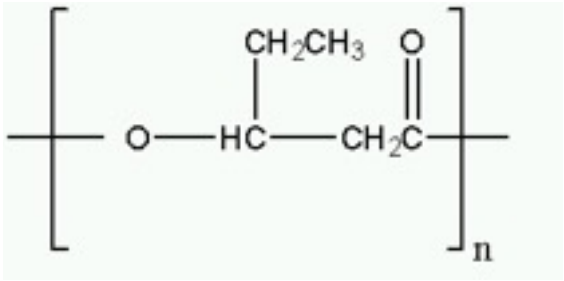
[133] Wang Y. et al., Evaluation of three- dimensional scaffolds prepared from poly(3-hydroxybutyrate-co- 3-hydroxyhexanoate) for growth of allogeneic chondrocytes for cartilage repair in rabbits. *Biomaterials* 2008;29:2858–68.

fig.3: Polyhydroxyalkanoates (PHAs)

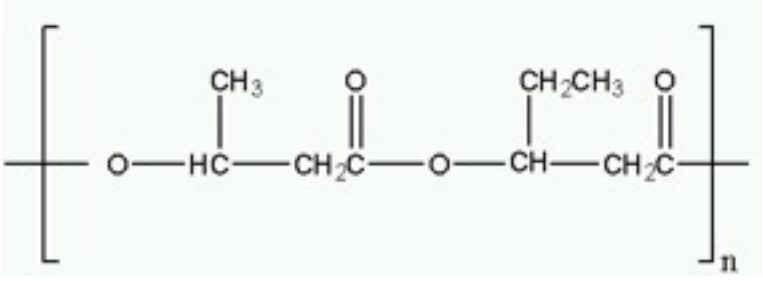
PHB



PHV



PHBV



3.1.2. SYNTHETIC POLYMERS

Research in the "first half of the 20th century with polymers synthesized from glycolic acid and other - hydroxy acids was abandoned for further development because the resulting polymers were too unstable for long-term industrial uses. On the contrary this instability leading to biodegradation,i s very important for medical uses.

Synthetic biomaterial guidance provided by biomaterials may facilitate restoration of structure and function of damaged or diseased tissues. Synthetic polymers are highly useful in biomedical field since their properties (e.g., porosity, degradation time, and mechanical characteristics) can be tailored for specific applications. They are often cheaper than biologic scaffolds, can be produced in large quantities with high batch-to-batch reproducibility, have a long shelf time and show physicochemical and mechanical properties comparable to those of biological tissues.

Synthetic polymers represent the largest group of biodegradable polymers, and they can be produced under controlled conditions. They exhibit, in general, predictable and reproducible mechanical and physical properties such as tensile strength, elastic modulus, and degradation rate .Polymers prepared from glycolic acid and lactic acid, have found a multitude of uses in the medical industry, beginning with biodegradable sutures. Since that time other medical devices, based on lactic and glycolic acid, as well as other materials, including poly(dioxanone), poly(trimethylene carbonate) copolymers, and poly(-caprolactone) homopolymers and copolymers, have been accepted for use as medical devices. In addition to these approved devices, a great deal of research continues on polyanhydrides , polyorthoesters , and other materials[134].

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[134] Pathiraja A.Gunatillake and Raju Adhikari, Biodegradable synthetic polymers for tissue engineering, *European cells and materials* Vol.5. 2003 (pages 1-16)

Short chain saturated aliphatic polyesters There is increasing attention towards aliphatic polyesters to solve “white pollution” problems caused by traditional non-biodegradable polymers and also for their use in the biomedical field .Polyesters are considered the most competitive biodegradable polymers commercialized up to now[135].

Poly(-hydroxyacids) are biodegradable synthetic polymers, widely investigated for the preparation of 3D scaffolds, in bone and cartilage TE. They include poly(glycolic acid) (PGA), poly(lactic acid) and poly(lactic- co-glycolic acid) copolymers. PLA exists in three forms: L-PLA (PLLA), D-PLA (PDLA), and racemic mixture of D,L-PLA (PDLLA)[136]. Poly(-hydroxyacids), PCL and their copolymers are the most widely used synthetic biodegradable polymers in the biomedical field, having FDA approval for various applications, such as sutures, wound dressings and stents. They can be easily processed and their degradation rate, physical and mechanical properties are adjustable by changing the molecular weight or copolymer ratio. Their degradation proceeds via a random, bulk hydrolysis of ester bonds in the polymer chain with subsequent possible premature fail of scaffold. A faster degradation is caused by the concentration of the acidic degradation products, leading to further stimulation of degradation and a subsequent lowering of pH. Acide degradation products and lowering in the pH can cause inflammatory tissue reactions in vivo, and would limit the clinical use of PDLLA;.the lowering of pH might be prevented combining PDLLA with basic compounds agents such as bioactive

[135]Díaz A. et al., Synthesis, Properties and Applications of Biodegradable Polymers Derived from Diols and Dicarboxylic Acids: From Polyesters to Poly(ester amide)s , Int. J. Mol. Sci. 2014, 15, 7064-7123

[136]Jagur-Grodzinski J. Biomedical application of functional polymers. *React Funct Polym* 1999;39:99–138.

gel particles (BAG) and calcium phosphates.[137] In this way we can also improve the osteocondutivity and mechanical properties of bone sostitutes[138].

A problem of poly(-hydroxyacids), is their hydrophobicity, disadvantageous in tissue regeneration applications due to the poor wetting and lack of cellular attachment and interaction[139].To overcome this issue can be used surface modification techniques, such as plasma treatment and coating with natural polymer . Natural materials (e.g., collagen, chitosan or N- succinyl-chitosan) were used to coat PLGA scaffolds to improve their interaction with osteoblasts. Collagen increased cell attachment and proliferation, but chitosan and N-succinyl-chitosan decreased them. Chitosan and N-succinyl-chitosan increased differentiation, but collagen decreased it[140].

An investigation has been conducted with scaffolds composed of poly(L-lactic acid) (PLLA) skeleton covered with bonelike apatite or apatite/collagen composite. Saos-2 osteoblast-like cells were used to evaluate the cellular behaviors on these biomimetic coatings. The results suggested that the apatite coating and apatite/collagen composite coating could improve the interactions between osteoblasts and the polymeric scaffolds. The apatite/collagen composite coating was more effective than apatite coating in improving such interactions[141]. The PLLA scaffold with apatite/collagen composite coating is promising as a candidate 3D substrate for bone tissue engineering.

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[137] Dunn AS et al., The influence of polymer blend composition on the degradation of polymer/hydroxyapatite bio- materials. *J Mater Sci Mater Med* 2001;12:673–7.

[138] Kim H-W et al., Effect of biphasic calcium phos- phates on drug release and biological and mechanical properties of poly(-caprolactone) composite membranes. J *Biomed Mater Res* A 2004;70A:467–79.

[139]Place ES et al., Synthetic polymer scaffolds for tissue engineering. *Chem Soc Rev*; 38:1139–51.

[140] Wu Y-C et al., Bone tissue engi- neering evaluation based on rat calvaria stromal cells cultured on modified PLGA scaffolds. *Biomaterials* 2006;27:896–904.

[141] Chen Y. et al., PLLA scaffolds with biomimetic apatite coating and biomimetic apatite/collagen com- posite coating to enhance osteoblast-like cells attachment and activity. *Surf Coat Technol* 2006;201:575–80.

For in vivo formation of porous biodegradable scaffolds has been proposed by Krebs et al., an injectable, biomaterial scaffold that solidifies in situ via phase inversion with microporous, interconnected architecture on the surface and within the bulk. This injectable system utilizes the biodegradable polymer poly(lactic-co-glycolic acid), a nontoxic FDA-approved solvent, and biocompatible porogens.

Scaffold porosity persisted until 8 weeks and mechanical properties were found not to vary significantly over time. Was confirmed *in vitro* capacity of preosteoblast cells to adhere to surface and to migrate throughout the scaffold. Its ability to form porous structures upon injection *in vivo* was confirmed via subcutaneous injections in mice[142].

A study has been conducted by Wei el al. with the objective to develop a three-dimensional (3D) porous tissue engineering scaffold with the capability of controlled releasing recombinant human bone morphogenetic protein-7 (rhBMP-7) for enhancement of bone regeneration. The rhBMP-7 delivering NS-scaffold has been demonstrated to induce ectopic bone formation throughout the scaffold after subcutaneous implantation in rats. By varying the composition and molecular weight of PLGA nanospheres which were immobilized onto the scaffold, rhBMP-7 release times from weeks to months were achieved from the 3D porous tissue engineering scaffold. The rhBMP-7 delivering NS-scaffold has been demonstrated to induce ectopic bone formation throughout the scaffold after subcutaneous implantation in rats[143]. The NS-scaffold system can provide varying BMP release rates to satisfy the needs of bone healing and regeneration at different sites and under different conditions , the porous scaffold provides a suitable microenvironment for cellular activity and tissue formation and the nano-fibrous structures have been demonstrated to improve

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[142] Krebs MD et al., Injectable poly(lactic-co-glycolic) acid scaffolds with in situ pore formation for tissue engineering. *Acta Biomater* 2009, doi:10.1016/j.actbio. 2009.04.035.

[143]Wei G. et al., The enhancement of osteogenesis by nano-fibrous scaffolds incorporating rhBMP-7 nanospheres. *Biomaterials* 2007;28:2087–96.

bone cell attachment and differentiation due to the structural similarity to type I collagen fibers which is a major extracellular matrix (ECM) component of natural bone.

In a study of Shen et al. was investigated the possibility of immobilization of basic fibroblast growth factor (bFGF) on polylactone-type polymer scaffolds via plasma treatment . Adhesion and growth of cells on PLGA scaffolds were greatly improved by immobilization of bFGF on them[144].

In a more recent study they studied immobilization of rhBMP-2 on polylactone-type polymer scaffolds via plasma treatment, resulted active in influencing mouse osteoblast-like cells[145].

Jacklenec et al. described sequential release of IGF-I and TGF-beta 1 from modular designed poly(l,d-lactic-co-glycolic acid) (PLGA) scaffolds, promoting chondrocytic differentiation of MSCs[146].

Niu et al. proposed porous nano-hydroxyapatite/collagen/poly(L-lactic acid)/chitosan microspheres (nHAC/PLLA/CMs) composite scaffolds containing different quantities of chitosan microspheres (CMs) prepared by a thermally induced phase separation method, biologically active in stimulating mesenchymal stem cells (MSCs) alkaline phosphatase (ALP) activity in rabbit[147].

Gupta et a. fabricated biocomposite nanofibrous scaffolds of poly(l-lactic acid)-co-poly(epsilon-caprolactone), gelatin and hydroxyapatite (HA) by combining the electrospinning and electrospraying techniques to create a better osteophilic environment for the growth and mineralization of osteoblasts.

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[144]Shen H.et al.Cell affinity for bFGF immobilized heparin-containing poly(lactide-co-glycolide) scaffolds. *Biomaterials* 32(13):3404-12 · February 2011

[145]Shen H. et al., The bioactivity of rhBMP-2 immobilized poly(lactide-co-glycolide) scaffolds, Biomaterials. 2009 Jun;30(18):3150-7. doi: 10.1016/*j.biomaterials*. 2009.02.004. Epub 2009 Feb 18.

[146]Jaklenec A. et al., Sequential release of bioactive IGF-I and TGF-beta 1 from PLGA microsphere-based scaffolds, *Biomaterials*. 2008 Apr;29(10):1518-25.

[147]Niu X. et al.,Porous nano-HA/collagen/PLLA scaffold containing chitosan microspheres for controlled delivery of synthetic peptide derived from BMP-2., *Journal of controlled release* 134(2009) 111-117.

These scaffolds showed better cell proliferation but also enhanced mineralization and alkaline phosphatase activity[148].

Poly-ε-caprolactone (PCL) is an aliphatic linear polyester susceptible to auto-catalyzed bulk hydrolysis. Compared with PGA or PLA, the degradation of PCL is significantly slower (years), due to its semi-crystalline nature and hydrophobicity. PCL is therefore most suitable for the design of long-term, implantable systems[149]. To increase degradation rate and improve processability it has been investigated copolymerization of PCL and poly (alfa-hydroxyacids), but obtaining poor mechanical properties[150].

Significant improvements in mechanical properties of polymers can be achieved via fibrous reinforcement. Phosphate-based glasses and fibers show great potential as reinforcing materials[151].

Composites materials of PCL/HAP, PCL/TCP, PCL/CaCO3, PCL/ wollastonite have been also proposed[152] [153] [154].

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[148] Gupta D. et al., Nanostructured biocomposite substrates by electrospinning and electrospraying for the mineralization of osteoblasts. *Biomaterials* 2009;30:2085–94.

[149] Pitt CG. Poly (-caprolactone) and its copolymers. In: Chassin M, Langer R, editors. Biodegradable polymers as drug delivery systems. New York: Dekker; 1990. p. 71–119.

[150] Ye WP et al., In vitro degradation of poly(caprolactone), poly(lactide) and their block copolymers: influence of composition, temperature and morphology. *React Funct Polym* 1997;32:161–8.

[151] Taddei P. et al., In vitro mineralization of bioresorbable poly([epsilon]-caprolactone)/ apatite composites for bone tissue engineering: a vibrational and thermal investigation. *J Mol Struct* 2005;744–747:135–43.

[152] Erisken C. et al., Functionally graded electrospun polycaprolactone and b-tricalcium phosphate nanocomposites for tissue engineering applications. *Biomaterials* 2008;29:4065–73.

[153] Fujihara K. et al., Guided bone regeneration membrane made of polycaprolactone/ calcium carbonate compos- ite nano-fibers. *Biomaterials* 2005;26:4139–47.

[154] Wei J, et al.,Preparation and characterization of bioactive mesoporous wollastonite-polycaprolactone composite scaffold. *Biomaterials* 2009;30:1080–8.

PCL based fibrous scaffolds were successfully produced without the occurrence of bead defects using electrospinning process obtaining good physical and biological characteristics[155]

In a study of Kim JH et al. PCL/polyethylenimine blend electrospun nanofibers were prepared to overcome the limitation of PCL ones because the PEI as a cationic polymer can increase cell adhesion and can improve the electrospinnability of PCL. The cell adhesion and cell proliferation of PCL nanofibers were increased due to the hydrophilic properties of PEI[156].

Savarino et al. have investigated the osteopromotive properties of cellular constructs composed of poly-epsilon-caprolactone and rabbit bone marrow stromal cells (BMSCs), or BMSCs engineered to express bone morphogenetic protein 4 (BMP4). Highly porous biodegradable PCL scaffolds were obtained via phase inversion/salt leaching technique. BMSCs and transfected BMSCs were seeded within the scaffolds by using an alternate flow perfusion system and implanted into non-critical size defects in New Zealand rabbit femurs. PCL without cells showed scarce bone formation at the scaffold-bone interface and scarce PCL resorption. Conversely, PCL seeded with autologous BMSCs stimulated new tissue formation into the macropores of the implant and neo-tissue vascularization. Finally the BMP4-expressing BMSCs strongly favoured osteoinductivity of cellular constructs, as demonstrated by a more extensive bone/scaffold contact.[157]

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[155] Li W-J.et al., Fabrication and characterization of six electrospun poly([alpha]-hydroxy ester)-based fibrous scaffolds for tissue engineering applications. *Acta Biomater* 2006;2:377–85.

[156] Kim JH et al., Electrospun nanofibers composed of poly([epsilon]-caprolactone) and polyethylenimine for tissue engineering applications. *Mater Sci Eng* C 2009;29:1725–31.

[157] Savarino L. et al., The performance of poly--caprolactone scaffolds in a rabbit femur model with and without autologous stromal cells and BMP4. *Biomaterials* 2007;28:3101–9.

Bioresorbable poly(uretane)s **(PURS)** are synthetic polymers that include urethane groups in their chains. They are produced through a complex synthesis between isocyanates (with at least 2 -N=C=O groups in the molecule) and polyols (with 2 or more hydroxyl groups, -OH, in the molecule) and are present in a variety of chemistry, molecular weights and ratio of the segments constituting their molecular structure. Therefore they have a broad range of mechanical, biological and physical properties, and possess greater elasticity in comparison to conventional biodegradable polymers. PURs can be prepared by reactive liquid molding, rendering them potentially useful as injectable biomaterials for non- invasive therapies To control their hydrolytic degradation and to improve their biocompatibility, have been developed PURs with different molecular structures[158]. To avoid carcinogenic or toxic degradation products linear diisocyanates in place of aromatic components are usually employed[159].

In a study of Gogolewski et al.[160] porous scaffolds were produced from newly designed biodegradable, segmented aliphatic polyurethanes of various chemical compositions and hydrophilic-to-hydrophobic segment ratios. The scaffolds were implanted into monocortical defects in the iliac crest of healthy sheep for 6 months. The defects implanted with porous scaffolds from polyurethanes were healed to varying extents with cancellous bone. New bone that was formed in the scaffolds with a higher amount of hydrophilic component contained more calcium phosphate deposit than the bone formed in the scaffolds with a lower amount of the hydrophilic component.

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[158] Guelcher SA. Biodegradable polyurethanes: synthesis and applications in regenerative medicine. *Tissue Eng* B 2008;14:3–17.

[159] Santerre JP et al., Understanding the biodegradation of polyurethanes: from classical implants to tissue engineering materials. *Biomaterials* 2005;26:7457–70.

[160] Gogolewski S. et al., Biodegradable polyurethane cancellous bone graft substitutes in the treatment of iliac crest defects.,*J Biomed Mater Res* A. 2007 Jan;80(1):94-101.

Kavlock et al.[161] synthesized a family of segmented degradable poly(esterurethane urea)s from 1,4-diisocyanatobutane, a poly(e-caprolactone) (PCL) macrodiol soft segment and a tyramine-1,4-diisocyanatobutane-tyramine chain extender. By systematically increasing the PCL macrodiol molecular weight from 1100 to 2700 Da, the storage modulus, crystallinity and melting point of the PCL segment were systematically varied together with mechanical properties.

Hofmann et al.[162] described a long-term culture environment that supports the survival, proliferation, and in vitro vasculogenesis of human umbilical vein endothelial cells (HUVEC) in a co-culture model of HUVEC and primary human osteoblasts (hOB) employing polyurethane scaffolds and platelet-rich plasma in a static microenvironment.

In a study of Boissard et al.[163] is reported the preparation of hydroxyapatite nanoparticles (nHA)/poly(ester urethane) composite scaffolds using a salt-leaching-phase inverse process with the aim of improving scaffold osteoconducivity.

Grad et al.[164] demonstrated that biodegradable polyurethane porous scaffolds seeded with articular chondrocytes support cell attachment and the production of extracellular matrix proteins, even if dedifferentiation of the chondrocytes was observed with prolonged time in culture.

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[161] Kavlock K.D. et al, Synthesis and characterization of segmented poly(esterurethane urea) elastomers for bone tissue engineering, Acta Biomater. 2007 Jul; 3(4): 475–484.

[162] Hofmann A, et al., The effect of human osteoblasts on proliferation and neo-vessel formation of human umbilical vein endothelial cells in a long-term 3D co-culture on polyurethane scaffolds. *Biomaterials* 2008;29:4217–26.

[163] Boissard CIR et al., Nanohydrox- yapatite/poly(ester urethane) scaffold for bone tissue engineering. *Acta Biomater* 2009, doi:10.1016/j.actbio.2009.05.001.

[164] Grad S. et al., The use of biodegradable polyurethane scaffolds for cartilage tissue engineer- ing: potential and limitations. *Biomaterials* 2003;24:5163–71.

Eyrich et al.[165] investigated a combination of long-term stable fibrin gels and polyurethane scaffolds for cartilage engineering, obtaining stability in cell culture for several months.

Henri et al.[166] demonstrated that porous scaffolds with regular topography are better tolerated in vivo compared to non-porous scaffolds, while increasing scaffold porosity promotes angiogenesis and cellular infiltration.

Poly(propylene fumarate) **(PPF)** is an unsaturated linear polyester with fumarate double bonds that can be crosslinked in situ. It is biocompatible, biodegradable, osteoconductive, and capable of both preformed and injectable applications . PPF scaffolds can be used to fill irregularly shaped defects with minimal surgical intervention[167].

Composite materials realized combining PPF and bioactive ceramics, such as Hap, TCP or $CaSO_4$, have shown to improve material osteoconductivity and mechanical properties[168]. Ceramic fillers also act as internal pH buffer inhibiting PPF autocatalytic degradation[169] .

Polyphosphazenes are inorganic/organic hybrid polymers. The polymer backbone consists of alternating phosphorus and nitrogen atoms and organic substituents are linked to the phosphorus atoms as side groups . The major

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[165] Eyrich D, et al. In vitro and in vivo cartilage engineering using a combination of chondrocyte-seeded long-term stable fibrin gels and polycaprolactone-based polyurethane scaffolds. *Tissue Eng* 2007;13:2207–18.

[166] Henry JA et al,, Structural variants of biodegradable polyesterurethane in vivo evoke a cellu- lar and angiogenic response that is dictated by architecture. *Acta Biomater* 2009;5:29–42.

[167] Timmer MD et al., In vitro cyto- toxicity of injectable and biodegradable poly(propylene fumarate)- based networks: unreacted macromers, cross-linked networks, and degradation products. *Biomacromolecules* 2003;4:1026–33.

[168] Kharas GB, et al. Synthesis and characterization of fumarate- based polyesters for use in bioresorbable bone cement composites. *J Appl Polym Sci* 1997;66:1123–37.

[169] Peter SJ et al., In vitro degradation of a poly(propylene fumarate)/b- tricalcium phosphate composite orthopaedic scaffold. *Tissue Eng* 1997;3:207–15.

precursor, polydichlorophosphazene, is extremely hydrolytically unstable but can be readily substituted with nucleophilic substituents to give a wide range of stable poly[(organo)phosphazenes] with an extremely wide range of properties . The properties of the resulting material are highly dependent on the side-substituents and their ratios[170].

Polyphosphazenes containing amino acid ester substituents as well as a pharmacon, are of considerable interest as bioerodible systems for controlled release of drugs. Due to their inorganic backbone and the proper choice of substituents the polymeric matrix degrades into products which are harmless at physiological concentrations and may favor cell adhesion and proliferation[171].

Larencin et al.[172] synthesized a degradable amino acid containing polymer, poly[(methylphenoxy)(ethyl glycinato) phosphazene], and a 3-D matrix system was prepared using a salt leaching technique. This 3-D polyphosphazene polymer matrix system was seeded with osteoblast cells for the creation of a cell-polymer matrix material. Osteoblast cells were found attached and grew on the 3-D-PHOS at a steady rate throughout the 21-day period studied in vitro,

Nair et al.[173] studied two novel biodegradable polyphosphazenes : poly[(ethyl alanato)1.0(ethyl oxybenzoate)1.0 phosphazene] (PN-EA/EOB) and poly[(ethyl alanato)1.0(propyl oxybenzoate)1.0 phosphazene] (PN-EA/POB). Both supported the adhesion and proliferation of primary rat osteoblast cells in vitro. Furthermore, the enzymatic activity of the osteoblast cells cultured on the polymers was confirmed by the alkaline phosphatase activity. Thus, these

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[170] Krogman NR et al., Miscibility of bioerodible polyphosphazene/poly(lactide-co-glycolide) blends. *Biomacromolecules* 2007;8:1306–12.

[171] Grolleman CWJ et al., Studies on a bioerodible drug carrier system based on a polyphosphazene: Part II. Experiments in vitro. *J Controlled Release* 1986;4:119–31.

[172] Laurencin CT et al., A highly porous 3-dimensional polyphosphazene polymer matrix for skeletal tissue regeneration. *J Biomed Mater Res* 1996;30:133–8.

[173] Nair LS et al., Synthesis, characterization, and osteocompatibility evaluation of novel alanine-based polyphosphazenes. *J Biomed Mater Re*s A 2006;76A:206–13.

polymers demonstrated excellent tissue compatibility and in vivo biodegradability.

Miscible polyphosphazenes/PLGA blends to combine the properties of the two classes of biocompatible polymers have been developed. Blends of poly[bis(ethyl glycinato) phosphazene] and PLGA were synthesized and it was demonstrated that diverse properties could be obtained via compositional changes of the pure polymers[174]. it was also shown that the acidic degradation products of PLGA could be neutralized by the degradation products of polyphosphazenes[175]. Due to the low glass transition temperature of poly[bis(ethyl glycinato) phosphazene] the mechanical properties of the polymer blends were compromised. Blends of PLGA and poly[(ethyl alanato)$_1$ (p-phenyl phenoxy)$_1$ phosp- hazene], that has a higher glass transition temperature, showed cell adhesion and proliferation comparable to PLGA. The presence of the polyphosphazene in the blends was able to increase the phenotypic expression and mineralized matrix synthesis of the primary rat osteoblasts in vitro[176].

Nair et al. evaluated the feasibility of developing nanofiber mesh from poly[bis(p-methylphenoxy)phosphazene] (PNmPh) by electrospinning. The electrospun nanofiber mats supported the adhesion of bovine coronary artery endothelial cells (BCAEC) as well as promoted the adhesion and proliferation of osteoblast like cells[177].

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[174] Ibim SEM et al., Novel polyphosphazene/poly(lactide-co-glycolide) blends: miscibility and degradation studies. *Biomaterials* 1997;18:1565–9.

[175] Ambrosio AMA et al., Degradable polyphosphazene/poly([alpha]-hydroxyester) blends: degradation studies. *Biomaterials* 2002;23:1667–72.

[176] Deng M. et al.,Miscibility and in vitro osteocompatibility of biodegradable blends of poly[(ethyl alanato) (p-phenyl phenoxy) phosphazene] and poly(lactic acid-glycolic acid). *Biomaterials* 2008;29:337–49.

[177] Nair LS et al., Fabrication and optimization of methylphenoxy substituted polyphosphazene nanofibers for biomedical applications. *Biomacromolecules* 2004;5:2212–20.

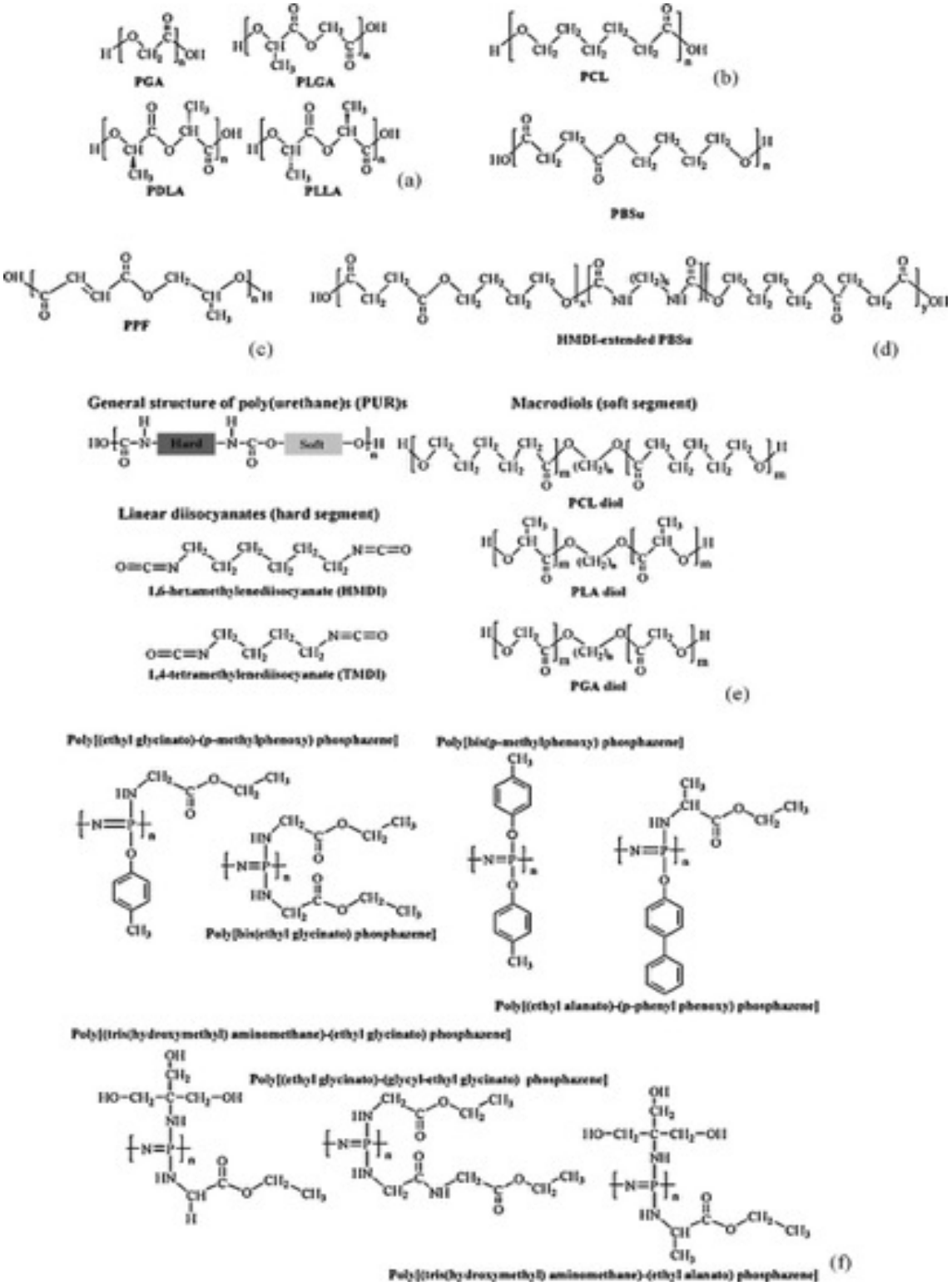
Poly(1,4-butylene succinate) **(PBSU)** is a biodegradable aliphatic polyester that can be easily synthesized by condensation polymerization of of succinic acid and butan-1,4-diol. It has good degradability and excellent processability. A study of Li et al.[178] showed that the PBSU was biocompatible as the osteoblasts could proliferate and differentiate on the PBSU plates and the hydrolytic degradation behavior of the PBSU films in the phosphate-buffered saline (PBS) was similar to that of the degradable poly(alpha-hydroxyesters). A composite scaffolds of PBSu/wollastonite/apatite for bone fabricated via electrospinning and biomimetic process has been proposed by Zhang et al[179].

[178] Li H, et al., In vitro Evaluation of biodegradable poly(butylene succinate) as a novel biomaterial. *Macromol Biosci* 2005;5:433–40.

[179] Zhang D et al., Fabrication of fibrous poly(butylene succi- nate)/wollastonite/apatite composite scaffolds by electrospinning and biomimetic process. *J Mater Sci Mater Med* 2008;19:443–9.

fig.4: Chemical structure of synthetic polymers:

from: D. Puppi et al. *Progress in Polymer Science* 35 (2010) 403–440



3.2. Degradation of Biodegradable Polymers

Degradation behavior of biomaterials follows several mechanisms and is controlled by different factors. Therefore is important to know their degradation kinetics in order to optimize their employment. The need for biomaterials with controlled and predictable degradations kinetics has led to research on the behavior of the biodegradable polymers.

The term biodegradation may be defined as the “gradual breakdown of a material mediated by a specific biological activity”[180]

Biodegradable polymers have a temporary function, therefore degradation should occur as a controlled mechanism. Specially in drug delivery applications, the polymer is required to degrade following a well defined kinetic in order to release the encapsulated drugs at specific times.
The degradation of a polymer can occur at different stages including also its preparation, processing and storage.

During polymer processing and fabrication some degradation can occur If processing techniques involve high temperature or high shear stresses inside the material, causing degradation of the starting polymer; on the contrary, some chain orientation caused by some process could alter the degradation time making it generally more resistant. Sterilization method may also have an effect on the material degradation causing crosslinking or polymer chain breakage.

After implantation in tissues the material is exposed to the body fluids the most important degradation mechanisms are oxidation and hydrolysis.

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[180]Williams, D.F. and Zhong, S.P., Biodeterioration/biodegradation of polymeric medical devices *in situ*, *Int. Biodeter. Biodegrad.*, 95, 1994.

Oxidation may be chemical and enzymatic. During inflammatory response to the implanted foreign materials, inflammatory cells produce highly reactive oxygen species, such as suoperoxide , hydrogen peroxidase, nitric oxide and hypochlorous acid , which can cause polymer chain scission[181] [182].

Hydrolitic degradation is the scission of chemical bonds in the polymer backbone to form oligomers and monomers by water attach directed to water-labile bonds by either direct access to the polymer surface or by imbibitions into the polymer matrix followed by bond hydrolysis[183].

It is influenced by the chemical structure of the polymer: covalent bonds in the backbone and no hydrolysable groups require longer times to degrade.

Hydrolysis reactions may be catalyzed by enzymes known as hydrolases, such as proteases, esterases, glycosidases and phosphatases.[184]

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[181] Labow, R.S. et al., The effect of oxidation on the enzyme-catalyzed hydrolytic biodegradation of poly(urethan)s, *J. Biomater. Sci. Polym. Ed.*, 13, 651, 2002.

[182] Lee, K.-H. and Chu, C.C., The role of superoxide ions in the degradation of synthetic absorbable
sutures, *J. Biomed. Mater. Res.*, 49, 25, 2000.

[183] Santerre, J.P. et al., Biodegradation evaluation and polyester-urethanes with oxidative and hydrolytic enzymes, *J. Biomed. Mater. Res.*, 28, 1187, 1994.

[184] Shalaby, W.S.W. and Park, K., Chemical modification of proteins and polysaccharides and its effect on enzyme-catalyzed degradation, in *Biomedical Polymers. Designed-to-Degrade Systems,* Shalaby, S.W., Ed., Hanser Publishers, Munich, 1994, chap. 9.

3.3. Fabrication techniques

Body, cells and tissue are organized into a three-dimensional architecture, therefore scaffolds have to be fabricated by different methodology to facilitate the cell distribution and guide their growth into three-dimensional space.

In this chapter are reported the different scaffold fabrication techniques:

Solvent casting combined with particulate leaching

This scaffold fabrication technique is simple, easy and inexpensive and does not require any technological complexity..

This technique involves polymer dissolution into a suitable solvent and then solution casting in a 3D mold filled with porogen particles (salt, wax or sugars). After the evaporation of the solvent, the particles are leached away using water or an aliphatic solvent to form the pores of the scaffold[185].

A drawback of this technique could be the presence of residual organic solvent in the scaffold that can alter its biocompatibility. In addition, it can be used only to produce thin membranes or 3D specimens with thickness up to 2mm. It is also possible to have porogen particles agglomeration leading to large pore size or porous architecture failure[186].

Gas Foaming

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[185] Mikos A.G. et al., Preparation and characterization of poly(L-lactic acid) foams, *Polymer* 35 (5) (1994) 1068e1077.

[186] Mikos,A. G. et al.. (2004). Synthetic Bioresorbable polymer scaffolds. In: An introduction to material in medicine, Ratner B D, Hoffman A S, Schoen F J, Lemons J E, (Ed.). pp 743, *Elsevier Academic Press*. USA.

In this technique, instead of organic solvents, high pressure carbon dioxide gas is used. The porosity and the structure of the scaffolds depend upon the amount of gas dissolved in the polymer. A polymeric matrix is exposed to carbon dioxide at high pressure (800 psi) to saturate the polymer with the gas. By reducing the gas pressure dissolved CO2 becomes unstable and separates from the polymer. As a result pore nucleation is obtained[187].

Freeze drying

The freeze-drying technique can be employed to produce highly porous scaffolds and drug delivery matrices. It is possible to use this technique to achieve a porosity level above 90% and at the same time to control the pore size for targeted applications. One of the main disadvantages is the possibility of obtaining a close-pore morphology[188]. The process involves using an appropriate solvent, usually water, to make a polymer solution that is then cooled down resulting in the formation of ice crystals. The system is kept at a low pressure to achieve the complete sublimation of the crystals that will lead to the formation of the pores[189].

Supercritical -fluid technology

A supercritical fluid is any substance at a temperature and pressure greater than its thermodynamic critical point. Above this critical point all

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[187] Sachlos, E. & Czernuszka, J. T.(2003). Making tissue engineering scaffolds work. Review on the application of solid free from fabrication technology to the production of tissue engineering scaffolds. *European cells and materials.* 5, 29-40.

[188]Y.S. Nam YS et al., Porous biodegradable polymeric scaffolds prepared by thermally induced phase separation, *J. Biomed. Mater. Res*. 47 (1) (1999) 8e17.

[189] Whang K. et al., A novel method to fabricate bioabsorbable scaffolds, *Polymer* 36 (4) (1995) 837e842.

materials in the liquid and gas phase have the same densities, resulting in the formation of a single phase.

CO2, a non-toxic gas with a relatively low critical point (Tc = 31°C, Pc = 73.8 bar is most widely used in the supercritical fluid field[190].In this form, it can diffuse through solid materials like a gas and dissolve them like a liquid. The advantage of technique in scaffold production is that does not require organic solvents that could damage the system. The drawback is that it yields a non porous surface and a largely unconnected porous structure.

Thermally-induced phase-separation technique

This class of techniques is based on changes in the thermodynamic state of a polymeric solution to induce its separation into a multiphase system[191]. When the phase separation occurs, the homogenous solution separates in a polymer-rich phase and polymer-poor phase. The polymer-rich phase solidifies and the polymer-poor phase is removed with the formation of an interconnected porous space. The drawback is a possible incomplete solvent removal with a consequent reduced biocompatibility and alteration of the incorporated active factors.. A limited range of pore size is generally achieved and is difficult to control the structure of the scaffold.

Wet spinning

The polymer is dissolved in a suitable solvent, and the resulting solution is extruded through a spinneret into a coagulation bath where a nonsolvent-induced phase separation transforms liquid polymer streams into solid filaments. At this point microfibers are usually drawn through a series of rollers to obtain the desired mechanical strength, elasticity and elongation. Fiber

[190] Kim SH et al,A biocompatible tissue scaffold produced by supercritical fluid processing for cartilage tissue engineering.*Tissue Eng* Part C Methods. 2013 Mar; 19(3):181-8.

[191] H. Lo H. et al,, Fabrication of controlled release biodegradable foams by phase separation, *Tissue Eng*. 1 (1) (1995) 15e28.

structure and shape are determined during the gelation of the polymer spin dope by complex interactions of many factors that affect the phase separation of the polymer solution.

This technique has been used to process naturally-derived polymers, such as chitin and chitosan[192], which cannot be formed by other spinning techniques.

As proposed by Zhang[193] PLLA/chitosan braid prepared from wet-spun PLLA fibers and wet-spun chitosan fibers can be a suitable candidate as an ideal osteoblast carrier for the repairing of damaged chest wall and bone large segments.

Fiber bonding

Fiber bonding technique for scaffold fabrication has been developed in 1993 (Freed et al. 1993; Mikos et al. 1993).

The fibers, bonded together to form a three-dimensional network, provide large surface area for cell interaction and growth.

Two different fiber bonding approaches have been proposed.

In the first method, developed by Mikos et al.[194], PGA fibers are immersed in a PLLA solution. When the solvent evaporates, PGA fibers are embedded in PLLA. The composite is then heated to weld together the fibers at the cross-points forming a highly porous foam. The PLLA is then removed by dissolution with methylene chloride. This fabrication technique results in foams with porosities as high as 81% and pore diameters of up to 500 mm.

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[192] Tuzlakoglu K., Reis RL., in Chitosan-based scaffolds in orthopedic applications ed.by Reis RL. (Woodhead; Cambridge, 2008), p. 357-373.

[193] Zhang X., In vitro degradation and biocompatibility of poly(L-lactic acid)/chitosan fiber composites, *Polymer* 48 (2007) 1005e1011

[194] Mikos, A.G. et al., (1993b). Preparation of Poly (glycolic acid) bonded fiber structures for cell attachment and transplantation. *Journal of Biomedical Materials Research*, 27, 183-189.

A second method for bonding PGA fibers uses atomization of PLLA or PLGA. PLLA or PLGA dissolved in chloroform is sprayed onto the PGA fibers[195]. Since PGA is only weakly soluble in chloroform, the fibers remain unchanged during this process. When the solvent is evaporated, the fibers are glued together with PLLA or PGA .Tubes made in this manner implanted in rats for 17 days, showed fibrous tissue ingrowth, indicating that constructs with these structural properties could support neotissue formation .

Although these techniques produce highly porous scaffolds with interconnected pores suitable for tissue regeneration they involve the use of solvents potentially toxic to cells if not completely removed. In order to extract these chemicals, the constructs must be vacuum dried for several hours, making it difficult to be used immediately in a clinical setting. Furthermore, the first method involves heating to high temperatures. The combination of toxic chemicals and high temperature presents difficulties if cells or bioactive molecules are to be included in the scaffold during processing.

Electrospinning

Electrospinning is a technique that uses a high voltage to create an electric field between a droplet of a polymer solution at the tip of a needle and a collector plate. One electrode of the voltage source is placed into the solution and the other is connected to the collector. This creates an electrostatic force opposing the surface tension of the drop.

The drop due to the increasing electrostatic force elongates forming a conical shape (Taylor cone). When the electrostatic force overcomes the surface tension , a charged, continuous jet of solution is ejected from the cone. The jet of solution accelerates towards the collector, whipping and bending. As the solution moves toward the collector, the jet rapidly thins and dries as the

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[195] Mooney, D.J.et al., (1996). Stabilized polyglycolic acid fibre-based tubes for tissue engineering. *Biomaterials*, 17, 115–124.

solvent evaporates. On the surface of the collector, a non-woven mat of solid nanofibers with mean diameter down to 50 nm is collected.

The findings of a study by Li et al.[196] strongly suggest that the PCL nanofibrous structure is suitable as a candidate tissue engineering scaffold for cartilage regeneration.

Smith et al.[197] in their study demonstrated that an electrospun nanofibrous architecture enhanced the osteogenic differentiation and mineralization of embryonic stem cells compared to a dense architecture in both two and three dimensional cultures.

Melt molding combined with particle leaching

Melt molding process involves the filling of a mould with PLGA powder and gelatin microspheres and heating above the glass transition temperature of PLGA while applying pressure to the mixture[198] [199]. In this way the PLGA particle attach together. Once the mould is removed gelatin microspheres are dissolved by immersing the mixture into water leading to the formation of pores and the scaffold is then dried. Scaffolds produced by this technique assume the shape of the mould.

Solid Freeform Fabrication

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[196] Lu, P. & Ding, B. (2008) Applications of electrospun fibers. *Recent Pat Nanotechnol*. 2(3),169-82.

[197] Smith, L.A.et al. (2006). Nano fibrous scaffolds and their biological effects. In: *Tissue Cell and Organ Engineering*, Kumar, C. (Ed.), pp 195, Wiley-VCH.

[198] Thompson R.C.et al.(1995a). Biodegradable polymer scaffolds to regenerate organs. *Adv Polymer Sci* 122: 245-274.aton, FL. pp 173-195.

[199] Thompson, R.C.et al.,(1995b). Poly (a-hydroxy ester)/short fiber hydroxyapatite composite foams for orthopaedic applications. In: *Polymers in Medicine and Pharmacy*, Vol 394. Mikos AG, Leong KW, Yaszemski MJ, (Ed.). pp. 25-30, Materials Research Society Symposium Proceedings, Pittsburgh USA.

Solid Freeform Fabrication (SFF), commonly known also as Additive Manufacturing (AM), consists in the production of specific parts and components without the need of custom molds and dies. They are manufactured in a freeform process with the use of computer files and carefully controlled equipment to build 3D scaffolds with a layer by layer process. Several methods are available, all of which rely on Computer Assisted Design (CAD) and Computer Aided Manufacturing (CAM)[200] [201].

The application of CAD strategies in conjunction with SFF fabrication allows scaffolds with highly uniform pore morphologies, unlimited range of pore sizes, porosities and full pores interconnectivity to be realized with great accuracy for patient specific applications .

[200] Leong KE, et al.,Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs. *Biomaterials*. 2003 Jun;24(13):2363-78.

[201] Suri S. et al.,Solid freeform fabrication of designer scaffolds of hyaluronic acid for nerve tissue engineering., *Biomed Microdevices*. 2011 Dec;13(6):983-93.

4. BONE TISSUE ENGINEERING

Fracture healing results in restoration of the original structure and function of the bone tissue. In this process, proper reduction and immobilisation are essential to achieve optimal bone healing. This can be achieved through the use of specific reduction techniques, surgical instruments, and orthopaedic implants .

Contact between the fragments is required for secondary osteons to advance from one fragment to another. Smaller defects heal spontaneously without the need for additional 'bone healing enhancers'.

Larger bone-defects, represent a great challenge in both human and veterinary orthopaedics as these defects do not show spontaneous closure and require additional means to enhance bony union . Current clinical treatments for large bone defects include autologous and allogeneic transplantations.

Autografts represent the gold standard[202]. They are histocompatible and non-immunogenic, and offer all of the requirements of a bone graft material. Specifically, autografts possess the essential components for osteoinduction (i.e., bone morphogenetic proteins (BMPs) and other growth factors), osteogenesis (i.e., osteoprogenitor cells) and osteoconduction (i.e., three-dimensional and porous matrix)[203].

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[202] Brinker WO et al.,(1997) Bone grafting. Small animal orthopedics and fracture repair. WB Saunders Company, Gainesville, pp 147–153

[203] Albrek T, Johansson C (2001) Osteoinduction, osteoconduction and osteointegration. Eur Spine J 10:S96–S101

Autografts involve harvesting bone from the patient and thus require a second operation at the site of tissue harvest and they may result in donor site injury and morbidity, deformity, scarring and they are associated with surgical risks, bleeding, inflammation, infection, and chronic pain. Furthermore autografts are not indicated when large volumes of tissue are required.

Allografts and xenograft involve transplanting donor bone tissue, generally from a cadaver[204]. Allogeneic bone is also likely histocompatible, and is available in various forms, including demineralized bone matrix (DBM)[205], morcellised and cancellous chips, corticocancellous and cortical grafts, and osteochondral and whole-bone segments, depending on the host-site requirements. In comparison to autografts, allografts and xenograft are associated with risks of immunoreactions and transmission of infections. They have reduced osteoinductive properties and no cellular component, because donor grafts are devitalized via irradiation or freeze-drying processing.

The mentioned clinical interventions can improve repair of bone, but none possess all of the ideal characteristics: high osteoinductive and angiogenic potentials, biological safety, low patient morbidity, no size restrictions, ready access to surgeons, long shelf life, and reasonable cost.

The field of bone tissue engineering (BTE) has attracted great interest, initiated nearly three decades ago. with an exponentially increasing number of studies and reviews published on the PubMed database. BTE with the collaborative efforts of scientists, engineers and surgeons, focuses on alternative treatment options to eliminate the issues of current clinically used treatments (i.e., donor site morbidity, limited availability, immune rejection, and pathogen transfer).

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[204] Khan SN et al.,(2005) The biology of bone grafting. *J Am Acad Orthop Surg* 13:77–86

[205] Jin DD (1991) Bone matrix gelatin. Clinical application in 38 cases. Chung-Hua Wai Ko Tsa Chih 29:312–314

The goal in bone tissue engineering is to obtain[206]:

- a biocompatible scaffold that closely mimics the natural bone extracellular matrix,
- osteogenic cells to lay down the bone tissue matrix,
- morphogenic signals that help to direct the cells to the phenotypically desirable type,
- sufficient vascularization to meet the growing tissue nutrient supply and clearance needs.

4.1. Bone Development

There are two pathways for bone formation: intramembranous and endochondral[207].

In intramembraneous bone formation mesenchymal progenitor cells differentiate into osteoblasts with the subsequent development of parts of the mandible, clavicle, and many cranial bones. Most bones in the body (i.e. all long bones and vertebrae) are formed through endochondral formation. In this process mesenchymal progenitor cells first differentiate into chondrocytes, responsible for depositing a cartilaginous template, later mineralized and replaced by bone. Several molecular regulators intervene in osteogenesis including Indian Hedgehog (Ihh), parathyroid hormone related peptide (PTHrP),

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[206] Velasco, M.A., et al.,“Design, Materials, and Mechanobiology of Biodegradable Scaffolds for Bone Tissue Engineering,” *BioMed Research International*, vol. 2015, Article ID 729076, 21 pages, 2015.

[207] Gilbert SF. Developmental Biology. 6th edition. Sunderland (MA): Sinauer Associates; 2000. Osteogenesis: The Development of Bones.

bone morphogenetic proteins (BMPs), vascular endothelial growth factors (VEGFs) and fibroblastic growth factors (FGFs).

Furthermore, in both processes, bone remodeling is required for normal bone formation, involving a balance between osteoclastic bone resorption and osteoblastic bone formation.

4.2. Bone Defect Repair

Bone repair is a process that recapitulates many of the events of both intramembraneous and endochondral bone formation, without the formation of scar tissue[208] [209].

First there is hematoma formation accompanied by an inflammatory response, and the recruitment of many of the signaling molecules involved in the new bone formation (i.e., ILs, TNF- α, FGFs, BMPs, PDGF, VEGF, etc.). Intramembranous bone formation begins at the cortex and periosteum. The external soft tissues stabilize the fracture by the formation of a callus, which subsequently undergoes chondrogenesis, and after a process similar to endochondral ossiflcation. The mechanical continuity of the cortex is achieved via subsequent remodeling of the newly formed bone[210].

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[208] Fazzalari NL. Bone fracture and bone fracture repair. *Osteoporos Int*. 2011;22(6): 2003–2006.

[209] Shapiro F. Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts. *Eur Cell Mater.* 2008;15:53–76.

[210]Amini AR. et al., Bone Tissue Engineering: Recent Advances and Challenges, Crit *Rev Biomed Eng*. 2012; 40(5): 363–408.

4.3. Bone Tissue Engineering

BTE should focus on both bone development and bone defect repair processes.

A key component in tissue engineering for bone regeneration is the scaffold. It serves as a template for cell interactions and the formation of bone-extracellular matrix and provides structural support to the newly formed tissue.

Scaffolds requirements include mechanical properties similar to those of the bone to repair, biocompatibility and biodegradability at a rate tailored to bone growth rate.

A scaffold serves as

- osteoconductive structure, since new bone is deposited from adjacent living bone .

- delivery vehicle of bioactive agents such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs) and transforming growth factors (TGFs) that transform recruited precursor cells from the host into bone matrix producing cells,

- providing osteoinduction. Osteoinductive biomaterials can induce ectopic bone formation by instructing its surrounding in vivo environment to form bone. The biological mechanisms of this phenomenon have not been fully elucidated, but it is recognized that these materials hold great potential for bone tissue regeneration. Different biomaterials have demonstrated osteoinductive properties, including natural and synthetic ceramics (i.e., hydroxyapatite (HA)[211].

[211] LeGeros R.Z., “Properties of osteoconductive biomaterials: calcium phosphates,” *Clinical Orthopaedics and Related Research*, no. 395, pp. 81–98, 2002.

- providing osteogenesis by seeding the scaffolds before implantation with cells that will create new centers for bone formation, such as osteoblasts and mesenchymal cells that have the potential to commit to an osteoblastic lineage. Genetically transduced cells expressing osteoinductive factors can also be used.

Combining scaffolds, cytokines and cells to generate ex vivo tissue-engineered constructs is hypothesized to provide more effective bone regeneration in vivo in comparison to biomaterial matrices alone.

Scaffolds for osteogenesis should mimic bone morphology, structure and function in order to optimize integration into surrounding tissue[212]

4.4. Materials

Ceramics, polymers and composites can be used as biomaterials. Ceramic materials are based on calcium phosphates and bioglasses. They have good osteoinductive properties but low mechanical properties and difficulties in forming process.

Polymers such as those derived from polyglycolic acid (PGA) and polylactic acid (PLA) have easy formability, good mechanical properties and biodegradability which may vary according to their molecular weight but low osteoinductive capacity[213].

Ceramic-polymer composite are biodegradable materials, with good mechanical strength, osteoinductive, osteoconductive, and formability properties combining the properties of each material family[214].

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[212] Vassilis Karageorgiou, Biomaterials, 26 (2005) 5474–5491.

[213] Devin JE et al., Three-dimensional degradable porous polymer-ceramic matrices for use in bone repair. *J Biomater Sci Polym* Ed. 1996;7(8):661–669.

[214] Gosain AK et al., A 1-year study of os-teoinduction in hydroxyapatite-derived biomaterials in an adult sheep model: part I. *Plast Reconstr Surg*. 2002;109(2):619–630.

4.4.1. Ceramics

Ceramic materials are a group of inorganic oxides and salts used for their similarity to the mineral component of bone in the case of calcium phosphate or because of their capacity of strength bonding to osseous tissues in the case of bioglasses .

Calcium Phosphates: The most common calcium phosphate for bone tissue regeneration is hydroxyapatite (HA) which is a crystalline calcium phosphate (Ca10(PO4)6(OH)2) present in bones[215]

Bioglasses: are a family of bioactive glasses, compound of SiO2, Na2O, CaO, and P2O5 in variable proportions. There are several types of bioactive glasses: conventional silicates, such as bioglass 45S5, phosphate-based glasses, and borate-based glasses[216].

4.4.2. Polymers

Biodegradable polymers for BTE may be of natural or synthetic origin. Among the natural polymers used for tissue regeneration are those materials inspired by the extracellular matrix like collagen. Among the synthetic polymers are polylactic acid (PLA), polyglycolic acid (PGA), and their copolymers.

Collagen: is the main component of connective tissue in mammals. Collagen type I is present in bone, and it is the most abundant in nature and

[215] S. Samavedi, A.R. et al.“Calcium phosphate ceramics in bone tissue engineering: a review of properties and their influence on cell behavior,” *Acta Biomaterialia*, vol. 9, no. 9, pp. 8037–8045, 2013.

[216] L. L. Hench L.L.et al., “Bioactive glasses: importance of structure and properties in bone regeneration,” *Journal of Molecular Structure*, vol. 1073, pp. 24–30, 2014.

most considered for biomedical applications. It has good biocompatibility, low antigenicity and the ability of crosslinking, therefore its mechanical and degradation properties can be tailored[217].

Poly(α-ester)s[218]:

Polyglycolide (PGA) is a highly crystalline polymer (45–55% crystallinity). It has high mechanical strength. The high rates of degradation and acidic degradation products limit his clinical applications , therefore, copolymers containing PGA units are being developed to overcome those disadvantages[219].

Polylactide (PLA) is a chiral molecule and its monomer exists in two optically active forms: L-lactide and D-lactide.Their polymerization forms a semicrystalline polymer and PLA behaves as crystalline or amorphous depending of these stereoisomers. The molar mass of the polymer as well as the degree of crystallinity has a significant influence on the mechanical properties[220]. Poly-L-lactide (PLLA) is a low rate degradation polymer, has good tensile strength and high Young's modulus , therefore, it is useful for load-bearing applications, such as orthopedic fixation devices]. It has been reported that high molecular weight PLLA can take between 2 and 5.6 years for total resorption in vivo[221].

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[217] Yang I. et al"Mechanical properties of native and cross-linked typeI collagen fibrils," *Biophysical Journal*, vol. 94, no. 6, pp. 2204–2211, 2008.

[218].Nairand L.S. et al., "Biodegradable polymers as biomaterials,"*Progress in Polymer Science (Oxford)*,vol.32,no.8-9,pp.762–798, 2007.

[219] Maurus P.B. and C. C. Kaeding C.C., "Bioabsorbable implant material review," *Operative Techniques in Sports Medicine*, vol. 12, no. 3, pp. 158– 160, 2004.

[220] Savioli M., "Poly (lactic acid) production for tissue engineering applications," in *Proceedings of the 20th International Congress of Chemical and Process Engineering (CHISA '12)*, pp. 1402–1413, August 2012.

[221] Bergsma J.E.,"In vivo degradation and biocompatibility study of in vitro pre-degraded as-polymerized polyactide particles," *Biomaterials*, vol. 16, no. 4, pp. 267–274, 1995.

Poly(lactide-co-glycolide) (PLG): L- and DL-lactides have been used for copolymerization with glycolide monomers to obtain different degradation rates. PLG degradation rate depends on a variety of parameters including the LA/GA (lactidyl/glycolidyl) ratio, molecular weight, and the shape and structure of the matrix[222].

Polycaprolactone (PCL): PCL is a semicrystalline polyester obtained by the ring opening polymerization of monomeric units of " -caprolactone''. It presents hydrolytic degradation due to the presence of hydrolytically labile aliphatic ester bonds; however, its rate of degradation is rather slow (2-3 years) with respect to polymers like PLA. PCL has low tensile strength and high elongation at breakage. It can be used in conjunction to other materials for load-bearing applications[223]

Biocomposites in recent years there has been a trend in the development of scaffolds made of ceramic/polymer composites. Ceramics like calcium phosphates have excellent osteoinductive properties but low degradability, low mechanical strength, and difficulty in forming processes for controlling the physical and geometrical characteristics required from the scaffold. Polymers exhibit poor osteoinductivity but better mechanical properties and degradability rates and can be formed by various manufacturing processes that allow better control of their geometric characteristics. Composites of collagen type I and calcium phosphates are widely used in bone tissue engineering due to the similarity to natural bone and capacity of enhancing osteoblast differentiation and accelerating osteogenesis The development of ceramic-polymer composites allows biodegradable materials with good

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[222] Gunatillake P. et al., "Recent developments in biodegradable synthetic polymers," *Biotechnology Annual Review*, vol. 12, pp. 301–347, 2006.

[223] Chim H. et al."A comparative analysis of scaffold material modiecations for load-bearing applications in bone tissue engineering," *International Journal of Oral and Maxillofacial Surgery*, vol. 35, no. 10, pp. 928–934, 2006.

mechanical and biological properties In table biocomposites used for bone tissue engineering[224].

[224] Chen Q., “Progress and challenges in biomaterials used for bone tissue engineering: bioactive glasses and elastomeric composites,” *Progress in Biomaterials*, vol. 1, no. 1, article 2, 2012.

Biocomposites used for bone tissue engineering

Biocomposite		Percentage of ceramic (%)	Compressive (*C*), tensile (*T*), flexural (*F*), and bending (*B*) strengths (MPa)	Modulus (MPa)	Ultimate strain (%)	Toughness (kJ/m^2)	Reference
Ceramic	**Polymer**						
HA fiber	PDLLA	2 to 10.5 (vol.)	45 (*F*)	1.75×10^3 to 2.47×10^3			Deng et al. ([2001])
	PLLA	10 to 70 (wt.)	50 to 60 (*F*)	6.4×10^3 to 12.8×10^3	0.7 to 2.3		Kasuga et al. ([2001])
HA	PLGA	40 to 85 (vol.)	22 (*F*)	1.1×10^3		5.29	Xu et al. ([2004]), Xu and Simon ([2004a,b])
	Chitosan	40 to 85 (vol.)	12 (*F*)	2.15×10^3		0.092	Xu et al. ([2004])
	Chitosan + PLGA	40 to 85 (vol.)	43 (*F*)	2.6×10^3		9.77	Xu et al. ([2004])
	PPhos	85 to 95 (wt.)					Greish et al. ([2005])
	Collagen	50 to 72 (wt.)					Rodrigues et al. ([2003])
β-TCP	PLLA-co-PEH	75 (wt.)	51 (*F*)	5.18×10^3			Kikuchi et al. ([1999])
	PPF	25 (wt.)	7.5 to 7.7 (*C*)	191 to 134			Peter et al. ([1998])
A/W	PE	10 to 50 (vol.)	18 to 28 (*B*)	0.9×10^3 to 5.7×10^3			Juhasz et al. ([2003a,b]), Juhasz et al. ([2004])
$Ca_3(CO_3)_2$	PLLA	30 (wt.)	50	3.5×10^3 to 6×10^3			Kasuga et al. ([2003])
Bioglass®	**PGA**	**2 to 1 (wt.)**	**0.5 to 2 (*T*)**	**0.5 to 2 (*T*)**	**150 to 600**		Chen et al. ([2010a]) Chen et al. ([2011b]), Liang et al. ([2010])
Human cortical bone		70 (wt.)	50 to 150 (*T*)	12×10^3 to 18×10^3			Keaveny and Hayes ([1993]), Moore et al. ([2001]), Nalla et al. ([2003]), Zioupos and Currey

table 1:from Q. Chen, C. Zhu, and G. A. Thouas, “Progress and challenges in biomaterials used for bone tissue engineering: bioactive glasses and elastomeric composites,” *Progress in Biomaterials*, vol. 1, no. 1, article 2, 2012.

5. BLOOD VESSEL TISSUE ENGINEERING

To allow the peripheral or coronary revascularization bypass surgery is commonly performed. Autografts are the standard clinical approach for the replacement of small diameter blood vessels (inner diameter < 6 mm), using saphenous vein, arm vein, mammalian artery, or radial artery. The drawbacks of this procedure are the considerable morbidity associated and the scarce availability due to diseases or previous organ harvesting. Arterial autografts are more indicated for coronary by-pass surgeries due to their higher mechanical properties. Synthetic materials, as polyethylene terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE), are successfully used for the replacement of medium-large diameter blood vessels (ID > 6 mm), when high blood flow and low resistance prevail. However, synthetic grafts used for below-the-knee vascular by-pass and coronary by-pass (ID < 6 mm) have unacceptable patency rates in the long term. Their use for small diameter blood vessels leads to several complications like aneurysm, intimal hyperplasia, calcification, thrombosis, infection, and lack of growth potential for pediatric applications.

5.1. Vascular Tissue Engineering

As reported by Couet et al.[225], vascular tissue engineering “aims to apply the principles of engineering and life sciences towards the development of a

[225] Couet,F. et al., “Macromolecular biomaterials for scaffold-based vascular tissue engineering,” *Macromolecular Bioscience*, vol. 7, no. 5, pp. 701–718, 2007.

vascular construction that demonstrates biological and mechanical properties as close as possible to those of a native vessel". The use of bio engineered vascular conduit is fundamental in small caliber vessel, meanwhile the possibility to obtain bioengineered large vessel replacement is actually less important due to the satisfactory result and still less expensive use of artificial or homograft conduit.

The basic strategy for vascular tissue engineering consists of the design and the production of appropriate scaffolds for vascular cell adhesion, proliferation, and differentiation and the choice of cell type. Requirements of an ideal TEVG (tissue-engineered vascular graft) in particular small diameter vessels are reported in table

Table 2:Requirements of an ideal Tissue-Engineered Vascular Graft, from Catto V.. et al, "Vascular Tissue Engineering: Recent Advances in Small Diameter Blood Vessel Regeneration," ISRN Vascular Medicine, vol. 2014, Article ID 923030, 27 pages, 2014.

Biocompatibility	Nontoxicity Nonimmunogenicity Nonthrombogenicity Nonsusceptibility to infection Ability to grow for pediatric patients Maintenance of a functional endothelium
Mechanical properties	Compliance similar to native vessel Burst pressure similar to native vessel Kink and compression resistance Good suture retention
Processability	Low manufacturing costs Readily available with a large variety of lengths and diameters Sterilizable Easy storage

There are two different approaches in vascular tissue engineering:.

One envisage the use of a bioreactor to generate physiological-like stimuli onto cell seeded scaffolds for in vitro TEVG maturation, before the in vivo implantation. A second approach is the direct implantation of cell seeded scaffolds in the body that acts as a bioreactor for TEVG maturation[226].

5.1.1. Natural Polymers in TEVG

Fibrin is an insoluble body protein involved in wound healing and tissue repair . Fibrin clot, obtained by fibrinogen polymerization due to thrombin, is a fibrillar network gel that provides a structural support for adhesion, proliferation, and migration of cells involved in the healing. Finally, fibrin clot is resorbed through the fibrinolysis. Fibrinogen may be purified from autologous blood and used for scaffold fabrication avoiding immunological problems[227].

Elastin, is one of the major extracellular matrix (ECM) proteins in the arterial wall that confers elasticity , resilience, and durability . It is an important autocrine regulator to smooth muscle cells (SMC) and endothelial cells (EC) activity, inhibiting migration and proliferation of SMCs and enhancing attachment and proliferation of ECs. Elastin, used as a coating of vascular devices made of expanded polytetrafluoroethilene (ePTFE) , polyethylene terephthalate (PET,) a copolymer of ePTFE and polyethylene, and a

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[226] Catto V.. et al, “Vascular Tissue Engineering: Recent Advances in Small Diameter Blood Vessel Regeneration,” *ISRN Vascular Medicine*, vol. 2014, Article ID 923030, 27 pages, 2014.

[227] Pankajakshan D. et al., “Scaffolds in tissue engineering of blood vessels,” *Canadian Journal of Physiology and Pharmacology,* vol. 88, no. 9, pp. 855–873, 2010.

polycarbonate polyurethane , has demonstrated low thrombogenicity with reduced platelet adhesion and activation[228].

Hyaluronan ,hydrophilic, nonadhesive, biocompatible, and biodegradable[229].

Silk fibroin shows excellent mechanical properties and biocompatibility, degrades slowly and proteolytically in vivo, maintaining more than 50% of its mechanical properties after 2 months[230].

Collagen demonstrates low antigenicity, low ingammatory response, biocompatibility, biodegradability, and excellent biological properties . Collagen type I is one of the main components of the vascular wall, whereas it is widely used as scaffold for vascular tissue engineering applications[231].

5.1.2. Biodegradable Synthetic Polymers in TEVG

Biodegradable synthetic polymers compared to natural polymers demonstrate tailorable mechanical properties, high reproducibility and possibility of large amounts production.

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[228] S. G. Wise S.G. et al.,,"A multilayered synthetic human elastin/polycaprolactone hybrid vascular graft with tailored mechanical properties," *Acta Biomaterialia*, vol. 7, no. 1, pp. 295–303, 2011.

[229] B. Zavan, B. et al., "Neoarteries grown in vivo using a tissue-engineered hyaluronan-based scaffold," *FASEB Journal*, vol. 22, no. 8, pp. 2853–2861, 2008.

[230] Vepari C.and Kaplan D.L., "Silk as a biomaterial," *Progress in Polymer Science*, vol. 32, no. 8-9, pp. 991–1007, 2007.

[231] B. Marelli B. et al., "Collagen-reinforced electrospun silk fibroin tubular construct as small calibre vascular graft," *Macromolecular Bioscience*, vol. 12, no. 11, pp. 1566–1574, 2012.

Polyglicolic acid (PGA) degrades rapidly in vivo by hydrolysis to glycolic acid, metabolized and eliminated as carbon dioxide and water, and completely degrades in vivo within 6 months. PGA is a FDA approved polymer for human clinical use[232].

Polylactic acid (PLA) demonstrates good biocompatibility and mechanical properties and the ability to be dissolved in common solvents for processing . It is is more hydrophobic than PGA, leading to a slower degradation rate[233].

Poly-ε-caprolactone (PCL) shows good mechanical properties, specihcally high elongation and strength, and good biocompatibility It degrades very slowly in vivo (more than 1 year to completely degrade) by enzymatic action and by hydrolysis to caproic acid and its oligomers . It is approved by FDA[234] .

Polyglycerol- sebacate (PGS) demonstrates good biocompatibility and good mechanical properties, specifically high elongation and low modulus, indicating an elastomeric and tough behavior]. It degrades in vivo by hydrolysis in 2 months[235].

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[232] L. V. Thomas L.V.,et al., “Tissue engineered vascular grafts—preclinical aspects,” *International Journal of Cardiology*, vol. 167, no. 4, pp. 1091–1100, 2013.

[233] Kim K. et al.,“Control of degradation rate and hydrophilicity in electrospun non-woven poly(D,L-lactide) nanofiber scaffolds for biomedical applications,” *Biomaterials*, vol. 24, no. 27, pp. 4977–4985, 2003.

[234] De Valence S.et al.,“Long term performance of polycaprolactone vascular grafts in a rat abdominal aorta replacement model,” *Biomaterials*, vol. 33, no. 1, pp. 38–47, 2012.

[235] Y. Wang, G. A. Ameer, B. J. Sheppard, and R. Langer, “A tough biodegradable elastomer,” *Nature Biotechnology*, vol. 20, no. 6, pp. 602–606, 2002.

5.1.3. Hybrid Scaffolds from Synthetic and Natural Polymers

Hybrid scaffolds, made of synthetic and natural polymers, has been recently studied to combine the mechanical properties of synthetic materials with the biological behavior of natural polymers. Here are reported some types of hybrid scaffold proposed in the most relevant and recent (2008–2013) studies: :

- Blend of PCL + elastin + collagen type I [236]
- Recombinant human tropoelastin (inner layer) + PCL (outer layer)[237]
- PGA + PLLA + collagen type I[238]
- Blend of PCL + collagen type I[239] [240]
- PCL/PLA + collagen type I[241]
- PLDLA + fibrin[242]

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[236] McClure M.J. et al., “A three-layered electrospun matrix to mimic native arterial architecture using polycaprolactone, elastin, and collagen: a preliminary study,” *Acta Biomaterialia*, vol. 6, no. 7, pp. 2422–2433, 2010.

[237] Wise S.G.et al., “A multilayered synthetic human elastin/polycaprolactone hybrid vascular grae with tailored mechanical properties,” *Acta Biomaterialia*, vol. 7, no. 1, pp. 295–303, 2011.

[238] Yokota T. et al.,“In situ tissue regeneration using a novel tissue-engineered,small-caliber vascular graft without cell seeding,” *Journal of Soracic and Cardiovascular Surgery*, vol. 136, no. 4, pp. 900–907, 2008.

[239]Lee S.J.et al., “Development of a composite vascular scaffolding system that withstands physiological vascular conditions,” *Biomaterials*, vol. 29, no. 19, pp. 2891–2898, 2008.

[240] Tillman B.W. et al., “The in vivo stability of electrospun polycaprolactone-collagen scaffolds in vascular reconstruction,” *Biomaterials*, vol. 30, no. 4, pp. 583–588, 2009.

[241] He W. et al., Z. Ma, W. E. Teo et al., “Tubular nanohber scaffolds for tissue engineered small-diameter vascular graes,” *Journal of Biomedical Materials Research A*, vol. 90, no. 1, pp. 205–216, 2009.

[242] S. Koch S. et al.,“Fibrin-polylactide-based tissue-engineered vascular grae in the arterial circulation,” *Biomaterials*, vol. 31, no. 17, pp. 4731–4739, 2010.

5.1.4. Scaffolds from Decellularized Matrices

Recently, some studies are focused on the possibility to directly implant acellular scaffolds in the body.

In 1986, Weinberg and Bell[243] generated the first tissue-engineered blood vessel substitute, consisting of cultures of bovine endothelial cells , smooth muscle cells and fibroblasts embedded in a collagen gel. This graft had not sufficient strength and was unsuitable for implantation. This construct was evaluated in vivo as an arterial implant after reinforcement with Dacron® as shown from Matsuda in 1995[244].

Various methods of improving the mechanical properties of collagen gels (e.g., cross linking agents such as glutaraldehyde) have been investigated, but none has proven to yield a structurally stable vascular graft as reported from Charulatha in 2003[245].

As an alternative to collagen for natural ECM-based scaffolds, fibrin holds particular promise for its ability to induce collagen and elastin synthesis and improved mechanical properties as shown from Swartz in 2005[246].

Encouraging result, even if in larger diameter vessel, was achieved by combining fibrin gels with biodegradable polymeric scaffolds followed by

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[243] Weinberg CB, Bell E. A blood vessel model constructed from collagen and cultured vascular cells. *Science*. 1986;231(4736):397–400.

[244] Matsuda T.et al., A hybrid vascular model biomimicking the hierarchic structure of arterial wall: neointimal stability and neoarterial regeneration process under arterial circulation, *J Thorac Cardiovasc Surg* 110:988-997.

[245] V. Charulatha and A. Rajaram, “Influence of different crosslinking treatments on the physical properties of collagen membranes,” *Biomaterials*, vol. 24, no. 5, pp. 759–767, 2003.

[246] Swartz D.D.et al.,, “Engineering of fibrin-based functional and implantable small-diameter blood vessels,” *Se American Journal of Physiology—Heart and Circulatory Physiology*, vol. 288, no. 3, pp. H1451–H1460, 2005.

seeding of autologous arterial-derived cells, from the group of Tschoeke[247] in 2009, as also endothelialized vessels have been successfully implanted in the carotid arteries of sheep from Koch[248] and his team in 2010.

Decellularized tissue, often in the form of a Xenogenic, can serve as a naturally available scaffold.

Examples of such scaffolds were developed by Lantz[249] in 1993, using the small intestinal submucosa (SIS) decellularized and then implanted in aorta, carotid and femoral arteries of dogs. The grafts resulted completely endothelialized at 28 days post-implantation. At 90 days, the grafts were histologically similar to normal arteries and veins and contained a smooth muscle media and a dense fibrous connective tissue adventitia. Follow-up periods of up to 5 years found no evidence of infection, intimal hyperplasia, or aneurysmal dilation. One infection-challenge study suggested that SIS may be infection resistant, possibly because of early capillary penetration of the SIS (2 to 4 days after implantation) and delivery of body defenses to the local site.

Kaushal[250] in 2001 has employed decellularized porcine iliac arteries seeded with endothelial progenitor cells (EPCs), implanted into ovine carotid arteries. These TEVG constructs remained patent out to 130 days and were remodeled into neovessel, whereas the unseeded control group occluded within 15 days.

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[247] Tschoeke B., Tissue-engineered small-caliber vascular graft based on a novel biodegradable composite fibrin-polylactide scaffold. *Tissue Eng* Part A. 2009 Aug;15(8): 1909-18.

[248] Koch S., Fibrin-polylactide-based tissue-engineered vascular graft in the arterial circulation. *Biomaterials*. 2010 Jun;31(17):4731-9.

[249] Lantz G.C.et al., Small intestinal submucosa as a vascular graft: a review. *J. Invest. Surg.* 1993;6:297–310.

[250] Kaushal S.et al., Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. *Nat Med*. 2001 Sep;7(9):1035-40.

Simon[251] in 2003, shown as elements of the ECM are exposed to physical and chemical stresses during the process of decellularization, which can adversely affect the biomechanical properties of the ECM. This deterioration might ultimately lead to degenerative structural graft failure. Additional drawbacks of decellularized materials included the inability to modify the ECM content and architecture, the variability among donor sources, and the risk of viral transmission from animal tissue.

In a study of Quint[252] in 2011 tissue-engineered vessels (TEVs) were grown from banked porcine smooth muscle cells that were allogeneic to the intended recipient, using a biomimetic perfusion system. The engineered vessels were then decellularized, leaving behind the mechanically robust extracellular matrix of the graft wall. The acellular grafts were then seeded with cells that were derived from the intended recipient—either endothelial progenitor cells (EPC) or endothelial cell (EC)—on the graft lumen. TEV were then implanted as end-to-side grafts in the porcine carotid artery, which is a rigorous testbed due to its tendency for graft occlusion. The EPC- and EC-seeded TEV all remained patent for 30 d in this study, whereas the contralateral control vein grafts were patent in only 3/8 implants. The cell-seeded TEV demonstrated less neointimal hyperplasia and fewer proliferating cells than did the vein grafts. These results indicate that a readily available, decellularized tissue-engineered vessel can be seeded with autologous endothelial progenitor cells to provide a biological vascular graft that resists both clotting and intimal hyperplasia.

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[251] Simon P,et al., Early failure of the tissue engineered porcine heart valve SYNERGRAFT in pediatric patients. *Eur J Cardiothorac Surg*. 2003;23:1002–1006.

[252] Quint C.et al.,Decellularized tissue-engineered blood vessel as an arterial conduit. *Proc Natl Acad Sci* U S A. 2011 May 31;108(22):9214-9.

Xiong[253] in 2013 has conduct a study to develop and investigate a biomechanically functional and biocompatible acellular conduit using decellularized porcine saphenous arteries (DPSAs), through a modified decellularization process using Triton X-100/NH4 OH solution and serum-containing medium. Histological and biochemical analysis indicated a high degree of cellular removal and preservation of the extracellular matrix. Bursting pressure tests showed that the DPSAs could withstand a pressure of 1854 ± 164 mm Hg. Assessment of in vitro cell adhesion and biocompatibility showed that porcine pulmonary artery endothelial cells were able to adhere and proliferate on DPSAs in static and rotational culture. After interposition into rabbit carotid arteries in vivo, DPSAs showed patency rates of 60% at 1 month and 50% at 3 months. No aneurysm and intimal hyperplasia were observed in any DPSAs. All patent grafts showed regeneration of vascular elements, and thrombotic occlusion was found to be the main cause of graft failure, probably due to remaining xenoantigens.

5.1.5. TEVGs without Scaffolds

Some studies have been performed on the realization of completely biological TEVGs without the use of scaffolds, to avoid problems related to synthetic materials, such as inflammation, stenosis, and infection, allowing for a complete graft integration and increase of the patency rate . Three methods have been investigated[254]:

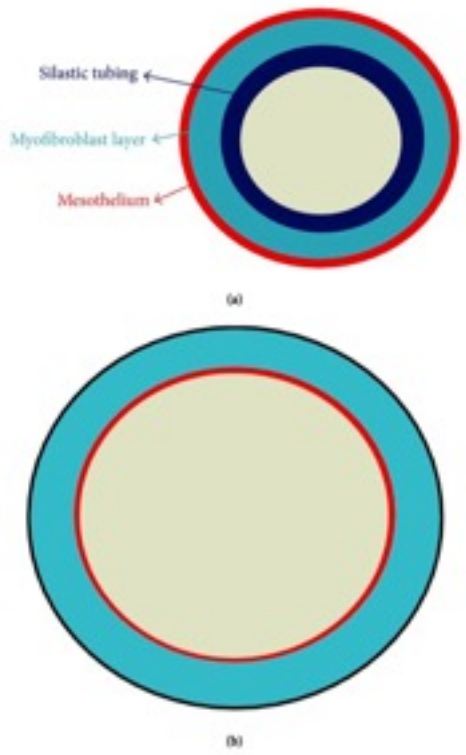
- ***in vivo bioreactor***, using the body as a bioreactor. A Silastic (silicone compound) tubing has been implanted in the peritoneal cavity inducing a

[253] Xiong Y.et al., Decellularized Porcine Saphenous Artery for Small-Diameter Tissue-Engineered Conduit Graft , *Artificial Organs* Volume 37, Issue 6, pages E74–E87, June 2013

[254] M. Peck M.et al.,“The evolution of vascular tissue engineering and current state of the art,” *Cells Tissues Organs*, vol. 195, no. 1-2, pp. 144–158, 2011.

foreign body reaction with the formation of a fibrous capsule around the mandrel composed of myofibroblasts, collagen matrix, and a single layer of mesothelial cells . After 2-3 weeks, the explanted tube of tissue was removed from the Silastic mandrel and reversed, so the mesothelium represented the inner layer . The limitations of this method are the requirement of a double surgery and the possible TEVG adhesion to the peritoneal wall during maturation with consequent damage to the mesothelium (the external layer)[255].

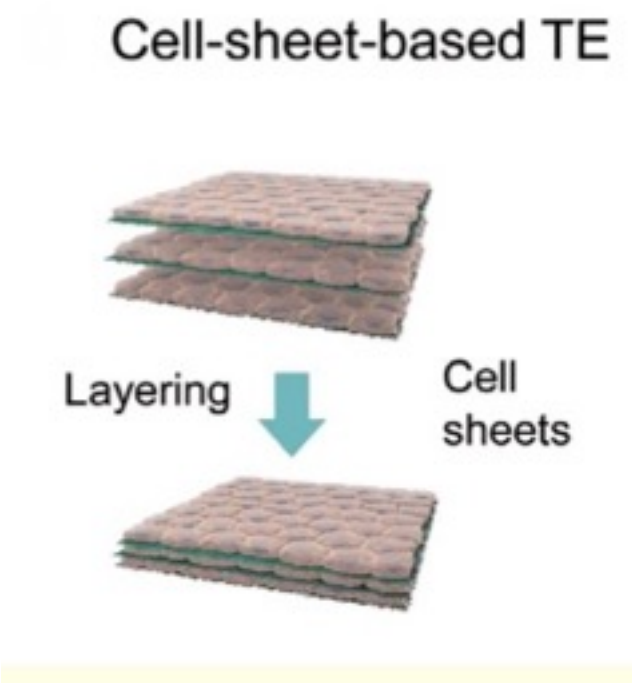
Fig.5: from Catto V. et al, Vascular Tissue Engineering: Recent Advances in Small Diameter Blood Vessel Regeneration, ISRN *Vascular Medicine* Volume 2014 (2014), Article ID 923030, 27 pages.
Sketch of the in vivo bioreactor approach; (a) fibrous capsule formed around a Silastic tubing in the peritoneal cavity; (b) after removal of Silastic tubing reversion of fibrous capsule, the mesothelium was the inner layer.



[255] Catto V. et al, Vascular Tissue Engineering: Recent Advances in Small Diameter Blood Vessel Regeneration, ISRN *Vascular Medicine* Volume 2014 (2014), Article ID 923030, 27 pages.

• ***cell sheet-based*** consists of the *in vitro* growing of cells with ascorbic acid in the culture medium to generate a large production of ECM . After a maturation period, cell sheets were detached from the culture flasks and rolled onto a mandrel The major limitation of this approach is the long time required for the *in vitro* TEVG growth. (6 to 9 months).

Fig.6: from:Shimizu T.Cell sheet-based tissue engineering for fabricating 3-dimensional heart tissues, *Circ J*. 2014;78(11):2594-603.



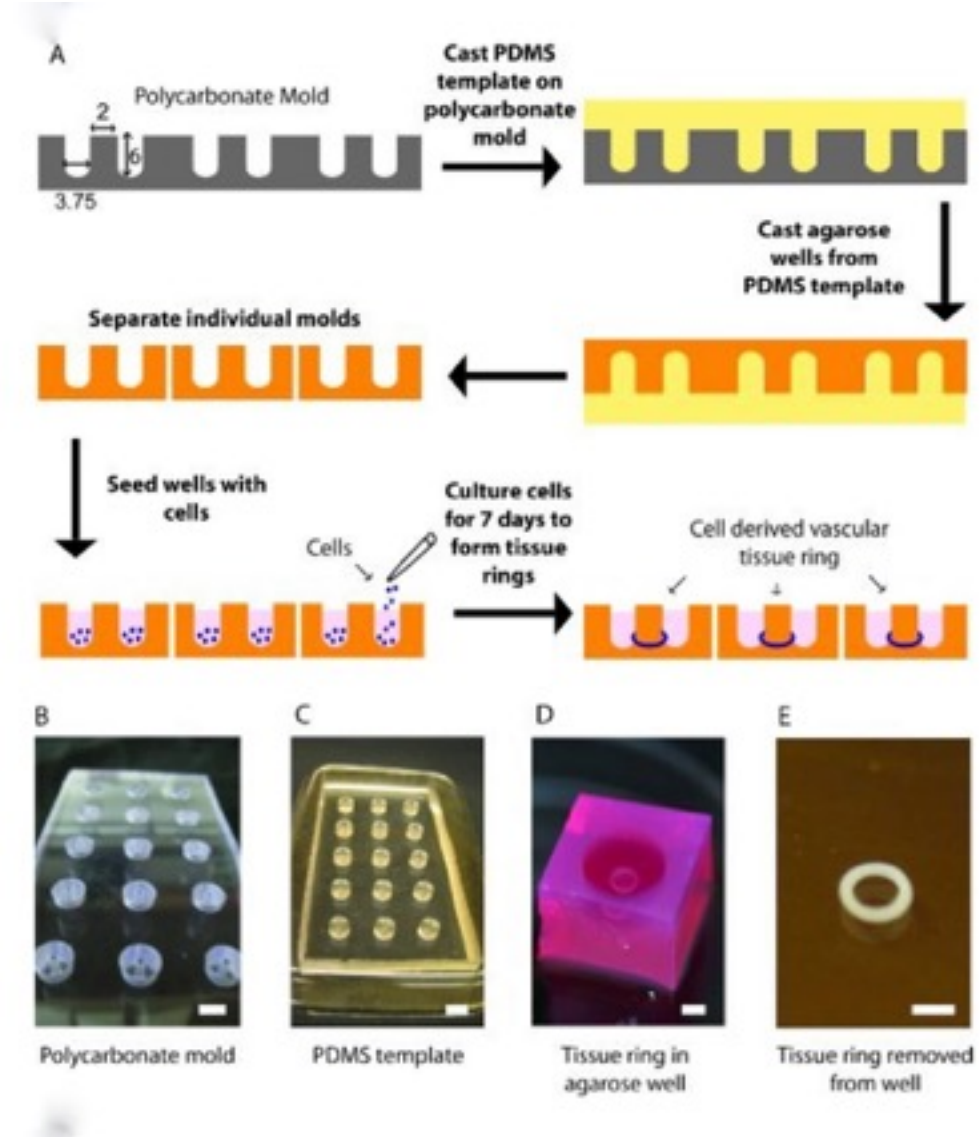
Celll sheets are harvested from temperature-responsive culture dishes and stacked for 3D tissue fabrication

- ***cell ring-based*** is described in a study of Gwyther[256] et al.: rat aortic smooth muscle cells seeded into annular agarose wells aggregated and formed thick tissue rings within 2 weeks of static culture . The tissue rings cultured for 7 days on silicone mandrels fused to form tubular constructs. Ring margins were visible after 7 days, but tubes were cohesive and mechanically stable, and histological examination confirmed fusion between ring subunits.

[256] T. A. Gwyther T.A.et al., “Engineered vascular tissue fabricated from aggregated smooth muscle cells,” *Cells Tissues Organs*, vol. 194, no. 1, pp. 13–24, 2011.

Fig.7: Schematic of the tissue ring generation process, from: Gwyter T.A.et al., Directed cellular self-assembly to fabricate cell-derived tissue rings for biomechanics analysis and tissue engineering, J Vis Exp. 2011 Nov 25;(57):e3366.

(A) Schematic of the tissue ring generation process.



(B) Custom polycarbonate mold with milled annular wells. Central post diameters are 2 mm. (C) PDMS template after it was peeled away from the polycarbonate mold. (D) Aggregated tissue ring cultured in an agarose mold with a 2 mm post. (E) Two mm diameter tissue ring in PBS. Scale bars = 6 mm (B, C) and 2 mm (D, E).

6. NEURAL TISSUE ENGINEERING

When a peripheral nerve is cut, either healing or permanent interruption of the neural pathways occurs. The final outcome depends on the severity of the injury and on its treatment

Healing and regeneration are likely to occur if the two ends are aligned in case proximity. The distal portion of the axon severed from the cell body degenerates and is phagocytosed by Schwann cells and by macrophages that migrate into the traumatized zone. Once the debris has been removed, the surviving Schwann cells, surrounded by the basement membrane, proliferate to form a regeneration tube and the part of the axon connected to the cell body begins to grow and exhibit amoeboid movement. The Schwann cells produce a variety of growth factors, cell-adhesion molecules that attract the growing axon tip, and the tube itself guide the regenerating axon to its proper destination.

For damage that results in gaps greater than approximately 3 cm, the current clinical gold standard treatment is the nerve autograft[257]. In this treatment a nerve is first removed from another part of the patient's body. The nerve is then used to bridge the gap and connect the two ends of the severed nerve, and to then guide the regrowth of the actual nerves . This treatment has a high rate of success, but there are drawbacks . Autologous grafts require a nerve taken from somewhere else on the patient's body. Not only the patient must have an additional operation in order to obtain this nerve, but the patient also must consider a possible loss of function at the location from which this nerve was taken . Moreover there are a limited number of locations that can be used as a donor site, as the two nerves must have a similar structure and, in addition,

[257] Deumens R. et al.,Repairing injured peripheral nerves: bridging the gap, *Prog Neurobiol*, 92 (2010), pp. 245–276

there is the possibility of the death of the donor tissue for an unsuccessful attempt to attach it to the damaged location .

To eliminate these problems graft methods that involve the use of extracellular matrix (ECM) of non-autologous tissue have been created[258]. However also in this technique there are drawbacks . The cells native to the non-autologous tissue must be completely eliminated to remove the risk of immunorejection, and this is difficult to obtain without the destruction of the ECM . It is vital that the ECM remain intact because it plays an important role in nerve regeneration. The ECM is the material that surrounds cells, and molecules within it can help guide, promote, or inhibit the growth of neurons. It has been difficult to create a graft that maintains the structure of the ECM while eliminating cellular debris because of limitations when using thermal, radiation, and chemical treatments,

Many new therapeutic strategies for improving nerve repair are being developed in basic, pre-clinical and clinical trials. Results have shown that hollow nerve guidance conduits (NGCs) and living cells are essential to provide trophic support and recreate the environment provided by the nerve autograft.

6.1. Scaffold for nerve tissue engineering

The goal in tissue engineering is to regulate the cell behavior and tissue progression through the development and design of synthetic extracellular matrix analogues to support three-dimensional cell culture and tissue regeneration. The properties required in a scaffold for nerve regeneration are biocompatibility, less inflammatory, controlled biodegradability with non-toxic degradative products, porosity for

[258]Badylak S.F.The extracellular matrix as a biologic scaffold material, *Biomaterials* Volume 28, Issue 25, September 2007, Pages 3587–3593

vascularization and cell migration and three-dimensional matrices with appropriate mechanical properties to mimic the extracellular matrix.

The ideal characteristics of a scaffold for neural tissue engineering are reported in figure 4:

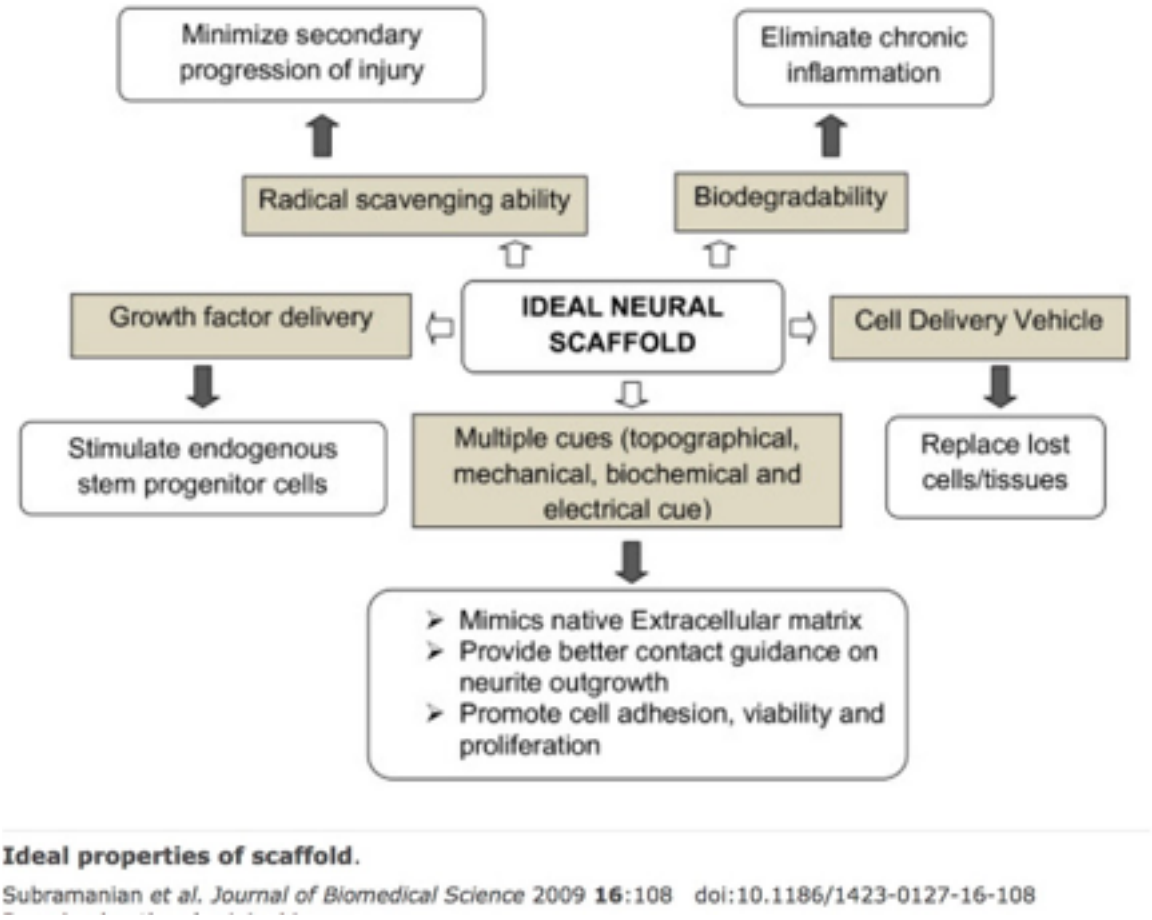


Fig.8: Schematic representation of requisites for an ideal neural tissue engineering scaffold

6.1.1. Natural materials for neural tissue engineering

6.1.1.1. Polysaccharides

Agarose, an alternating co-polymer of 1,4-linked 3,6-anhydro-alfa -L-galactose and 1,3-linked -beta-D-galactose, forms a solid below its gel-solution temperature. It has been fabricated into nerve guidance scaffolds with uniaxial channels[259]. Axonal growth can be further enhanced by adding *brain-derived neurotrophic factor (*BDNF), either through adsorption by rehydration of the agarose, or by loading the channels with bone marrow stromal cells genetically engineered to secrete BDNF.

Chitin and Chitosan. Researchers have shown that chitin and chitosan are ideal materials for biodegradable nerve guides and they are good environments for nerve cell adhesion and neurite outgrowth (7, 27, 28). In a study Chitosan nano/micro-fibrous tubes were transplanted as bridge grafts into a rat sciatic nerve gap. Chitosan nano-fibers resulted in the proliferation of C17.2 neural stem cells (NSCs) and a confluent layer of cells with extensive neurite outgrowth were detected[260].

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[259] Gao M.et al.,Templated agarose scaffolds for the support of motor axon regeneration into sites of complete spinal cord transection. *Biomaterials*. 2013 Feb; 34(5):1529-36.

[260] Jin H, XiuMei W, Myron S, FuZhai C. Scaffolds for central nervous system tissue engineering. *Front Mater Sci.* 2012;**6**(1):1-25.

6.1.1.2. Proteins

Collagen, can be processed into hydrogels and fabricated into different types of scaffolds. Their ability to promote nerve growth is influenced by the dimensions of the scaffolds and the presence of Schwann cells. Hollow guides of collagen gel have been employed to investigate the effect of Schwann cell implantation in a thoracic spinal cord lesion[261]. In vivo pure collagen guides did not support axonal elongation or Schwann cell entry . The inclusion of Schwann cells significantly improved the response; cell-seeded collagen channels promoted axonal growth by 14 days and myelination by 28 days after implantation.

Gelatin, the hydrolysis product of collagen, has also been been investigated. In a study of Du BL [262] graft of gelatin sponge scaffold containing genetically-modified neural stem cells has demonstrated to promote cell differentiation, axon regeneration, and functional recovery in rat with spinal cord transection.

Collagen and gelatin enables Schwann cell attachment and axonal extension, but their drawback is that these ECM proteins must be isolated from animal or human sources with the risk of pathogen transmission via graft materials.

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[261] Paino, C.L. , Bunge, M.B., Induction of axon growth into Schwann cell implants grafted into lesioned adult rat spinal cord. *Exp Neurol* 114 (2), 254-257 (1991).

[262] Du BL et al, *J Biomed Mater Res* A. 2015 Apr;103(4):1533-45

6.1.2. Synthetic materials for neural tissue engineering

Poly L-Lactic Acid (PLLA) is considered as an ideal cell environment for tissue engineering of the nervous system[263]. Several studies have shown that neural stem cells (NSCs) can develop on PLLA scaffold and this supports neurite outgrowth[264]. Some disadvantages of this polymer are its poor biocompatibility, discharge of acidic degradation products, poor process ability and early failure of mechanical features during degradation[265].

Poly D, L-lactic-co-Glycolic Acid (PLGA) has been extensively used as a scaffold material for tissue engineering. For the deficiency of natural adhesion sites on this polymer cell adhesion and growth can be impeded. For this reason, many techniques have been studied to modify PLGA scaffolds and promote cell adhesiveness, such as hydrolysis, aminolysis, blending and covalent attachment of adhesive peptides[266]. Recent evidence showed that PLGA nano-fibers are appropriate scaffolds for nerve tissue engineering application and their biological properties can be promoted by applying poly-L-lysine (PLL) in the polymer matrix[267].

Combination of Poly-Glycolic Acid (PGA) and Poly-Lactic Acid (PLA) that can react to make the copolymer, poly lactic-co-glycolic acid . After implantation, if hydrolysis of the ester bonds that develop the backbone of the

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[263] Vasita R, Katti DS. Nanofibers and Their Spplications in Tissue Engineering. *Int J Nanomed.* 2006;**1**(1):15-30.

[264] Tierney CM, Haugh MG, Liedl J, Mulcahy F, Hayes B, O'Brien FJ. The effects of collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering. *J Mech Behav Biomed Mater.* 2009;**2**(2):202-9.

[265] Vindigni V, Cortivo R, Iacobellis L, Abatangelo G, Zavan B. Hyaluronan benzyl ester as a scaffold for tissue engineering. *Int J Mol Sci.* 2009;**10**(7):2972-85.

[266] Yang, Y. *et al.*, Neurotrophin releasing single and multiple lumen nerve conduits. *J Control Release* 104 (3), 433-446 (2005).

[267] Kramer M, et al., Promotion of neurite out- growth in corporation poly-l-lysine into aligned PLGA nanofi- ber scafolds. *Europ Cell Mater .* 2011;**22**:53.

polymer occurs, it would lead to the degradation of scaffolds into metabolite producing glycolic acid and lactic acid. These acidic products can be absorbed by the host tissue and may cause pH decrease around the implantation location and also aseptic inflammation. PLLA is more hydrophobic and less crystalline than PGA and degrades at a slower rate so the degradation rate of the amorphous copolymer can thus be easily controlled by altering the ratio of PLA to PGA in the formulation[268].

Polylactide (PLA) and poly(lactide-co-glycolide) (PLGA) have been used to deliver Schwann cells, genetically modified Schwann cells, and neural stem cells in nerve tissue engineering. A study of Evans et al. study attempted to enhance the efficacy of peripheral nerve regeneration using poly(L-lactic acid) (PLLA) conduits by incorporating them with allogeneic Schwann cells (SCs). Although equivalent nerve regeneration to autografts was not achieved, this study provides promising results for further investigation[269].

Poly(hydroxybutyrate) (PHB) is a bacteria-synthesized polymer that degrades into a non-toxic metabolite in mammals. Implanting neonatal Schwann cells using PHB fibers enabled axonal elongation into the rostral and caudal ends following a cervical spinal cord laminectomy[270]. As also demonstrated in other studies, transplantation of hollow PHB conduits seeded with Schwann cells appears to improve peripheral nerve regeneration[271].

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[268] Timnak A,et al.,Fabrication of nano-structured electrospun collagen scaffold intended for nerve tissue engineering. *J Mater Sci Mater Med.* 2011;**22**(6):1555-67.

[269] Evans, G.R. *et al.*, Bioactive poly(L-lactic acid) conduits seeded with Schwann cells for peripheral nerve regeneration. *Biomaterials* 23 (3), 841-848 (2002)

[270] Novikov, L.N. *et al.*, A novel biodegradable implant for neuronal rescue and regeneration after spinal cord injury. *Biomaterials* 23 (16), 3369-3376 (2002).

[271] Erba P., Regeneration potential and survival of transplanted undifferentiated adipose tissue-derived stem cells in peripheral nerve conduits. *J Plast Reconstr Aesthet Surg.* 2010 Dec;63(12):e811-7.

Poly(acrylonitrile-co-vinylchloride) (PAN/PVC) is a co-polymer with substantial tensile strength that has also been used to investigate the potential therapeutic effects of Schwann cells. PAN/PVC guidance channels seeded with Schwann cells were also used to determine that regenerated CNS axons responded to electrical stimulus and conduct action potentials after spinal cord transection. Although PAN/PVC is not biodegradable, its presence did not preclude axonal extension, myelination, or re-establishment of electrical connections[272].

Polypyrrole (PPy) is a conductive polyacetylene derivative recently applied in many fields such as drug delivery, nerve regeneration and biosensor coatings for neural probes. Conjugation in the molecular backbone of PPy leads to its rigidity, insolubility and poor process ability. Therefore, it is very difficult to apply it alone as a structural material and it must be changed into a mechanically manageable and processable material. PPy can support cell adhesion and growth of a number of diverse cell types, has a good biocompatibility and can be an appropriate substrate for bridging peripheral nerve gaps. In recent studies it has not demonstrated evidence of toxicity, pyretogenesis, allergenesis, haemolysis and mutagenesis. There was a better migration of Schwann cells and the neural growth from dorsal root ganglia on glass with PPy membrane than those on glass without this membrane[273].

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[272] Pinzon, A.,et al. , Conduction of impulses by axons regenerated in a Schwann cell graft in the transected adult rat thoracic spinal cord. *J Neurosci Res* 64 (5), 533-541 (2001).

[273] Ghasemi-Mobarakeh L. et al., Application of conductive polymers, scaffolds and electrical stimulation for nerve tissue engineering. *J Tissue Eng Regen Med.* 2011.

6.1.3. Biomaterials for the controlled delivery of Neurotrophin

The failure of cells to activate regeneration-associated genes and the accompanying lack of neurotrophic support are important factors hindering nerve repair[274]. The application of neurotrophins can induce the growth of specific subpopulations of axons, facilitate synaptic transmission, and improve functional recovery[275] [276]. The ability of growth factors to improve regeneration has been assessed by directly injecting the molecules to the injury site, or by delivering them via cells or polymer vehicles.

Biomaterials have the potential to achieve local prolonged delivery of neurotrophic factors by encapsulating, facilitating electrostatic interactions, or covalently incorporating them. A biomaterial delivery matrix can protract activity of growth factors, which have a very short half life in the body[277] [278]. The use of biomaterials may increase the amount of available neurotrophic factor by concentrating it to the desired site of delivery and by preventing its degradation

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[274] Taylor, L., Jones, L., Tuszynski, M.H., & Blesch, A., Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. *J Neurosci* 26 (38), 9713-9721 (2006).

[275] Thuret, S., Moon, L.D., & Gage, F.H., Therapeutic interventions after spinal cord injury. *Nat Rev Neurosci* 7 (8), 628-643 (2006)

[276] Zhang, N., Yan, H., & Wen, X., Tissue-engineering approaches for axonal guidance. *Brain Res Brain Res Rev* 49 (1), 48-64 (2005).

[277] Putney, S.D. & Burke, P.A., Improving protein therapeutics with sustained- release formulations. *Nat Biotechnol* 16 (2), 153-157 (1998).

[278] Krewson, C.E., Dause, R., Mak, M., & Saltzman, W.M., Stabilization of nerve growth factor in controlled release polymers and in tissue. *J Biomater Sci Polym Ed* 8 (2), 103-117 (1996).

in the body. The ability to deliver multiple growth factors in a controlled manner is also important, as a variety of neural and inflammatory cells and factors are involved after a nerve injury.

7. EXPERIMENTAL STUDY IN THE TREATMENT OF CRITICAL BONE DEFECTS[279] [280]

7.1 Animals

Forty-two 4-month-old male New Zealand white rabbits, weighing 2/3 kg, were selected and kept in controlled stabulation (temperature, humidity and light/dark cycle), maintained on a standard diet with a and allowed free mobilization. In a first experience two different scaffolds (PCL and PCL-HA) for three different observation times (4, 8 and 12 weeks) were tested. Six groups of six animals in a random mode were created (*PCL 4w, *PCL 8w, *PCL 12w, *PCL-HA 4w, *PCL-HA 8w and *PCL-HA 12w). After this first evaluation in terms of bone regeneration, a group of six animals was created to evaluate a longer observation time (PCL 24 weeks). All procedures followed were approved by the local ethic committee of Pisa University.

[279]Dini F., Puppi D., Chiellini F., Franceschi R., Coli A., Miragliotta V., Giannessi E., Stornelli M.R., Briganti A., Gabellieri P., Carlucci F., Barsotti G., Biogradable polymeric threedimensional supports for bone regeneration: results of a long-term study in vivo. *Atti conv. S.I.S.VET* 2014

[280]Dini F., Barsotti G., Puppi D., Coli A., Briganti A., Giannessi E., Miragliotta V., Mota C., Pirosa A., Stornelli M.R., Gabellieri P., Carlucci F., Chiellini F., Tailored star poly(ε-caprolactone) wet-spun scaffolds for in vivo regeneration of long bone critical size defects. *Journal of Bioactive and Compatible Polymers* 1-16. 2015

7.2 Surgical procedure

Premedication before anaesthesia was performed by an intramuscular injection of medetomidine (20 µg/kg), ketamine (5 mg/kg) and fentanyl (0.1 mg/ 2 mL, 10 µg/kg).

After cleaning and disinfection a local anaesthetic cream containing Lidocaine (Lignocaine) and prilocaine was applied on the marginal ear vein to produce full thickness skin anaesthesia and after a venous catheter was inserted for Intravenous inducing anaesthesia and administration of fluid therapy.

Induction anaestesthesia was performed by propofol (4–6mg/kg in bolus; 0.7–0.9mg/kg/min in continuous infusion), and every rabbit was maintained with oxygen in mask. A catheter was inserted into the central auricular artery to measure blood pressure.

The right limb of each rabbit was shaved, subjected to trichotomy and the skin prepared with antiseptic solution (10% povidone-iodine solution).

A loco-regional anaesthesia with ropivacaine 0.5% (1mg/kg) was performed in the brachial plexus.

A constant monitoring of heart rate, breathing, blood pressure, oxygen saturation and temperature was performed.

Antibiotic prophylaxis was subcutaneously given (enrofloxacin 10 mg/kg).

The animals were restrained in right lateral recumbency. A skin incision 40 mm long was made on the antero- medial aspect of the right radius carpal joint. The muscles were separated and the radius bone was exposed. A length of 20 mm was marked in the central diaphysis of the radius and the marked piece of the bone was cut with the help of an orthopedic heck saw, and removed to create a segmental defect. For each segmental bone removed was

measured length, width and thickness. The segmental defect in each animal was filled with a PCL or PCL-HA scaffold with similar dimensions. No additional fixation was used.

When the implanting surgery was done, muscles, fascia, and skin were closed separately in routine ways.

To minimize post-operative discomfort a protective bandage was applied for 5 days, buprenorphine (30µg/kg q8h for 2 days), carprofen (4 mg/kg q24h for 4 days) and metoclopramide (0,2 mg/kg q8h for 2 days)were administered after surgery. Antibiotic prophylaxis was performed with enrofloxacin 10 mg/kg q24h for 7 days. The rabbits were kept in single cages and observed for signs of infections, pain and proper activity. For each group of animals at 4, 8, 12 and 24 weeks post implantation euthanasia was performed with an overdose of pentobarbital sodium.

Surgical skeletization of treated limbs was immediately performed, and the regeneration site was macroscopically evaluated in order to assess the characteristics of peri-implant tissues and newly formed bones. Specimens of 40mm were explanted (scaffold, 10-mm proximal and distal radius bone and ulna) and put in 4% buffered formalin solution.

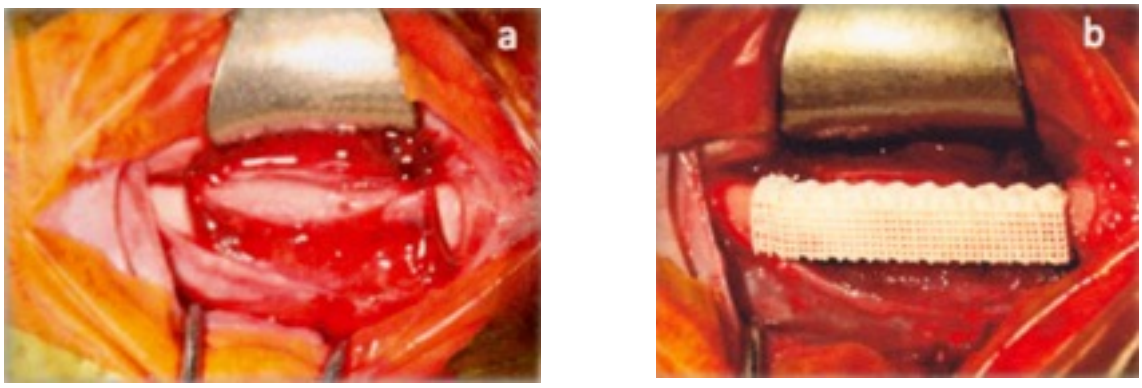


fig.9: a) segmental defect b) implanted scaffold

7.3 Radiological evaluation

For each animal medio-lateral and antero-posterior X-rays of treated forelimbs were executed under general anaesthesia every 4 weeks to evaluate the phases of organization of the callus, and its progression. A first examination was performed after scaffold implantation to evaluate the length of osteotomy and radiopacity of each scaffolds. Evaluation of bone evaluation was performed using a modified scoring system for defect bridging and bone formation[281] [282] [283](tab.)

Table 2: Scoring system for quantitative X-ray evaluation modified from Lane & Sandhu 1987 e Bodde et al 2007.

Scoring system point for X-ray evaluation	Points
Bone formation	
No evidence of new bone formation	0
Bone formation occupying <25% of defect	1
Bone formation occupying from 26% to 50% of defect	2
Bone formation occupying from 51% to 75% of defect	3
Bone formation occupying from 76% to 100% of defect	4
Union with host bone (for each side) (×2)	
Non-union (full fracture line)	0
Union <25%	1
Union 26%–50%	2
Union 51%–75%	3
Union >75% (absent fracture line)	4
Remodelling	
Absence of remodelling	0
Intramedullary canal	2
Full remodelling of cortex	4
Total score	16

[281] Bodde EWH et al. Closing capacity of segmental radius defects in rabbits. *J Biomed Mater Res A* 2008; 85: 206–217.

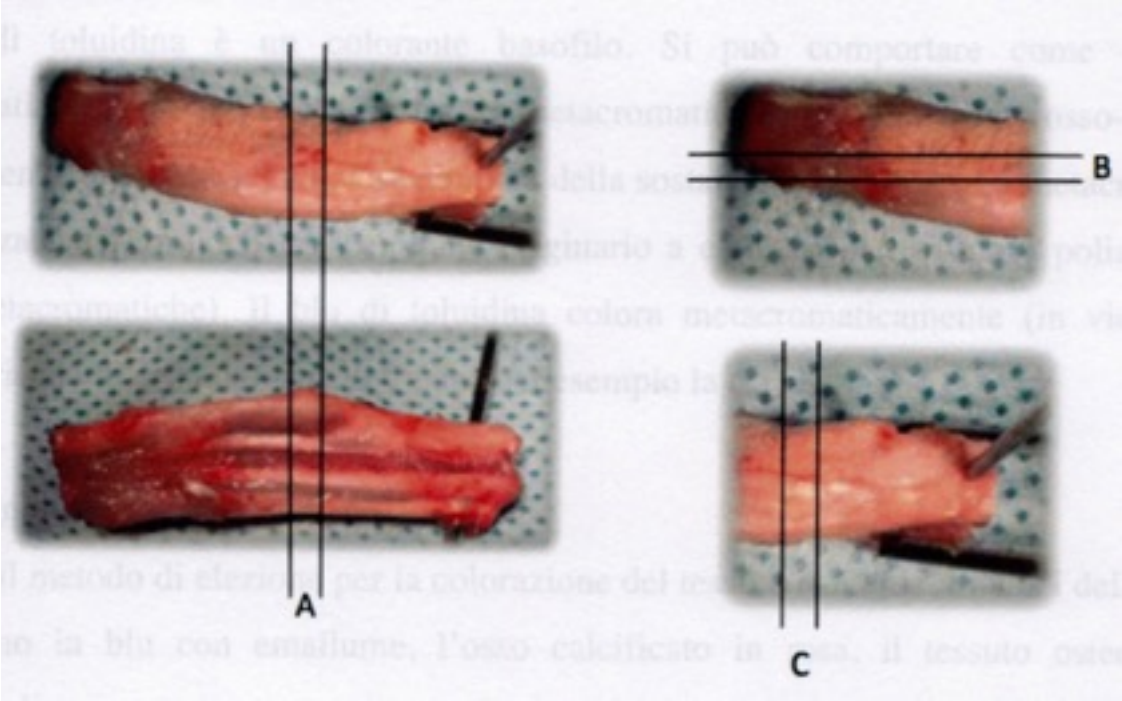
[282] Hedberg EL, et al. Methods: a comparative analysis of radi- ography, microcomputed tomography, and his- tology for bone tissue engineering. *Tissue Eng* 2005; 11: 1356–1367.

[283] Lane JM, Sandhu HS, Current approaches to experimental bone grafting, *Ortop Clin North Am* 1987; 18:213-225

7.4 Histological procedures

All the explanted specimens placed in 4% buffered formalin solution, after 48–72 h were transferred to a commercial decalcifying solution for 10-15 days or until the bone became mechanically flexible. After washing in tap water for 12 h, the specimens were cut into 3mm fragments as shown in the photos below.

Fig.10: first cross section (A), a second longitudinal section (B), third cross section (C).



Each fragment was processed for paraffin embedding, cut in the microtome at thicknesses varying from 4 to 6 μm and mounted on a microscope slide, and stained with haematoxylin–eosin, Mallory trichrome, toluidine blue and Congo red

7.5 Results

7.5.1 *Macroscopical evaluation.*

All rabbits showed a good and quick recovery of forelimb function without complications. Only one animal exhibited signs of discomfort in the first 24h after surgery, showing resting on the back of the phalanges, self-trauma and mutilation of a phalanx of the treated limb. This complication was ascribed to radial nerve edema post nerve block and treated with systemic gluco- corticoid (methylprednisolone hemisuccinate 1 mg/kg q12h for 4 days and after q24h for other 4 days) with complete and quick recovery of function of the limb. It was not excluded from the study.

Fig. 11: Functional recovery 15 days after-surgery

7.5.2 Radiographs.

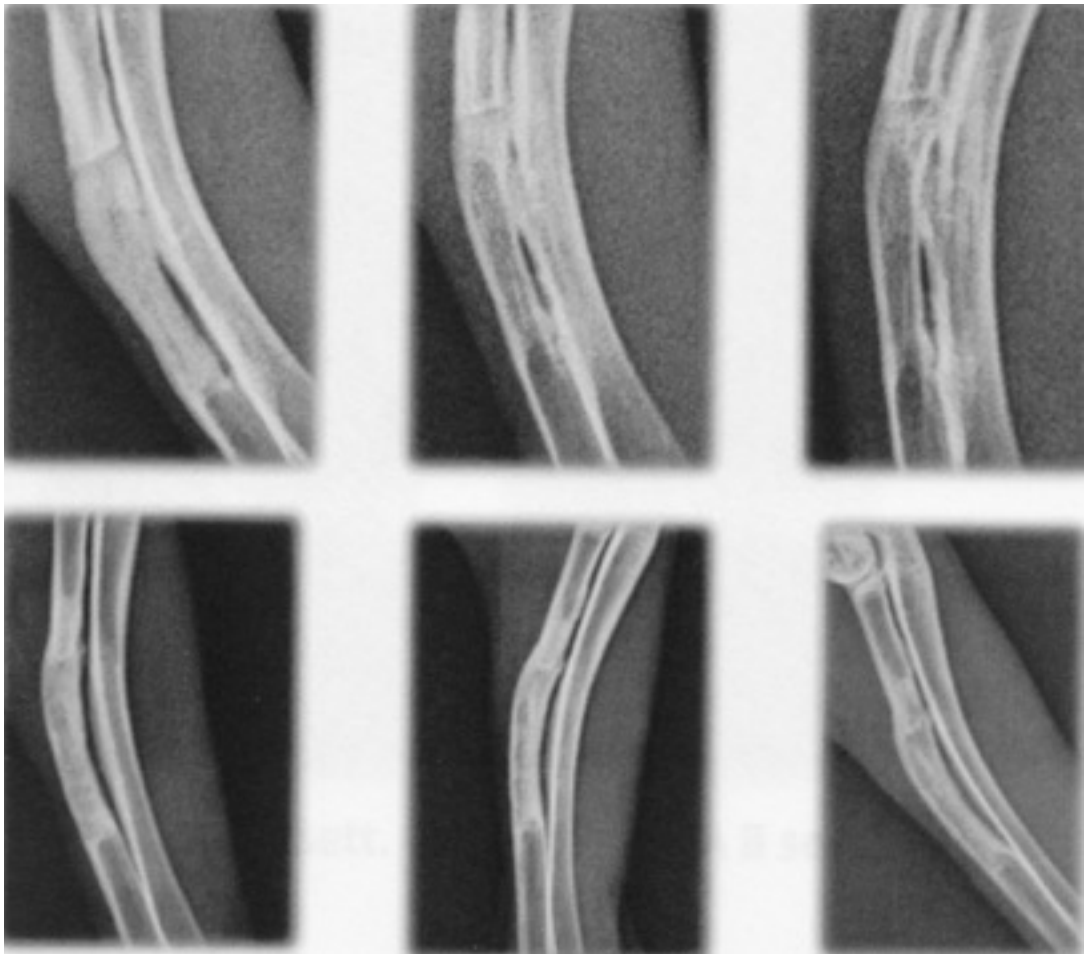
The X-rays performed immediately after surgery confirmed scaffolds radiotransparency and critical size defects (20,73 ± 0.89 mm).

The X-rays performed before the removal of the scaffolds are described below:

- PCL 4w group. In only one subject signs of bone remodelling were detected, two animals showed new bone area percentages covering the scaffold between 25-50%, three animals between 50-75%, only one 75-100%. The mean total score was 7.75 (±3.8).
- PCL-HA 4w group. In nobody signs of bone remodelling were detected, one animal showed new bone area percentages covering the scaffold between 25-50%, three animals between 50-75%, two 75-100%. The mean total score was 7.41 (±1.39).
- PCL 8w group. In two subjects signs of bone remodelling were detected, one animal showed new bone area percentages covering the scaffold lower than 25%, one between 25-50%, three between 50-75%, one 75-100%. The mean total score was 9.33 (±2.71).
- PCL-HA 8w group.In two subjects signs of bone remodelling were detected, one animal showed new bone area percentages covering the scaffold between 25-50%, three between 50-75%, two 75-100%.The mean total score was 9.58 (±2.45).
- PCL 12w group. Bone remodelling was evident in one animal, one animal showed new bone area percentages covering the scaffold lower than 25%, two 25-50%, one 50-75%, two 75-100%. The mean total score was 7.66 (±4.72).

- PCL-HA 12w group. Signs of bone remodelling were detected in two animals. One animal showed new bone area percentages covering the scaffold lower than 25%, one 25-50%, four 75-100%. The mean total score was 10.08 (±3.92).
- PCL 24w group. A good bone regeneration was detected in five animals. The mean total score was 12.6 (±4.5). Only one animal showed poor regeneration with a total score of 3.5. with a mean total score of 12.

Fig. 12: Radiographs showing the evolution of bone regeneration at 4, 8 and



12 weeks.

7.6 tissue explants evaluation

7.6.1 *Visual assessment.*

Inflammatory reaction signs were not macroscopically evident in tissues around the scaffolds in all treated rabbits. A newly formed bone was observed , on the lateral side of the scaffold near the ulna in all cases in which high and medium radiological scores were present.

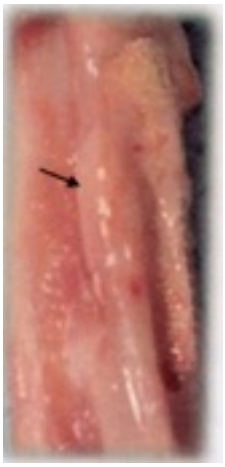


Fig.13: newly formed bone on the lateral side of the scaffold near the ulna (arrow: →)

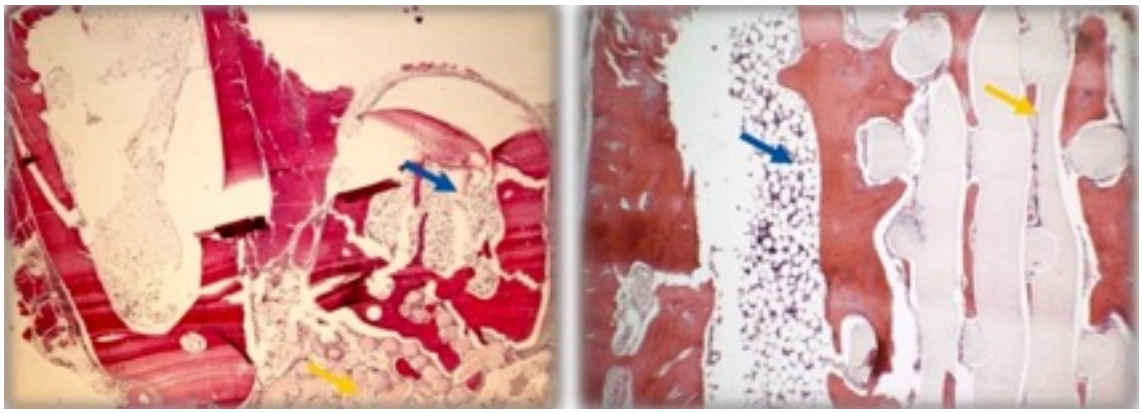
7.6.2 Histology.

Histological examination confirmed radiological observations, without significant differences between all PCL and PCL-HA groups. bone

Samples with a medium and high radiological score, had the surgical gap filled with a new bone segment that appeared either as a bridge from ulna to the junction between radius and the scaffold or as ossification cores between the fibres of the scaffold. The newly formed lamellar or woven bone invaded the scaffold resembling the radius diaphysis, in most cases fused with the ulnar diaphysis forming together the specie's physiological angle (Figure 6(d)). Many small cavities surrounded by mature bone confluent to a medullary cavity were observed. These cavities were sometimes occupied by scaffold fibres, fat cells and mononucleated elements such as bone marrow cells (Figure 6(d) to (f)). The bone tissue never completely invaded the scaffold but only the lateral edge of the scaffold, near the ulna.The remaining part of the scaffold appeared rich in fibrous connective tissue.

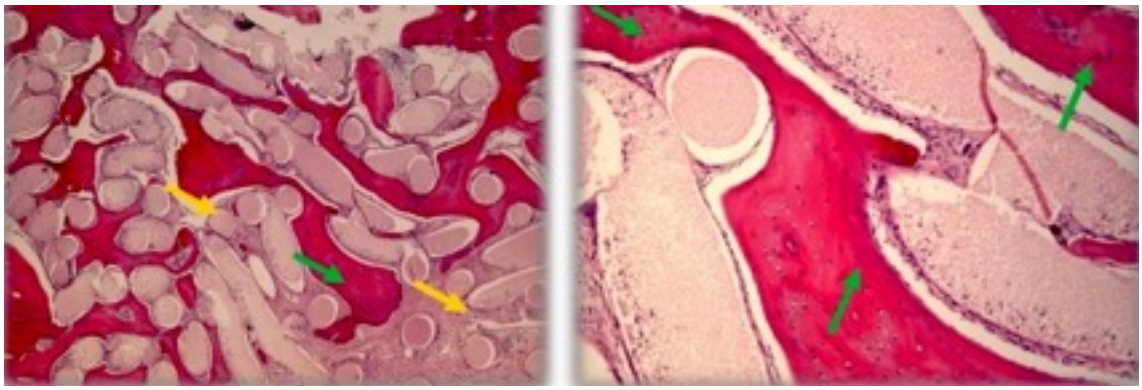
Samples with a low radiological score showed small quantities of newly formed bone tissue never filling the surgical gap; connective or granulation tissue and inflammatory infiltrate and fat cells were present between the scaffold fibres.

Fig.14: **Photomicrographs of histological samples (**Haematoxylin–eosin-stained)



A) longitudinal section B) transversal section

yellow arrow indicates scaffold fibres., blue arrow indicates medullary cavity

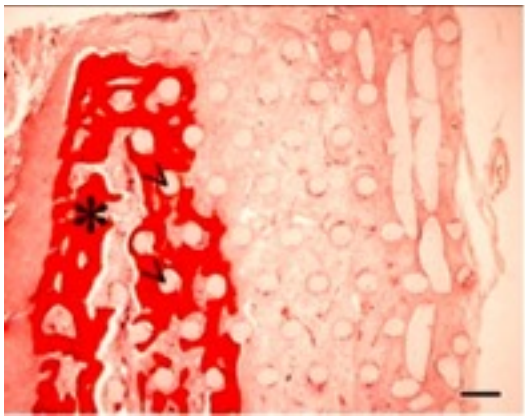


C) newly formed bone between scaffold's fibres D) lamellar or woven bone

yellow arrows: scaffold's fibres, green arrows: newly formed bone

Fig.15: **Photomicrographs of histological samples**

A)

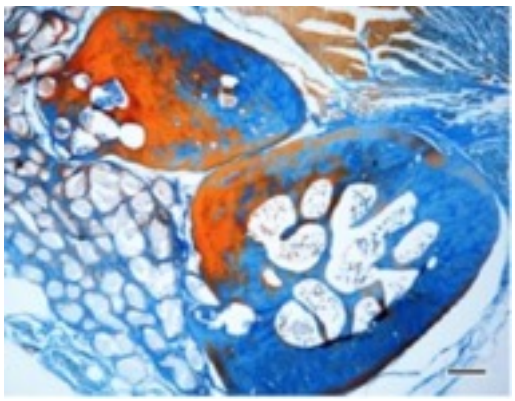


B)

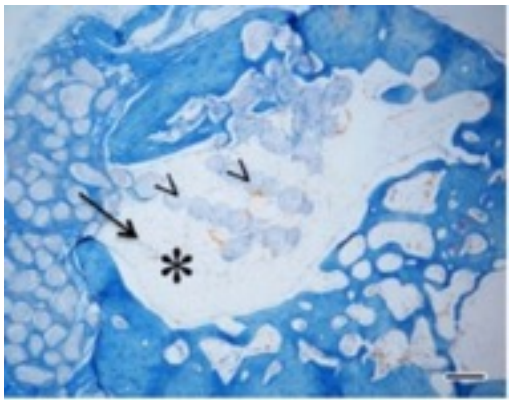
Haematoxylin–eosin-stained longitudinal section at 8 weeks.

A. mononucleated inflammatory infiltrate (arrowheads: >) and fat cells (arrows: →) among scaffold fibres. Asterisks (*) indicate the ulnar diaphysis.

B. bone tissue (asterisk*) growing into the implanted scaffold (arrowheads >).



C)



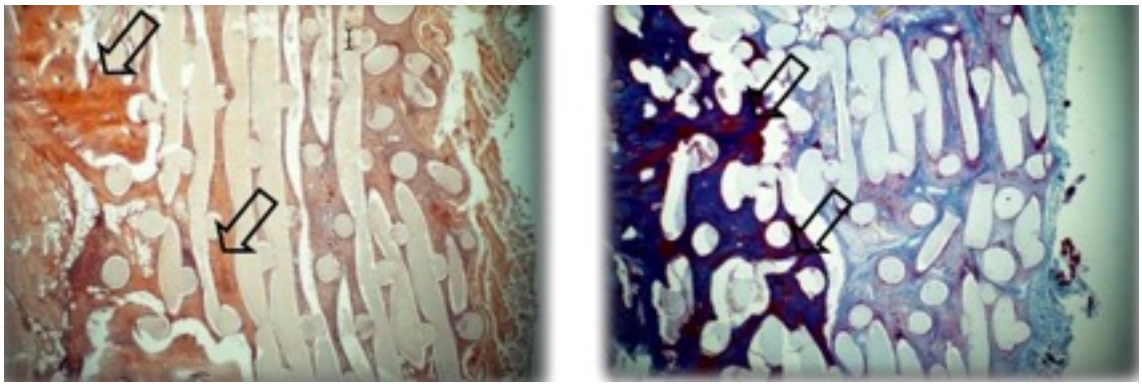
D)

Mallory–stained transversal section at 24 weeks.

C. Two bone outlines instead of a single diaphysis;

D. Medullary cavity (asterisk: *) of the neoformed radius occupied by scaffold fibres (arrowheads: >) and fat cells (arrow: →)

Fig.16: **Photomicrographs of histological samples**



A) Congo Red Staining B) Mallory–stained

bone tissue growing into the implanted scaffold

8. DISCUSSION

Tissue engineering refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues. Its goal is to assemble functional constructs that restore, maintain, or improve damaged tissues or whole organs.

It is included in a broad field called *Regenerative medicine* that also incorporates research on self-healing ,where the body uses its own systems, sometimes with help foreign biological material, to recreate cells and rebuild tissues and organs.

In clinical practice the terms “tissue engineering” and “regenerative medicine” have become largely interchangeable.

In addition to medical applications, in this field are included non-therapeutic applications as the use of biosensors to detect biological or chemical threat agents, and tissue chips to test the toxicity of an experimental medication.

In this study we have performed a review of the state of art in the field of tissue engineering and regenerative medicine, starting from history until the new experience with biomaterials.

We have also reported a clinical experience with biocompatible and biodegradable scaffolds made of star poly(ε-caprolactone) (PCL)or star poly(ε-caprolactone)– hydroxyapatite (PCL-HA) composite material.

Poly(ε-caprolactone) is an aliphatic linear polyester susceptible to auto-catalyzed bulk hydrolysis. Its degradation is significantly slow (years), due to its semi-crystalline nature and hydrophobicity. PCL is therefore most suitable for the design of long-term, implantable systems.

Hydroxyapatite (HA) is a synthetic ceramic similar to the natural bone apatite, the main component of bone matrix. HA is defined as bioactive for its capacity to support bone growth and osteointegration of orthopaedic, dental or maxillofacial implants and it is degraded in vivo through an osteoclast-mediated mechanism by simultaneous resorption and phagocytosis.

Studies on bone tissue engineering have reported the development of composite materials by means of inclusion of HA particles in a biodegradable polymeric matrix in order to improve scaffold mechanical properties and increase the osteoconductivity. In addition, the inclusion of HA particles create a basic pH that can contrast the products of the acid degradation of the polymer matrix.

In this study New Zeland white rabbits have been used because they provide a good animal model for bone healing studies; its bone has Haversian system similar to human.

In this experience has been created a defect in the radial diaphysis of New Zealand white rabbits that represents a critical size bone defect. Its size (20 mm) is greater than two times the diameter of the radius segment and studies regarding bone regeneration in radial diaphysis of New Zealand white rabbit demonstrated that no-treated 15-mm-long bone gaps did not show signs of regeneration.

The X-ray data reported in this study showed that in all groups with a medium and high radiological score (from 4 to 24 w), bone regeneration along the scaffold was present, without differences in radiological scoring results with the two types of scaffolds (PCL and PCL-HA). In all these groups signs of bone remodelling were detected with different new bone area percentages covering the scaffold and a significant mean total score.

Histological examination confirmed radiological observations, without significant differences between all PCL and PCL-HA groups.

Samples with a medium and high radiological score, had the surgical gap filled with a new bone segment that appeared either as a bridge from ulna to the junction between radius and the scaffold or as ossification cores between the fibres of the scaffold. The newly formed lamellar or woven bone invaded the scaffold resembling the radius diaphysis, in most cases fused with the ulnar diaphysis forming together the specie's physiological angle. Many small cavities surrounded by mature bone confluent to a medullary cavity were observed. These cavities were sometimes occupied by scaffold fibres, fat cells and mononucleated elements such as bone marrow cells. The bone tissue never completely invaded the scaffold but only the lateral edge of the scaffold, near the ulna.The remaining part of the scaffold appeared rich in fibrous connective tissue. The percentage of visible scaffold depended on the degree of bone regeneration, but a part of it was always evident, also in those animals in which bone bridging was complete, including rabbits of PCL 24w group. Therefore, we can deduce that the bone regeneration process requires longer time to be completely accomplished and that a scaffold is still needed 24 weeks after implantation to guide tissue growth and fill the defect gap.

Samples with a low radiological score showed small quantities of newly formed bone tissue never filling the surgical gap; connective or granulation tissue and inflammatory infiltrate and fat cells were present between the scaffold fibres.

In this study PCL-HA scaffolds showed no significant differences in terms of bioactivity in comparison to PCL scaffolds. These results confirm an in vitro investigation showing that HA loading did not significantly improve enhancement of proliferation, differentiation and extracellular matrix production of preosteoblast cells grown on PCL-based scaffolds by computer-aided wet-spinning.

A possible explanation for these results could be that the wet-spinning process does not allow a proper concentration of HA on the outer surface of the fibres to achieve an enhancement of scaffold osteoconductivity.

9.CONCLUSION

Surgical treatment of major pathology includes transplantation of tissue from one site to another in the same patient (autograft) or from one individual to another (transplant or allograft). These procedures have been revolutionary, but problems exist with both techniques.

The field of tissue engineering and regenerative medicine aims to regenerate damaged tissues, instead of replacing them, by developing biological substitutes that restore, maintain or improve tissue function.

In this study we have performed a review of the state of art in this field, starting from history until the new experience with biomaterials, describing natural and synthetic materials and scaffolds production techniques, focusing in medical fields of application such as bone tissue, blood vessel, neural tissue engineering.

We have also reported an experience in the use of PCL scaffolds in bone tissue repairing.

This study has confirmed the biocompatibility of PCL scaffolds. No signs of local or systemic toxicity were detected in the site of implant and no foreign body reaction was found. Histological examination of samples confirmed the absence of necrosis, inflammation or fibrosis areas.

The radiological and histological evidence of newly formed bone tissue penetrating into the scaffolds structure validates the osteoconductivity of both tested scaffolds. Osteoinductivity may have been influenced by the close proximity between radius and ulna (the regenerated bone was mainly evident on the lateral site, near the ulna where a periosteal activation loading to join the newly formed bone with the ulna was observed.

This study confirm the possibility to use scaffolds as an alternative to standard bone grafts for the treatment of bone critical size defects. Bone tissue engineering, but also blood vessel, neural tissue and other fields of tissue engineering are promising areas of research for a new therapeutic approach to the treatment of traumatic and degenerative pathologies.

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