DESIGN, DEVELOPMENT AND CHARACTERIZATION OF NOVEL BIOMATERIALS FOR PERIODONTAL TISSUE ENGINEERING

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DESIGN, DEVELOPMENT AND CHARACTERIZATION OF NOVEL BIOMATERIALS FOR PERIODONTAL TISSUE ENGINEERING

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CHAPTER I

INTRODUCTION

1.1 DENTAL ANATOMY

In order to design and to develop biomaterials substitutes for the replacement of dental and bone tissues, it is mandatory to understand how the tooth and the surrounding tissue, which supports and stabilizes it, are composed. Craniofacial and, in particular, dental and mandibular tissues are very interesting and complicate to replicate, since they are a combination of hard and soft tissues that have to communicate and work together.

1.1.1 Tooth development

Tooth development, namely odontogenesis, is a complex process that occurs in the 6th week of embryonic life and continues to 15th year of birth, when the roots of the permanent 3rd molars reach their completion[1].Tooth development involves a series of interactions, and differentiations of epithelial cells, from the mucosal lining of the oral cavity, and ectomesenchimal cells, which originate at the ectodermal junction of the developing brain[2–4]. These cells instruct the overlying ectoderm to start tooth development, which begins in the anterior portion of the future maxilla and proceeds posteriorly[2,5,6]. The early stage of tooth formation involves three sequential phases: bud stage, cap stage and bell stage[5,7]. Briefly, in the cap stage, tooth formation starts with formation of the dental lamina. The localized proliferation of cells in dental lamina forms oval swellings, the tooth bud which grows into the mesenchyme, the focal bud-like thickenings determine the site of the future teeth (20 for the deciduous teeth and 32 for the permanent one)[1,6]. The deep surface of each ectodermal tooth bud becomes invaginated and the ectomesenchymal cells which are within this concavity form the dental papilla that will become the soft tissue core of the tooth, the dental pulp. The ectodermal, cap-shaped covering over the papilla is called enamel organ since it will produce the future enamel of the teeth. The outer cellular layer of the ectodermal enamel organ is named outer enamel epithelium, the inner layer coating the cap is the inner enamel epithelium, and the cell region between the above layers forms the bulk of the cap and is called the stellate reticulum[3,8,9]. As the enamel organ and the dental papilla form, the surrounding condenses to become the dental sac, which lathers forms the cementum and periodontal ligament[5]. The formation

of a concavity along the inner surface and the continuous growth of the tissue of the cap leads to bell stage. In this phase the inner enamel epithelium develops into enamel-forming ameloblasts, while dental papilla cells differentiate into odontoblasts, which produce predentin and deposit it adjacent to the inner enamel epithelium. The predentin is a matrix of collagen fibers, that subsequently calcifies to become dentin. Dentin has a tubular structure, due to cytoplasmic processes of single odontoblasts, which recede from the dentine-enamel junction and leave behind a cytoplasmatic extension in the deposited dentinal matrix. Whereas the development of dental pulp cells occurs after a stimulus of the inner enamel epithelium, differentiation of the inner enamel epithelium in ameloblasts occurs only after dentin deposition[7,9,10]. After a tiny layer of dentin is formed, ameloblasts produce enamel in form of prisms or rods over the dentin layer, thus helping to form the outer layer of the tooth, namely the crown. The enamel matrix is a high mineralized tissue that, approximately, consists of 95% of minerals. Meanwhile odontoblasts form dentin and ameloblasts form enamel, inner and outer enamel epithelia cells proliferate together in the neck region to surround the dental papillary part, forming the so called epithelial root sheath, or Hertwig epithelial root sheath[11]. In this region, the inner enamel epithelium stimulates dental pulp cells to differentiate into odontoblasts instead of ameloblasts, in order to generate the dentin of the root[3,4,7,12]. As the dentin increases, the pulp cavity becomes smaller and a tight canal for vessels and nerves is formed at the bottom of the root.

After formation of dentin in the root region, root sheath undergoes disintegration, allowing ectomesenchymal cell penetration from the dental sac to the root surface, which will differentiate into cementoid-depositing cementoblasts over the root dentin, converting it into calcified cementum[2,13,14]. As the teeth develop, jaws ossify and ectomesenchymal cells from dental follicle become active in bone and in other periodontal tissues formation. Some cells from dental follicle differentiate into periodontal fibroblasts, which will form the periodontal ligament, while others become osteoblasts involved in alveolar bone development in which fibers of the periodontal ligament, will get anchored ensuring tooth stability[15,16]. It has been hypothesized, even if not yet proved, that ectomesenchymal cells remain in the mature periodontium and take part in the tissue turnover [17].

The subsequent crucial event is represented by the completed tooth crown eruption into the oral cavity; as the tooth erupts and passes through the oral epithelium, the incisal part of the reduced dental epithelium is destroyed, but the present epithelium interacts with oral epithelium to become the junctional epithelium, and the tooth reach the final position[10]. Deciduous teeth usually erupt between the 6th and 24th months after birth, indeed permanent teeth develop later, but in a similar manner to the deciduous one and, as they grow, the root of the corresponding deciduous tooth is

resorbed by osteoclasts[10]. Permanent teeth eruption usually starts during the sixth year of life and keeps going on until early adulthood.

1.1.2 The components of tooth

The tooth is divided in two zones, the crown and the root. The upper part, exposed to the mouth environment, is the crown, while the root is usually embedded within the bone.

There are four tissues that make up a tooth: enamel, dentin, cementum (hard tissue) and pulp (soft tissue).

The *Enamel* constitutes the outer surface of crown, it is formed by ameloblast and its structure is the hardest in the body[18]. Thus, it makes the tooth able to withstand cyclic and high stresses, the chewing process and changes in pressure and temperature. The enamel does not have the ability to regenerate itself, or to further grow after it is completely formed; however, it does have the ability to re-mineralize, allowing the tooth structure to be protected, to regain minerals and to stop caries, if proper nutrition and oral cares are followed[18–20]. Furthermore, the enamel covers the second tissue that makes teeth, the *dentin,* which is the most abundant component of the tooth[5,21,22]. The dentin is softer than the enamel, but harder than bone, because it is composed by microscopic canals named dentinal tubules, which contain dentinal fibers. These fibers, confer mechanical strength and transmit stimuli and nutrition throughout the tissues[23]. The dentin is formed by odontoblast cells and, unlike enamel, it has the ability to grow during life. For this reason, three types of dentin exist: primary dentin is the tissue that forms when a tooth erupts, secondary dentin is the result of primary dentin growth and the third type, named reparative dentin, is formed as a response to irritation and trauma (erosion or caries)[21,22,24,25]. The root of tooth is covered by a tissue that is not as hard as enamel or dentin, but it is still harder than bone: it is the *Cementum.* It contains attachment fibers that anchor the tooth at the bone, so its role at the interface with alveolar bone is crucial in order to give stability to the tooth[26,27]. The cementum could be divided in primary cementum, which covers the entire length of the root and does have the ability to grow and cellular cementum, which continues to form on the apical half of the root. The inner tooth tissue is the *pulp,* a soft tissue located in the center of tooth, surrounded by dentin. The pulp composition includes connective tissue, blood vessels, lymph vessels and nerve tissue; which are responsible for signal transmission of pain and sensitivity; and dentin-producing cells, having a role in repairing structural damages[10,28]. The pulp tissue is retained in two zones in the pulp chamber, which are the crown and the pulp canals located in the root; the amount of pulp tissue decreases with the growth of dentin[1].

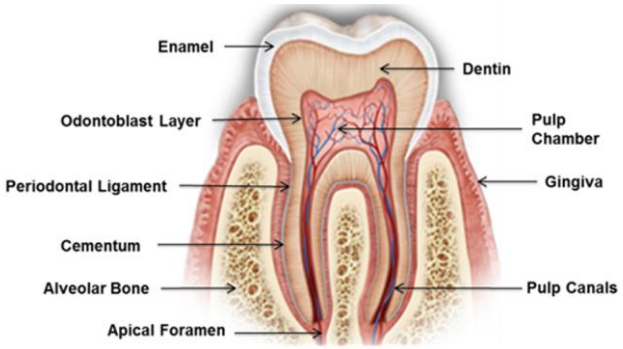

Figure 1. Dental anatomy[29]**.**

1.1.3 The components of periodontium

The Periodontium is a combination of hard and soft tissue and its principle function is to anchor the tooth to the jaw bone tissue. It is formed by two units, the **gingival** and the **attachment unit**[8,30]. Gingival unit is formed by two different tissues, *gingiva* which is a soft tissue that surrounds the teeth and the *alveolar mucosa*, which consists of the soft tissue that constitutes cheeks, lips, soft palate and the underside of tongue. The gingiva is steadily bound to the underlying bone and it is connected with the alveolar mucosa by the mucogingival junction. The gingiva consists of a free gingiva that is unattached to the underlying bone and an attached gingiva, that extends from the base of the free gingiva to the mucogingival junction[30–33]. A crucial role is played by the cemento-enamel junction, also called epithelium junction, since it seals off the periodontal tissue from the oral cavity. A healthy periodontium depends on the integrity of this junction, in fact a failure of the epithelium junction is the starting point of many periodontal pathologies [30,34,35].

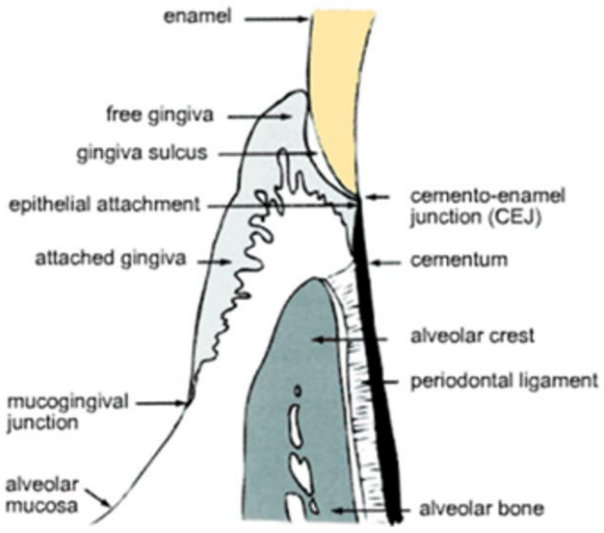

Figure 2. Periodontium structure[36]**.**

The attachment unit is composed by the *cementum*, the *periodontal ligament* and the *alveolar process*. *Cementum* is a biphasic material, composed of about 50% mineral substituted apatite and 50% organic matrix, which contains around 90% collagen type I, and trace of other collagens (III, V, VI,XII, XIV) and non-collagenous proteins (such as bone sialoprotein, dentin matrix protein 1, dentin sialoprotein, fibronectin, osteocalcin, osteonectin, osteopontin, tenascin)[30,37]. This hard, avascular connective tissue that covers roots of teeth, serves primarily to attach the periodontal ligament fibers, and could be divided in two different types: the acellular extrinsic fiber cementum, also named primary cementum[8], and the cellular intrinsic fiber cementum. Most of the periodontal ligament fibers are inserted in the primary cementum, which is highly mineralized and develops very slowly, because it is considered acellular since cells that form it remain on the surface[30,33]. The cellular intrinsic fiber cementum is less mineralized and with cementoblast cells entrapped in the lacunae within the matrix that they produce[30]. The cellular intrinsic fiber cementum is produced as a repair tissue for root fractures and to fill resorptive defects.

Concerning *periodontal ligament*, it is a soft tissue consisting of specialized connective tissue, which ranges in width between 0.15 and 0.38 mm, with a decrease of thickness with aging[8,30,38]. Its principal function is to support teeth in their socket and, at the same time, to permit them to withstand the considerable forces of mastication[30,33,39]. Furthermore, the periodontal ligament could act as a sensor for the positioning of jaws and, most important function, is that it is a reservoir of cells for tissue regeneration[30].

The *alveolar process* is the portion of the maxilla and mandible that forms and supports the tooth socket (alveoli). It forms when the tooth erupts to provide the osseous attachment to the forming periodontal ligament. It consist of an external plate of cortical bone formed by Haversian bone and compacted bone lamellae. The inner socket wall of thin, compact bone called the alveolar bone, contains a series of openings through which neurovascular boundless link the periodontal ligament with the central component of the alveolar bone and the cancellous bone and trabeculae, between these two compact layers, which acts as supporting alveolar bone[30,40].

1.2 TISSUE ENGINEERING CONCEPT

1.2.1 The History of Tissue Engineering

Tissue engineering can be defined as the use of a combination of cells, engineering materials, and suitable biochemical factors to improve or replace biological functions in an effort to improve clinical procedures for the repair of damaged tissues and organs. The first definition of tissue engineering is attributed to *Drs. Langer* and *Vacanti* who stated it to be "*an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ*" in the 1993[41].

The ambitious objective of tissue engineering is to create functional constructs able to restore, maintain and improve the damaged tissues or whole organs. Since ancient times, artificial creation of tissues and organs was a dream and a desire of human mankind. The first historical reference to tissue engineering, is usually referred to the famous painting "Healing of Justinian" by Fra Angelico, which depicts the brothers Saints Damien and Cosmos transplanting a homograft limb onto a wounded soldier (278 AD). Many others examples that could be referred to the field of earlier tissue engineering could be find in history, from both literature and arts, which reveal the desire of human to create by himself living individuals or, at least, parts of them. In the early 1500, Paracelsus, a Swiss physician, alchemist and astrologer, tried to find a formula to create life starting from a mixture of chemical substances closed in a defined environment. Two hundred years later, Johann Wolfang von Goethe, in his literature's work "*Faust*", posed as central theme the desire to create life as a mean to be powerful. The creation of the Homunculus in *Faust* drama, could be considered a precursor of the modern technology, such as cloning, genetic or stem cell technique. Besides literature, art and mythological reports, various people performed pioneering works to replace body parts combining materials, usually metallic, and the first knowledge in clinical medicine and biology. The first example of tissue substitution, was in dentistry[42]. Since the Galileo-Roman period, it was quite usual to substitute lost teeth or parts of skull with metallic implants or with homologous transplantation, as it is detailed in Ambroise Parè's work published in the 1564 "*Dix livres de la chirurgie*", where he provided information about instrumentations, measures and materials needed to reconstruct teeth and maxillofacial parts[43]. It was in the middle of 18th Century that, in parallel with clinical studies animal experiments began. The first to perform studies on the fate of transplantation using animals was John Hunter, posing the basis for the future transplantation medicine[44]. But hundred years had to be passed for the first successful transplantation, performed by Heinrich Christian Bünger, who transplanted the first skin graft within 1817[45]. He used the method to restore the nose of a woman who had suffered for fifteen years from a skin eruption that

caused the loss of her nose and affected other areas of the face so that a forehead or cheek graft was inadvisable. Christian Heinrich Bünger chose as a donor site the superior lateral surface of the upper thigh, taking an oval piece of skin which he trimmed to shape and applied to the freshened nasal region one and a half hour after removal, a delay caused by attempts to stanch excessive bleeding in the freshened area. The graft was partially successful and an year later other procedures were carried out on the upper lip and nose with a flap from the arm. A new era was being born, more related to science and experimental proofs, even more the link between medicine and engineering technique became strong, in particular between dental technique and clinical medicine which led the formation of a Maxillofacial and Plastic Facial Surgery discipline at the Westdeutsche Kieferklinik in Düsseldorf. Furthermore, the darkness time during the First and Second War was, from the side of the early tissue engineering, a huge field for generate knowledge and experience.

The milestone breakthrough was reached when scientists understood that the tissue regeneration is dependent on the cell proliferation, in particular the first reference could be attributed to Rudolf Virchow, in his publication "*Zellularpathologie*". But the art became science, in 1950s when R.G. Harrison first, maintained frog neural tissue outside of the body for weeks, and it was in those years that investigators understood the requirements of cells in order to maintain the viability, avoiding particular bacterial contamination with the addition of antibiotics[46].

This long history, started thousand years ago, brought to the recent and first definition of tissue engineering that, in 1987, set as fundamental the relationship between tissue and substitute, a cooperation between biology and engineering to create or restore human tissues[47]. The concept of "scaffold" as a substrate to culture and grow cells, started to be the basis concept of this new agreed multidisciplinary science. Key point in tissue engineering was given by a co-working between Boston Children's Hospital and MIT, in particular between Dr. Joseph Vacanti and Prof. Robert Langer, who generated skin grafts from a culture of dermal fibroblasts and keratinocytes grown on protein scaffolds, and used it for regeneration of burn wounds [48]. Their article in Science[41] (1993), could be considered as the beginning of a new biomedical discipline and they could be considered as the founders of modern Tissue engineering, which is supported on three pillars, the three paradigms of tissue engineering: c*ells, matrix(scaffold materials) and regulators* [41,49,50].

Cells are the building blocks of tissues, and tissues are the basic units of function in the body. Generally, groups of cells make and secrete their own support structure, the so called extra-cellular matrix (ECM). This matrix, acts as a support for cells, furthermore it receives messages in form of signaling molecules from many sources that constitute the local environment (ECM). Each signal can start a chain of responses that determine what happens to the cell. By understanding how individual cells respond to signals, interact with the ECM, and organize into tissues and organs,

researchers have been able to manipulate these processes to restore damaged tissues or even create new ones.

The process often begins with building a from a wide set of possible sources, from naturals (e.g. proteins) to synthetics (e.g. polymers, ceramics). Once scaffolds are created, cells with or without regulators, called growth factors, can be introduced. If the environment is right, a tissue develops.

1.2.2 The Three Paradigms of the Tissue Engineering

Tissue engineering is an interdisciplinary field where biology and engineering are combined together to develop substitutes that could replace, maintain and eventually improve the damaged functions of human tissues. Unlike drug therapy or organ transplantation therapy, tissue engineering approach provides implants specifically designed to treat the disease state, trying to avoid complications due to rejection, immune response or systemic drug therapy issues. Tissue engineering, is based on the use of a combination of cells, engineered materials and methods, as well as suitable biochemical and physio-chemical factors to improve or replace biological function of the tissue[41,50,51]. There are two approaches in order to generate a substrate that allows the tissue regeneration:

- Development and growth of human tissues *in vitro*, for future implantation in the body to replace tissue loss;
- *In vivo* implantation of cell-free devices or three dimensional constructs with cells, in order to induce regeneration of tissue loss directly in the site of implantation.

The ideal approach of tissue engineering is to first isolate cell samples through a biopsy in the patient, to subsequently seed and culture them on a 3D scaffold under specific and controlled accompanied conditions, until a new tissue layer is formed and the scaffold is degraded and, finally, to implant the novel tissue into the wound.

Since tissue formation results from cellular action, *cell paradigm* is a key factor aimed at regenerating tissues. In particular, three different strategies are commonly adopted in order to generate a new tissue: cell injection, cell induction and cell-seeded scaffolds[52]. Cell injection approach involves injection of scaffold cell-free in the defect site[53]. Unfortunately, this kind of strategy shows limited effects, since a low engraftment and an inadequate localization of the injected cells, in particular in tissue characterized by a continuous movement (such as cardiac tissue). In order to improve this approach, cells have to be injected using a carrier which acts as vehicle. This improvement allows an adequate localization, the prevention of a direct contact with the immune system and a proper cell proliferation and differentiation[53,54]. Due to their characteristics, stem cells are becoming an important tool of tissue engineering and are the most

successful candidate for cell injection approach[55]. Stem cells could be divided, according to their potency in: totipotent (a single cell divides and produces all of the differentiated cells in an organism), pluripotent (cells that have the potency to differentiate into any of the three germ layers: endoderm, mesoderm, and ectoderm), multipotent (cells that have the potential to differentiate into multiple, but limited cell types), oligopotent (potency to differentiate into a few cell types) and unipotent cells (one stem cell has the ability to differentiate in only one cell type)[56]. Stem cells can be divided into different types of cells, according to their potential ability, and could be classified as Embryonic stem cells (totipotent, pluripotent) and adult stem cells (multipotent, unipotent)[55]. Embryonic stem cells (ESCs) are derived from the 5-7 days old blastocyst, prior to germ layer formation and, as such, ESCs are thought to be pluripotent and to give rise to progenitor cells from all of three germ layers. The clinical application of ESCs is limited, due to ethical issues as well as their enhanced possibility to form teratomas after implantation[57]. Adult stem cells can be found in adults and are thought to be tissue specific[58]. For example, bone marrow contains mesenchymal stem cells (MSCs) that are capable of differentiating into cartilage (chondrocytes), bone (osteocytes), muscle (myoblast), tendon (fibroblast) and other connective tissues.

The second strategy, cell induction therapy, involves the use of an engineered *matrix* (biomaterial) that induces tissue regeneration without the use of any exogenous biological factor. In fact, a biomaterial should mimic the ECM, should serve as a compatible interface with the biological system and promote cell functions such as cell adhesion, migration, proliferation, and differentiation[59,60]. Furthermore, the designed material should avoid an immune system response. The structural, chemical and mechanical properties of biomaterials are also important, as they could influence cell functions and the foreign body response *in vivo.* Moreover, an applied biomaterial should maintain the shape of the defect and prevent distortion of surrounding tissues. Biocompatibility, adequate mechanical property, biodegradability, porosity and inter pores connectivity, depot for sustained release of biomolecules (e.g. growth factor) and cells as well as post process and sterilizing ability, are the fundamental characteristics that a material used to induce regeneration in a wound tissue should have[51,52,61]. Usually, in cell induction therapy a biomaterial is coupled with *regulators,* since a biomaterial, on its own, is not enough able to induce stem cell differentiation and, in most cases, it is recognized as a foreign body by the immune system, which tries to isolate it[52,62,63]. Growth factors are biochemical regulators that include proteins, peptides and signaling molecules that bind to receptors residing on the cell surface, with the primary result of activation or inhibition of cellular functions such as proliferation and differentiation. Many growth factors are versatile, stimulating cellular function in numerous cell types, while others are specific to a particular cell type. A tissue engineering implant is a combination of biological cues and

biomaterials, where the biomaterial could be used as carrier for growth factor and its delivery *in vivo* enhances tissue regeneration[64–66]. The signaling molecules could be incorporated into the scaffold during or after fabrication and the degradation rate of the material, as well as the pore size and the interconnectivity control the time of releasing[67–70].

The third strategy, cell-seeded scaffold, combine all the previous described paradigms: cells, matrix and regulators[52,71]. A synergic effect of all components create an engineered tissue, which promotes tissue regeneration without immune or inflammatory responses. A complex study of all variables is needed, chemical and physical properties of biomaterial should be designed and developed for every specific tissues as well as the type of regulators and the type of cells (the most likely candidate for such therapies are the MSCs)[72]. There are a lot of concerns about this therapy, regulatory and industrialization steps are difficult to be defined, but a construct made by combination of materials, cells from a patient and growth factors, ready to be implanted, is the real goal of tissue engineering[73].

The following paragraph will focus on craniofacial tissue engineering, in particular dental and periodontal tissue engineering, with particular emphasis on the state of the art of stem cells, regulators and materials.

1.3 CRANIOFACIAL TISSUE ENGINEERING

Craniofacial region is an unique tissue composed by a multitude of parts, including bone, cartilage, nerve, blood vessels and soft tissues. This melting pot of tissues, makes it one of the most difficult part of the body to regenerate and requires biological and engineering knowledges in order to prepare a correct tissue engineered construct. Furthermore, craniofacial tissues play important physiological and anatomical roles and, in addition, have an important aesthetic function[52,74]. Many congenital defects, diseases and injuries could affect this tissue equilibrium and require specific surgical procedures and therapy in order to replace functionality[75–77]. The most used strategy in order to replace the lost tissue, is the use of autologous material, therefore the insufficient host tissue and the morbidity of the donor site result in a second surgical procedure, with economical and psychological consequences[78]. Nowadays, clinical approach involves more and more the use of engineered construct made with natural or synthetic materials, coupled with growth factors that could stimulate new tissue regeneration[59,60,79,80]. Researchers are working with even more effort, in order to create constructs using stem cells from the body of the patient and to implant a formed tissue that reduces immune system responses and inflammation. The use of stem cells is still far from a clinical use, because regulatory and practical issues are still to be clarified, but the great potential of this tool need all the efforts in order to get clinical available.

There are three different approaches for the regeneration of craniofacial region, each one different in terms of levels of complexity: conduction, induction by bioactive regulators, and cell transplantation[81]. In this research work, we focus on bone tissue engineering, which serves several important functions in the craniofacial region. In craniofacial, bone, maintains the mechanical integrity of head, protects the soft tissues and the cranial cavity. Many pathologies and injuries result in bone deficiencies, such as resorption and loss of alveolar bone, scoliosis of the mandibular arch, mandibular asymmetry and defects following removal of sinus. Gold standard strategy for bone replacement involves the use of autologous tissue from cranium, iliac crest or rib[79]. This approach is associated with some issue, such as bone resorption, problems in the harvesting process and site donor pain[82]. Furthermore, in the case of large bone defect, limited autogenous bone does not permit to fill the defect site in a properly manner. A tissue engineering approach, that involves the use of synthetic or natural materials, bioactive molecules, cells or a combination of the three, has the opportunity to conduct regeneration, without creating uneasiness to the patient. Conductive approach involves the use of an engineered natural or synthetic matrix alone[59,83–87]. Several materials have been developed and some of them are already being used for craniofacial bone regeneration, such as synthetic polymers (Poly-lactic acid, Poly(methyl metacrylate), poly(dioxane-co-glycolide), poly(propylene fumarate), PEO/PBT)[88–91], ceramic (hydroxyapatite, β-tricalcium phosphate, coralline hydroxyapatite, orto-calcium phosphate)[92–96], natural materials (collagen, chitosan, alginate, pectin, hyaluronic acid)[97–99]and combinations of them[100,101]. The implanted material in the defect site, acts as a passive three dimensional scaffold on which cells could adhere, proliferate and differentiate. In craniofacial bone tissue engineering, and in particular in periodontal regeneration, some materials are used as barrier, (guided tissue regeneration, GTR), in order to promote proliferation and infiltration of only certain type of cells[33]. For example, in alveolar bone regeneration, porous osteoconductive scaffolds and membrane barriers are usually used, in order to promote bone growth and to avoid fibroblast infiltration from the gingiva tissue, respectively[102,103]. Materials used should have a degradation rate compatible with formation of new tissue, should mechanically sustain the surrounding tissues until the new bone has replaced the matrix, produce biocompatible by-products from the degradation process, promote cell migration as well as vascular infiltration and should not stimulate an immune response.

However, it is desirable not to only have conduction of tissue growth, but also stimulation and this is the case of the induction approach. With this strategy, cells and, subsequently the tissue, are controlled and forced to migrate into the scaffold by specific regulators such as peptide, proteins, growth factor and signaling molecules. The encapsulation of growth factors into the matrix, reduces their degradation and acts as a storage, facilitating the interaction with receptors on the cell surface.

There are many growth factors and hormones used to guide osteoid matrix deposition by osteoblasts and mineralization: bone morphogenetic proteins (BMPs), basic fibroblast growth factor (bFGFs), insulin-like growth factor (IGFs), transforming growth factor β (TGF-β), platelet derived growth factor (PDGF), growth hormone (GF) and parathyroid hormone (PTH)[64,104].

The strategy involves the release of these bioactive molecules into the defect site, which can influence new bone formation through their effect on bone cell recruitment, proliferation and differentiation. Many research works, showed the effect of growth factors *in vitro* and *in vivo* on bone mineralization; for example, the BMP family members BMP-2 and BMP-7 have been shown to stimulate formation of periodontal tissue (bone and cementum), in studies in animals including rodents, dogs, and non-human primates[51,65,105,106]. Many inductive approaches are still undergoing testing and clinical trials prior to approval by the Food and Drug Administration (FDA), however the use of this approach in clinical surgery could help in the treatment of large bone defects where there is a lack of cellular and vascular invasion from the surrounding tissues, due to pathologies such as cancer.

The third strategy, namely the cell transplantation approach, could involve seeded cells (usually MSCs) into an osteoconductive scaffold that acts as carrier, in order to recruit and differentiate bone-forming cells[107]. In this technique, typically the cells are taken by a biopsy from a donor, isolated, expanded and seeded onto a scaffold. The cells adhere and migrate into the scaffold, proliferate and differentiate into specific cell lineages and form a new tissue. This construct then could be implanted in the defect site[108]. However, this technique seems to be promising, there are many issues, in particular regarding the type of cell used. The best choice are cells from the patient himself, since they do not generate any immune response, but this approach takes long time to get a construct ready to be implanted in the defect site. Cells from other sources can be more readily but have the drawback to generate immunological problem. Regulatory and practical issues make this technique nowadays far from the clinical application, but stem cells from the oral facial tissue are interesting and many research groups are working on it. There are many sources of stem cells: Dental Epithelial stem cells (EpSC), Dental follicle Precursor cells (DFPCs), Dental Follicle Stem cells (DFSC), Stem cells from Human Exfoliated deciduous Teeth (SHED), Stem cells from Apical papilla (SCAP), Dental Pulp stem cells and Periodontal stem cells[75]. These cells have great potential in the transplantation technique and the combination of osteoconductive scaffolds, growth factors and stem cells could allow, in the future, new tissue ready to be implanted to be generated and to be implied in large defects without any immunological response, with the intended outcome to generate new tissue.

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CHAPTER II

PERIODONTAL TISSUE ENGINEERING

2.1 CURRENT APPROACHES IN PERIODONTAL TISSUE ENGINEERING

The main objective of periodontal tissue engineering is to regenerate tooth's supporting tissues. Tooth loss is a possible consequence of trauma or periodontal disease, such as gingivitis, periodontitis or tissue decay. Periodontal tissue regeneration involves formation of new connective tissue (cementum and periodontal ligament) and new alveolar bone. The American Association of Oral and Maxillofacial Surgeons reported that 69% of adults aged 35 to 44 have lost at least one permanent tooth and, by age 74, almost 26% of adults have lost all of their permanent teeth[1]. More than 300.000 dental implants are placed per year, and until 2020 this number is expected to increase[2–4]. The restoration of tooth by using titanium dental implants is nowadays a quite common procedure, furthermore the rate of success of dental implant is around 98%, but despite this encouraging number the positive fate of a surgical procedure that involves an insertion of titanium screw depends on the quality and quantity of alveolar bone which is present in the extraction site[5]. In the last decades beyond hard tissue reconstruction, correction of soft tissue defects has gained increasing attention, involving prevention of advanced periodontal defects related to mucogingival anomalies, as well as satisfying the increasing aesthetic demands of patients[6].

Periodontal regeneration is one of the earliest clinical disciplines that has achieved the therapeutic application of tissue engineering-based technology[7]. Many strategies have been studied, some of them are already commercially available, while others are under clinical trial investigation. Biomaterials, growth factors and stem cells are the three elements that are involved in the treatment of periodontal disease.

Current therapy for periodontal regeneration could be divided in four main groups: conservative therapy, radicular conditioner, bone graft and bone substitutes and guide tissue regeneration, or a combination of these last two therapies.

2.1.1 Conservative therapy

Conservative therapy involves a surgical debridement of the periodontal pathogenic bacteria, mineralized deposits on the root surface and infected cementum and removal of all the containing

toxin tissue parts[8,9]. This approach is still one of the most used methods for periodontal healing, and an essential step before any type of regenerative technique is applied. This approach is based on the concept that fibroblast cells from the epithelium possess the fastest growing, so increasing by surgery the distance between epithelia layer and the wound allows slower connective tissue formation and avoid achieving the radicular surface before the osteogenic cells. The first author that reported the importance of the debridement and the morphology of the bone defect was Prichard, and his article was published in 1957 [10]. After this first publication, many other clinical studies have been developed and, the results, confirm the importance to achieve a clinical situation characterized by no inflammation and a controlled bacterial plaque, in which the periodontal tissue could find an ideal condition to completely develop its regenerative capacity[11–13].

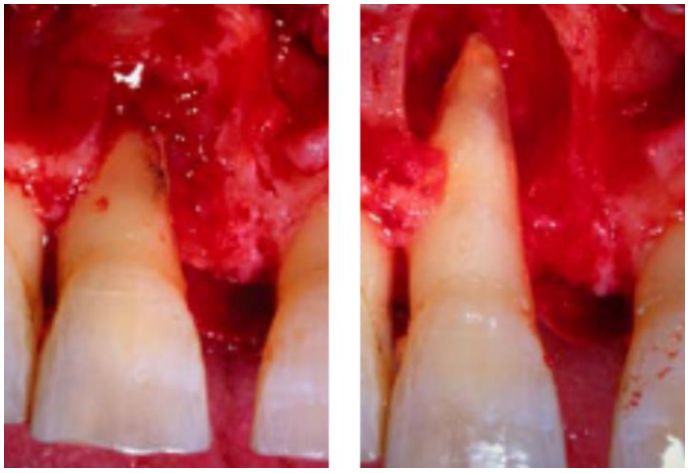

Figure 3. Debridement of periodontal zone. This therapy is an important step before any type of regenerative technique used [8].

2.1.2 Non –Surgical disinfection

After a surgical procedure, or due to a periodontal disease, the root surface could be exposed to the oral cavity, which per definition is full of various type of bacteria, that see the radicular surface as an adequate substrate for adhesion and biofilm formation, increasing the inflammatory response[14]. In these cases, the use of chemical conditioners is a common approach, in order to obtain a more biocompatible surface, especially in clinical practice[8,15]. Citric acid, EDTA and tetracyclines are the most common used acids, in facts their role is to decontaminate the surface from bacteria and endotoxins, furthermore the collagen fibers are exposed by their surface to the etching of detergents, allowing attachment of tissue-regenerating cells[8]. Like surgical debridement, the nonsurgical disinfection therapy gave controversially results, which led to the conclusion that there are no

evidences for the use of these chemical agents as providers of clinical benefits for patients, compared with conventional surgical procedure [16].

2.2.3 Bone graft materials and guided tissue regeneration membranes

Conventional technique alone promotes a periodontal repair, instead of regeneration; in order to achieve a good repopulation of the defect site, researchers developed the so called bone graft materials, which have to be used after debridement of the root surface and which promotes bone formation[17]. Current approaches are based, in particular, on bone graft materials and guided tissue membranes, or a combination of them and on the use of specific growth factor. A more recent strategy under development also involves the use of stem cells[18,19]. Stem cell therapy is a fascinating approach and their potential could overcome the well-known drawback of the nowadays used biomaterials, such as the lack in the induction tissue formation. Despite their great potential, there are many regulatory and ethical issues concerning the use of stem cells, furthermore they are still far from the practical use. Nevertheless, it is important to understand which are the current approaches and which kind of tools are used in order to achieve periodontal regeneration. Periodontal regeneration depends on four basic paradigms.. Scaffold materials act as a guide and form a three-dimensional template in which new tissue could grow. Cells are the principle actresses of the tissue regeneration process, since from their proliferation and differentiation a good periodontal regeneration could be achieved. Growth factors target cells activity, promoting proliferation and differentiation, as well as new matrix production towards the developing tissue. The blood supply provides nutrients for tissue growth and guarantees the homeostasis inside the three dimensional scaffold. Before the introduction of tissue engineering and of regenerative medicine concepts, the traditional approach to achieve the healing of the wound after surgical debridement of the periodontal tissue, was by repair[7]. Repair is defined as the healing of a wound by tissue that does not fully restore the architecture and, consequently, the function of the lost tissue[20,21]. Regenerative medicine used tissue engineered constructs in order to restore the original function and composition of the tissue[22]. Current approaches in periodontal tissue engineering, include the use of barrier membranes and bone grafting materials to encourage the growth of key surrounding tissues, while excluding unwanted cell types such as epithelial cells[23].

The native periodontium is formed by alveolar bone, cementum, junctional epithelium and a gingival connective attachment and, based on its embryonic origin, is formed by the interaction of mesenchymal and epithelial cells[24,25]. Periodontal regeneration follows a series of independent but linked sequence of events: osteogenesis, cementogenesis and connective tissue formation[26]. During healing process, cells respond differently to a variety of stimuli, and the quality of healing critically

depends on the type of cells that repopulate the wound first[23]. During a periodontal regeneration, there are four type of cells that compete: periodontal ligament cells, alveolar bone cells, cementoblasts and epithelial cells. The first three types of cells are capable of generating periodontal tissue, whereas the epithelial cells are responsible for the soft tissue regeneration, and usually migrate ten times faster than other periodontal cell types, this is the reason why periodontal therapy typically results in the formation of long junctional epithelium[27]. Infiltration of epithelial cells inside the defect promotes repair and formation of unusual architecture with a loss of function[28]. Current approaches in periodontal tissue engineering, involve the use of guided tissue membranes, which have the function to exclude infiltration of the epithelial cells[29]. If epithelial cells could be excluded long enough from the wound, allowing other cell types with regeneration potential to become established, epithelial down-growth could be prevented[23]. A combination of bone graft material, which acts as osteoconductive material to promote migration and differentiation of osteoblast cells, and GTR is the most used approach aimed at achieving periodontal regeneration[30,31]. Conventional techniques have the great disadvantage that results which could be obtained are not predictable. In the last three decades, the efforts of researchers in the field of periodontal tissue have been directed to find procedures that could regenerate tissue in a predictable manner. Many experiments and trials have been done and two types of strategies with the combined use of grafts and guided tissue membranes (GTR) have been successfully developed. The combination of bone grafts and guided tissue membranes has been demonstrated to stimulate alveolar bone regeneration. This synergic effect is based on the biological performance of bone grafts and on the "Melcher hypothesis", which proposed that the nature of the attachment in periodontal healing depends on the origin of cells which repopulate the area of the defect and, furthermore, the only cells that could achieve complete periodontal regeneration are cells originating from the periodontal ligament and from the perivascular bone cells [8,32,33].

The biological principles induced by bone grafting materials, in order to achieve bone lost regeneration, could be divided in three interrelated, but not identical, healing processes: osteointegration, osteoconduction and osteoinduction [34].

Osteointegration[35]

Osteogenesis is achieved by using the so called autologous bone graft. In this case the osteoblastic cells and Harvesian system have been replaced by the primitive, undifferentiated, and pluripotent cells derived from the graft material itself, which are somehow stimulated to develop into the bone-forming cell lineage, for example, osteoblast, which form new bone [36].

Osteoconduction

The ability of a material to recruit immature cells and to operate as a scaffold to guide the tissue regeneration. The material induces these cells to develop into pre-osteoblasts and osteoblasts from the surrounding tissue at the graft-host site, which results in bone growth, and replaced the graft[34,37].

Osteoinduction

Osteoinduction is conditioned by the presence of growth factors on the site and is the ability of a material to support the growth of bone over a surface[38]. In that case, the graft material, excludes the connective undifferentiated cells and induces the differentiation and proliferation of osteoblast cells, into newly bone formation[34,39,40].

Melcher hypothesis

Melcher hypothesis, involves the use of barrier materials, which enable cells migration from the connective tissue, in order to avoid repair process[23,31,41]. The healing process of periodontal tissue wounds, is characterized by three correlated steps. Firstly, the internal face of flap is epithelized forming the so called long epithelium attachment; more apically, the second step involves the maturation of the connective tissue which forms the so called connective attachment and, at the end, at the level of alveolar bone, in the deepest point of injury, the recovery of bone architecture and of the periodontal ligament takes place. Morphologically, the structure of the new tissue formed in the wound could be classified as repair or regeneration [7]. Repair process is a formation in a bone defect of a part of connective tissue formed by cells and fibroblasts, which replace, in part or totally, the osteoblasts, inhibiting the deposition of osteoid matrix and, consequently, new bone formation[20,21]. On the other side, regeneration involves the completely recovery of the structure and also the function of the periodonatal tissue[7,42]. Practically, the Melcher hypothesis is achieved through the application of barrier membranes[43]. Many animal experiments have already proved the efficacy of the GTR procedure and, for this purpose, different guide tissue membrane materials have been used, both non-resorbable and resorbable[31,41].

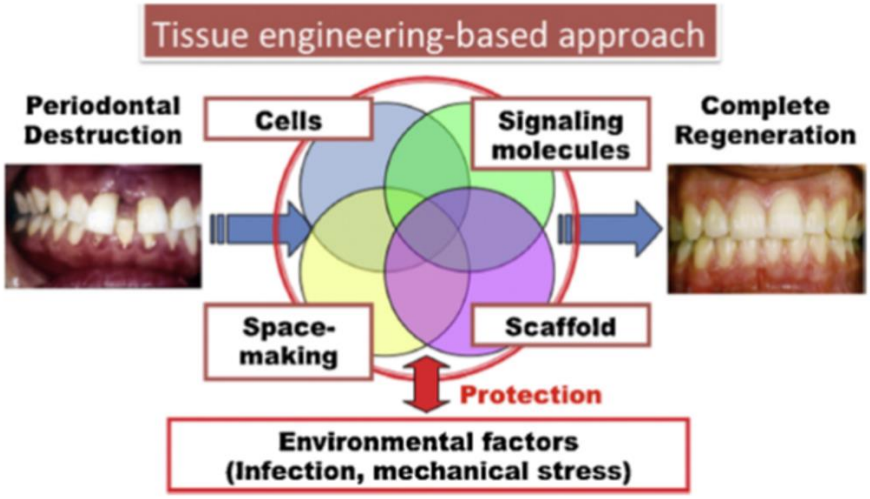

Figure 4. Steps to achieve periodontal regeneration[7].

Despite the implementation of this approach, reaching an optimal periodontal regeneration depends on the capacity to control the infection, that derives from the presence of microbial pathogens that contaminate periodontal wounds[44–46]. Periodontal defects are often the result of the removal of necrotic tissue or of a zone with an acute infection, where there is a high risk of bacterial growth and re-infection of new tissue[47,48]. Considering dental defects, bacterial infection possibility increases, at the interface with a lot of bacteria from different sources, owing to the natural function of mouth and teeth[49]. Hence, it is important to implement appropriate strategies in order to regenerate the periodontal tissue and to restrain bacterial growth. The current available strategy, aimed at reducing the risk of wound infection, involves the use of a conventional systemic antibiotic therapy, which can cause systemic toxicity, with associated renal and liver complications, resulting in the need of hospitalization for monitoring[50–53]. However, several research groups are working on complex systems in order to release low dosage of antibiotics direct *in situ*[54–57]. Antibiotic drugs used in periodontal tissue engineering, should struggle against bacteria, as well as should be biocompatible, nor damage the surrounding host tissue[58–60]. There are many research groups that implement antibacterial properties on bone graft materials or on the implant surface, in order to avoid bacterial adhesion. Current approaches involve the coating of titanium implants with several antibiotic drugs, modification of allograft with chemical groups which inhibit the adhesion of bacteria, and the use of specific signaling molecules which act as bactericidal substances[55,61–63].

2.2 BIOMATERIALS IN PERIODONTAL TISSUE ENGINEERING

2.2.1 Types of bone grafting materials

Bone graft materials are widely used in periodontal tissue engineering, since they are used in order to augment the alveolar bone and to provide a mechanical sustain for a future implant. Material used as bone grafting could be divided in: autografts, allografts, xenografts and alloplastic materials. Different are also the formulations in which bone graft could be produced: particles, three-dimensional scaffolds, injectable pastes and in combination with polymeric materials. In the last years, one of the most devastating periodontal disease was due to microbial infection, so many materials with antibacterial properties have been developed, in order to control the infection and to reduce the failure of the implant.

2.2.1.1 Autograft

Autogenous grafts are considered the current gold standard bone replacement graft material[64]. Autograft is a tissue transplanted from one part of the body to another in the same patient. Typically, if the material needed to fill the defect is small, the site from which autograft materials are obtained are intraorally, in particular from the extraction sockets, edentulous ridges, ramus, symphysis, tuberosity, or from the surrounding buccal plate. In large bone defects, the material needed is larger and is typically obtained from extraorally areas, such as the iliac crest or the tibia. For autologous bone grafts, the implant survival rate varie from 76% to 100%, with worse results for iliac crest bone compared to calvaria bone[65–68]. On the other side, other studies reported that the results obtained with intraoral grafts were similar to those obtained with extraoral sources, making this source more favorable since the material available is larger[67,69]. This multitude of controversial results depend on donor site variability and complications, which allow assessing that there is no scientific evidence to indicate which technique is the best[70].

The great advantages in using this approach are represented by the fact that these grafts are osteogenic, prevent disease transmission and are low cost. Clinical tests show excellent periodontal regeneration with new cementum formation. *Schallhorn et al*. used iliac crest grafts to treat infrabony defects and reported up to 4 mm gain in bone healing[71]. However, the main complication is that they require a second surgery and it is important to consider the possible donor site complications, such as infection and pain[72]. Furthermore, the limited supply of autograft materials is an additional issue that makes this approach always less attractive.

2.2.1.2 Allograft

An allogenic material is a graft derived from a donor of the same species, but genetically dissimilar. Allograft materials allow overcoming the issue of a second surgical procedure and the limited supply source[6]. The graft is typically obtained from tissue banks that process the donor tissues and, on the base on which the tissues are processed, allografts could be divided into freeze-dried bone allografts (FDBA) and decalcified freeze dried bone allografts (DFDBA). These types of graft have the great disadvantage to potentially include a foreign body immune response and the possibility of diseases to be transmitted; furthermore, a high risk of graft contamination during processing is present[73–75]. Despite these drawbacks, allografts have relatively high success rates and, depending on the remaining proteins into the matrix, they could act as osteoconductive or osteoinductive materials[76].

In particular, the decalcification process exposes bone morphogenic proteins which act as inductive molecules for bone regeneration, while ,on the other hand, this type of process causes a higher rate of resorption, resulting in less effective scaffolding properties. Several studied compared FDBA and DFDBA allograft materials. *Yukna et al.* performed animal studies in which both FDBA and DFDBA were placed into surgically created infrabony defects and evaluated by histological analysis[77]. After three months, FDBA allograft showed more substantial new bone formation than DFDBA. Excellent results in new bone formation, using FDBA graft, were also reported from *Altiere et al.* and *Blumenthal et al.*[78,79]. The great variability of these types of graft, is demonstrated by the study of *Mellonig et al.*, where DFDBA report similar bone filling percentage compared to FDBA in one study and show higher bone filling percentages in a previous study using FDBA mixed with an autologous graft material[80,81]. Another disadvantage of these kinds of approach is the high material cost, because many commercially available bone graft materials are xenograft[82].

2.2.1.3 Xenograft

Xenograft materials are bone grafts obtained from other species (typically bovine and porcine) and transplanted in human. Tissue banks usually choose these graft materials, because it is possible to extract larger amount of bone with a specific microstructure as compared to auto or allografts. Since the main disadvantage of xenografts is their antigenicity, tissues are carefully processed to remove all organic constituents. There are many commercially available products based on this process, such as Geistlich BioOss® particles from bovine source, which is considered the gold standard graft material in dental application. However, despite the positive results obtained from studies conducted on xenograft materials, the tissue/bone regeneration with this graft material might be unpredictable[83]. In one study, where defects were treated with bovine derived bone grafts, at 1 years

follow up, 78% of defect healed successfully, even if no difference was detected with the no treated lesion[84]. Furthermore, in another study, eight infrabony defects were filled with xenografts and the results show that 7 defects went thought successful healing, but ones defect healed by repair [85]. Great advantage of these types of graft is that only one surgical procedure is necessary, on the other hand many people are put off by the fact that for this type of procedures animal bone is used.

2.2.1.4 Alloplast

The graft materials described above have different disadvantages, such as second surgery procedures, risk of diseases transmission, limited availability and unpredictable results. In the last decades, synthetic materials have aroused even more interest because they can be implanted in the host tissue in a large amount, showing diverse advantages in filling large bone defects, such as controlled degradation properties, osteoconductive or osteoinductive characteristics, biocompatibility and customized mechanical properties[86]. The development of alloplast grafts is divided in two classes: ceramic and polymers, and a mix of the two[64,87]. Ceramic-based materials include calcium phosphate, calcium sulfate and bioactive glasses[88,89]. Natural polymers include modified polysaccharides, polypeptides and synthetic polymers such as poly(glycolic acid), poly(L-lactic acid). Natural or synthetic polymers are typically used in addition to the ceramic graft, in order to provide osteoinduction properties (peptides), or to increase mechanical properties, and to tune the degradation rate and cell adhesion.

Alloplastic materials are the most commonly investigated grafting materials for periodontal regeneration, and many positive results have been reported[64,86,90].

2.2.2 Ceramic hard materials

2.2.2.1 Calcium phosphate

Calcium phosphate (CaP) materials have a long history, in fact the first studies about them appeared in the early 1920[91] and, from that date, numerous studies were conducted and an enormous amount of data related to biological actions, chemistry and formulations were produced[92–94]. Calcium phosphate materials are widely used in bone and dental tissue engineer, since they are the principal compound of inorganic phase in native bone. This kind of composition induces a biological response, which is similar to the one generated during bone remodeling, that is resorption of the old bone and, in parallel, formation of new bone. Since the calcium phosphate graft materials are chemically similar to the natural bone, the degradation product of the synthetic material are non-toxic and naturally metabolized without any accumulation of calcium and phosphate in sensible organs, such as liver and kidney[95]. Calcium phosphate materials could be formed in different

geometry: three-dimensional porous or bulk scaffolds, granules and pastes or cements. The most used calcium phosphate materials as bone grafts in periodontal tissue engineering are hydroxyapatite, tricalcium phosphate materials and the combination of them.

2.2.2.2 Hydroxyapatite

Hydroxyapatite (HA), ($Ca_{10}(PO_4)_6(OH)_2$), is the most abundant inorganic component of native bone, around 65% of inorganic phase, and it has found many application as bone filler in clinical practice. Many studies have been conducted on the interaction between HA implants and bone[96–100]. It is well-known that a chemical bond directly occurs between bone and the HA graft, giving rise to a kind of bone matrix on the implant surface that is either composed of globular deposits or an organized network of collagen fibers, which may enhance the bonding between bone matrix and HA[101]. Osteoblastic cells are found on the HA surface, starting mineralized osteoid formation that matures into fully mineralized bone on the HA surface[102]. Apatite crystals appear on the surface of implanted HA grafts and, these one, are similar to the apatite found adjacent to the alveolar bone[103]. However, from a chemical point of view, hydroxyapatite has a great potential as bone filler, since osteoconductive properties have been proved, but it has a very slow degradation rate, which limits its use alone[104].

2.2.2.3 Tri-calcium phosphate

In recent years, due to the limitation of hydroxyapatite in terms of degradation rate, the interest on tricalcium phosphate materials (TCP) has been rapidly increased. The most studied TCP phase is the β one, which has shown good biocompatibility and osteoconduction properties in many studies[90]. βTCP materials have been studied in periodontal tissue engineering and have shown regenerative properties similar to those of autogenous bone[105]. However, βTCP has poor mechanical properties and its application as material is recommended just in low bearing applications and in particle form. Furthermore, the resorption rate could be too fast in certain applications, such as in large bone defects. The mechanism of βTCP resorption is based on two main hypothesis. The first manner is due to the dissolution by biological fluids, while the second mode hypothesizes that the resorption is due to the action of osteoclast cells[106]. The reality is that the two ways are related, in fact more soluble is the material, more extensively it is resorbed by osteoclasts[106]. However, a very soluble material might inhibit active resorption, indeed calcium ions inhibit osteoclast activity[107,108]. The dissolution of the material depends on many parameters, such as sintering process, micro- and macro-porosity, and purity of the row material[109]. The best way to

control the degradation rate is to mix the Ca-P materials in a rate which fits the needed characteristics[110,111].

2.2.2.4 HA/βTCP bi-phasic calcium phosphate

A grafting material used in periodontal tissue engineering should possess peculiar characteristics: osteoconductivity, good mechanical properties in order to sustain the load applied on the defect site, a resorption rate comparable with formation of new bone and, finally, the degradation products should not be toxic for the surrounding tissue[64,112,113]. In the last years many research works have been based on the combination of HA and βTCP materials, being aimed at managing the important properties and at achieving an excellent and predictable periodontal regeneration. The balance between HA and βTCP is a key point to obtain both mechanical strength and degradation and to stimulate excellent osteointegration. Several studies were performed in order to understand the best HA:βTCP, however the results are difficult to compare, since there are many variables that affect the results from one to another group of researchers[110,114,115]. For example, the degradation rate and the mechanical properties, as well as the biological properties, strongly depend on the sintering parameters, on the mixing technique, on the row material source and on the type of final geometry (3D scaffold, porosity, granules, paste, etc.). An interesting study conducted in 1997 by *Yamada et al*., compares different Ca-P ceramic materials in terms of osteoclastic resorption; HA and βTCP was maintained the same, and the percentage of ratio in the bi-phasic material was changed from 100 % of βTCP, to 100% HA, through 75/25 βTCP/HA and 25/75 βTCP/HA[106]. The results show that resorption lacunae were observed on pure βTCP and 75/25 βTCP/HA material; instead, osteoclasts did not resorb the material with a prevalence of HA in the composition. The lobulated lacunae detected on the Ca-P biphasic material surface were similar to those formed on natural bone, furthermore the high dissolution rate of pure βTCP and the totally absent osteoclast activity on the pure HA, suggest that HA/ βTCP biphasic material allows osteoclast to act in a more natural way. As osteoblastic bone formation is strongly related with osteoclast resorption during bone remodeling, biphasic Ca-P promotes a surface similar to that of the native bone, hence presumably the dissolution/precipitation process which occur during osteoclast resorption promotes chemical bonding between bone apatite and similar apatite formed on the ceramic surface. By changing the ratio between HA and βTCP, it is possible to manage the degradation rate of the bone graft, and consequently the bone formation. Mechanical properties of grafting materials, depend on the amount of βTCP and HA inside the biphasic material; in particular, as expected, poor mechanical properties are associated to the material with a high percentage of βTCP. In a recent study, of *Morra et al*.[116], a biphasic granulate bone filler was developed with a HA/βTCP ratio of 75/25, and animal study showed an excellent new bone formation without any inflammatory response.

Furthermore, the mechanical resistance not only depends on the composition, but also on the geometry and process parameters (i.e. sintering). These kinds of wide range of possibility make these bone grafts highly interesting for periodontal tissue engineering.

2.2.2.5 Bioactive Glass

A bioactive material, in tissue engineering field, has been defined as a material that undergoes specific surface reactions when implanted in the body, promoting the formation of an apatite-like layer which allows a strong bond formation between bone and grafting material[87]. This ability is different from the osteoinduction stimulation, since the only materials that could promote induction are the autogenous grafts. Osteoinduction is the property to induce osteoprogenitor cells and other tissues to not only migrate into the structure of the graft material, but to also promote differentiation, through affecting gene expression of undifferentiated cells[34]. In the case of bioactivity, it is more correct to speak in terms of osteostimulation, as the definition given by *Schepers and Ducheyne* [117]. Bioactive materials could be added to the autologous graft in large bone defects, where the material needed is high[118]. Bioactive glasses showed osteostimulation and high mechanical strength. There are three main different bioactive glasses: silicate, borate and phosphate. The most used and with a 40-year history, is the silicate bioactive glass, named Bioglass® (45S5), for example the key compositional features responsible for the bioactive of 45S5 glass are its low SiO_2 content, high Na_2O and CaO content, and high CaO/P_2O_5 ratio [119]. This composition promotes formation, on the surface of glass, of the carbonate-hydroxyapatite-like layer, which is similar to the mineral constituent of bone, therefore firmly bonds between bone and tissue are obtained[120,121]. In clinical evaluation PerioGlas, a commercially available bioactive glass, has shown the ability to inhibit the downgrowth of epithelial cells, promoting the regeneration of bone[122,123]. More recent studies have proposed borosilicate glass as a bioactive material, although further studies are needed, but a first encouraging result was obtained with small animal models, in which borate glasses were demonstrated to be non-toxic. Despite bioactive glasses' brittleness, which constitutes their main disadvantage, their unique properties to release ions in the surrounding tissue, which stimulate new bone formation, make these materials interesting for periodontal tissue engineering.

2.2.2.6 Calcium sulfate

Calcium sulfate, also called plaster of Paris, was used as augmentation material for the first time in 1892, in cavities caused by tuberculosis[124]. From that date, more than thousands of articles have been published, on the use of calcium sulfate as bone filler[125]. There are three different forms of calcium sulfate, based on the amount of water molecules inside the crystalline structure: anhydrate, dehydrate and hemihydrate, the latter state found in medical grade products[126,127]. Calcium sulfate is

a biocompatible, osteoconductive and biodegradable material, which is completely dissolved into its component elements and recedes leaving behind calcium phosphate deposits that stimulate bone growth[125,128]. Furthermore, its porosity and hygroscopic properties allow adsorption and infiltration of platelets, in order to stimulate bone formation and enhance angiogenesis. No immunogenic, or adverse reactions have been reported in literature and, due to its degradability, it is classified as a short term space maintainer. Calcium sulfate material is used, in dentistry, preferably in forms of paste or putty, and numerous publication report clinical effectiveness and safety as bone substitutes in periodontal defect[129–131]. Furthermore, calcium sulfate has also been used as a barrier membrane to prevent ingrowth of soft tissues, no improvement was reported if it is used with a barrier membrane[132,133].

It has been reported a study in which calcium sulfate pellets were impregnated with a drug and used as a system for antibiotic release *in situ*[134]. Despite many studies showed great potential for the application of calcium sulfate as a graft material, its enhanced solubility in contact with blood and saliva reduces its conversion from paste to rigid matrix, making the fate of the material unpredictable[128,135]. This reason limits its use as graft material but, in order to overcome this drawback, the researchers have been trying to combine calcium sulfate with other materials, such as calcium phosphate materials, aiming at stabilizing the structure, and controlling the degradation rate[130,131,133]. Other approaches involve production of a bi-phasic calcium sulfate, in which both dehydrate and hemihydrate forms are mixed, in order to manage the solubility and to obtain a rigid matrix after implantation[136]. The primary use of calcium sulfate and composite of calcium sulfate bone grafts are as injectable bone fillers for different applications, such as sinus augmentation or alveolar bone regeneration, with the great advantage that the use of a barrier membrane is not necessary, since the barrier properties of calcium sulfate enable epithelial cell downgrowth.

2.2.3 Natural soft materials

Non rigid materials are widely used in periodontal tissue engineering as barrier membranes, hydrogels and in combination with ceramic materials. Natural soft materials could mimic the extracellular matrix (ECM) of bone, allowing cell infiltration, proliferation and new bone formation. Elasticity, hydrophilicity, biodegradability, mouldability and biocompatibility are the most important properties of these materials. On the other hand, their disadvantages include source variability, immunogenicity if they are not pure, limited range of mechanical properties and lack of control over pore size. The most used natural biomaterials in periodontal tissue engineering include: collagen, chitosan, pectin[137] and alginate. A briefly introduction for each of them is reported as follows.

2.2.3.1 Collagen

Collagen is the most used natural material in the field of tissue engineering, since it proven biocompatibility and ability of promoting wound healing[138]. It could be extracted from several allogenic sources as it is the most prevalent structural protein found in the extracellular matrix of different connective tissues like cartilage, bone, tendon, muscle, skin etc.[139]. Several studies and commercially available products are based on type I collagen, the most abundant form of collagen present in the body and the major component of periodontal connective tissue[138,139]. It offers advantages of biocompatibility, cellular adhesion, migration and growth. Collagen materials degrade via enzymatic action and the by-products do not cause any inflammatory response[140]. Collagen is widely used in barrier membranes, as guiding materials for tissue regeneration, but it could be also used in hydrogel sponges and as coting material on ceramic scaffolds or dental implants, mimicking the natural component of ECM of bone, thus providing a biomimetic surface[79,141]. Collagen material has a good tensile strength, which could be enhanced by crosslinking agents, such as EDC, glutaraldehyde and tannic acid. Crosslinking of collagen fiber enhances mechanical and degradation properties, but could influence the cell response since most of this chemical crosslinking agents are toxic[142,143].

2.2.3.2 Chitosan

Chitosan is a natural polysaccharide derived from chitin that can be extracted from the crustaceous exoskeleton and is composed by β–(1,4)–glucosamine and N-acetyl-D-glucosamine[144]. Owing to its biocompatibility, its intrinsic antibacterial nature, its ability to not induce any foreign body reaction and to promote cell adhesion, proliferation and differentiation, chitosan has aroused great interest in tissue engineering and pharmaceuticals, finding many applications especially in bone tissue engineering[145,146]. Furthermore, chitosan has a backbone similar to that of glycosaminoglycan, the major component of bone ECM[147]. The application of chitosan in patients with periodontitis showed reduction of the gingival inflammation, due to its antimicrobial properties[148]. Many applications involve combination of ceramic materials with chitosan, for example Ca-P grafts mixed with chitosan in order to produce porous sponge or paste[149,150]. Chitosan could be extracted also from white mushrooms, eliminating the immunogenic issue due to the animal source.

Another important characteristic of chitosan materials is its poly-cationic nature, which allows ionic interactions with other poly-anionic materials to be created and to generate the so called polyelectrolyte (PEI)hydrogel[144,151–153]. These properties were used to produce materials for drug release, in particular for drug release in the intestinal track, since this ionic bond is pH sensible and the drug could be released more or less depending on the pH of the organ[151,154].

2.2.3.3 Pectin

Pectin is a natural anionic polysaccharide, a major component of citrus cell walls or apple peel by-product, consisting in a poly D-galacturonic chain with carboxyl groups, in part methoxylated[155,156]. Pectin is already widely used in the food industry and, in the last decades, it has found application in bone tissue engineering, in particular as a drug carrier[157,158]. Pectin carboxylic groups could be ionically crosslinked by calcium ions (Ca^{2+}) forming the so called "egg box" structure, where a divalent cation is bonded with different carboxylic anions[159,160]. Furthermore, ionic interactions occur with poly-cationic polysaccharides, to form a well-known polyelectrolyte structure (PEI)[161] . The limit of pectin is its great water-solubility, which causes fast dissolution and, when used as a drug carrier, a burst release of the therapeutic molecules occurs[153]. To overcome this problem, many research groups have been trying to combine pectin with other materials, such as chitosan, to stabilize and increase the resistance in water[152,154,162–164]. Pectin polysaccharide has been used in combination with Ca-P particles, since it could mimic the extracellular matrix and guide cell proliferation and as a coating material in different medical applications, such as chirurgic mesh as antiadhesive material[165–169].

2.2.3.4 Alginate

Alginic acid or alginate, is a natural material extracted from a brown algae, or synthetized through bacteria biosynthesis, which allow obtaining an alginate with more controllable structure. Alginic acid is a linear copolymer containing block of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate residues (G) [170]. The blocks are composed of consecutive G and M residues, whose different amounts define the characteristics of the alginate material, since they affect the physical properties of the material, for example only the G residues could be ionically crosslinked by calcium ions[171]. Alginate is an anionic natural polymer that has been widely investigated and used for many biomedical applications, because of its biocompatibility, low toxicity, relatively low cost and simplicity of gelation by addition of divalent cationic ions, such as Ca^{2+}[113]. Alginate, like all other natural polymers, has a low mechanical strength; for this reason, it is usually coupled with other materials, such as pectin, chitosan, or ceramic tricalcium phosphate or bioactive glasses[152]. Alginate hydrogels and alginate/bioactive glasses composite hydrogels have been used in periodontal tissue engineering, having shown an induction in osteoblast-like cell differentiation and enhanced alkaline phosphatase activity of human periodontal ligament fibroblast cells[172,173].

2.2.3.5 Hyaluronic acid

Hyaluronic acid (HyA) is one of the most used natural material, along with collagen, in the biomedical field and, in particular, in periodontal tissue engineering, since it is one of the

glycosaminoglycans present in the extracellular matrix of connective tissue, with an excellent potential as scaffold for tissue regeneration[174]. Hyaluronic acid is a linear polysaccharide and, in dentistry, it has shown anti-inflammatory and anti-bacterial effects in the treatment of periodontal diseases[175]. Furthermore, due to its tissue healing properties, it could be used as an adjunct in the treatment of periodontitis. The precise chemical structure of hyaluronic acid, is a repeating unit of d-glucoronic acid and N-acetyl-d-glucosamine[176,177]. The most important characteristics of HyA are: a hygroscopic nature, which allows the material to adsorb huge amount of water maintaining conformational stiffness[177,178], viscoelastic properties, which allow the material to maintain the space and to protect the surface in periodontal regeneration[179]. Furthermore, the viscoelastic nature makes unable virus and bacteria penetration. Recent studies have showed a bacteriostatic effect of HyA, making this material a suitable tool for barrier membranes, in order to reduce the bacterial contamination of surgical wounds, thus reducing the risk of post-surgical infections and promoting a more predictable regeneration[180–182].

2.3 MORPHOLOGY OF BONE GRAFT MATERIALS

Bone grafting materials could be shaped in different forms and formulations, depending on the application and on preference of the dental technician[30]. Bone graft materials should fill the void, avoiding collapse of the defect, promoting platelet, vessels and cells infiltration, should be easy to manage and to degrade, with the same rate in which the new bone growth. Hence, the important characteristics such as mechanical properties, porosity and degradability depend in part on the chemical composition of the scaffold and, in part, on the final material morphology. Porosity is the most important feature, because all other parameters depends on it[183]. Many studies and lot of works have been done to understand which pore size and porosity are the best, in order to promote osteoblast proliferation and bone ingrowth[184].

2.3.1 Porosity of bone graft materials and osteogenesis

The scaffolds used as bone graft, have primarily the function of osteoconduction and serve as template in which the cells could proliferate and differentiate[185,186]. In order to achieve an optimal osteointegration, the material should mimic bone morphology and allow blood vessel infiltration, nutrients transportation and the degradation rate should follow the new bone regeneration. Natural bone is composed of hydroxyapatite crystals deposited in a collagen matrix[187]. The morphology of bone is characterized by a trabecular bone, which is typically a porous environment within 50% to 90%, and pore sizes in the order of 1 mm and, surrounded by dense cortical bone, with a porosity between 3–12%[188–190]. Bone is a dynamic tissue, in constant remodeling, with osteoblasts producing

and mineralizing new bone matrix, and osteoclasts resorbing the matrix. The mechanical properties of bone vary by age and from the place in the body, for example in the femoral zone, the Young's modulus could vary from 7 to 17 GPa[190]. Of course, a scaffold material could not reach these properties, because if it promotes mineralization *in vivo*, properties will increase and allow a mechanical sustainment. Porosity and pore size of biomaterial scaffolds play a critical role in bone formation *in vivo* and *in vitro*[184]. The morphology of the bone graft influences the osteogenesis, as well as the mechanical properties and the degradation rate[191]. Porosity is defined as the percentage of void space in a solid and is independent from the nature of the material[192]. Pores are fundamental for bone tissue, because they allow osteoblast migration and proliferation as well as vascularization; furthermore, the porosity allows a better stability of the implant, since a better interlocking between the biomaterial and the surrounding tissue occur.

There are many techniques that could be used to obtain porous materials, such as gas foaming[193], salt leaching[194], phase transformation[195], freeze-drying[196], and sintering[197]. Of course, the process technique depends on the material used and, in the case of ceramic materials, the most used technique is a sponge replication, which allows an interconnected porosity to be obtained, with a controlled pore size[119].

The importance of porosity in bone regeneration has been shown by *Kuboki et al*. using a rat ectopic model, in which porous and dense particles of hydroxyapatite were implanted[191]. The results show that no new bone has formed on the dense particles, while porous particles promote new bone formation. The percentage of void (porosity) and the size of these voids (pore sizes), have been studied for long time from many research groups. Microporosity allows *in vitro* osteogenesis through suppressing cell proliferation and forcing cell aggregation; however, *in vivo* lower porosity promotes osteochondral differentiation, since hypoxia conditions are favored. Macroporosity promotes *in vivo* osteogenic differentiation, since it allows cell infiltration, bone ingrowth and vascularization; on the other side, macroporosity reduces the mechanical properties of the scaffold. *Hulbert et al*. define, as the lowest limit for osteogenic promotion, 100 μm of pore size. They used calcium aluminate material, with different pore size and a constant porosity of 46%, and then they implanted the scaffolds in a femoral dog[198]. The results show that no bone ingrowth was present in the scaffold with a pore size lower than 100 μm, while the highest rate of new bone formation was detected for the sample with a pore size between 150 and 200μm. These results are in agreement with the diameter of the Harvesian system, which reaches values around 100–200 μm, but in contrast with other research group works, which demonstrated no significant variation between samples with pores lower than 100 μm and samples with pores higher than 100μm. Bone ingrowth was similar in all of the pore sizes of the material implanted in rabbit femoral defects under non-

loading bearing condition. These results demonstrated that 100µm are not a critical size for non-loading bearing applications.

The effect of pore size and porosity, depends on the study, because if it is conducted *in vitro* or *in vivo,* different results are obtained in the two cases and what is demonstrated in one study is usually totally opposite in the other study[191,198,199]. This is mainly due to the different complexity of the two systems: *in vivo* osteogenesis depends on many variables and, in particular, from vascularization, furthermore there is a concurrence between osteochondral and osteogenic formation, and pore size and porosity influence in a heavier way these processes.

2.3.2 Porosity and pore size in vitro

The effect of porosity and pore size, was evaluated *in vitro* using osteoblasts and mesenchymal cells[200,201]. Small size pores allow cells aggregation and inhibit cells proliferation *in vitro*, resulting in an increase of expression of alkaline phosphatase activity and osteocalcin[202]. Macroporosity allows cell proliferation, since large pore size and higher porosity enhance nutrients and oxygen transportation. In general, *in vitro,* osteogenesis is not affected by pore size, but it is enhanced by lower porosity[184]. *Akay et al*. studied the effect of pore size in a primary rat osteoblast culture and showed that more cells were found in smaller pores than in large pores, where however cells migrate faster, but the mineralization was not affected by pore size[201].

2.3.3 Porosity and pore size in vivo

In vivo condition is characterized by many variables, for example osteogenesis depends, in particular, on the degree of vascularization, which is promoted by higher porosity and large pore size. Furthermore, small pores (around 100µm) promote chondrogenesis before osteogenesis and low porosity does not permit nutrients transportation[203,204]. *In vivo* it is demonstrated that high porosity and large pores promote osteogenesis by recruitment of cells, which are stimulated to migrate into the scaffold, as well as vascularization is enhanced and promote new bone formation[184]. Furthermore, this assumption is supported by the fact that no clinical results on the positive effect of pore lower than 40µm are present. In particular, higher porosity showed an increase in bone formation and bone ingrowth when a scaffold with a higher porosity is implanted. Pore size plays a critical role in bone formation and many studies were conducted to understand the better pore size range for bone regeneration *in vivo.* Porous hydroxyapatite scaffolds, with different pore size, were implanted subcutaneously in rats and the results showed that an increase between 300–400 µm in the expression of alkaline phosphatase activity was calculated for the material with a pore size range, furthermore capillary infiltration was detected just above this range[204,205]. However, there are many other articles that do not observe any statistical significant difference

between samples with different ranges of pore size[199,206]. Another interesting aspect about porosity and pore size, is the impact on the progression towards osteogenesis. Large dimension pores promote osteogenesis, small pores, in contrast, promote chondrogenesis first[203,204,207]. Furthermore, large pores allow vascularization and oxygen ah nutrients transportation promoting direct bone formation[203]. Pore geometry also affects bone regeneration: long channel pore promotes infiltration of cells and bone ingrowth, on the other side curved pores on the surface of the scaffold, without interconnection, hinder osteoblast precursor cells penetration and capillaries infiltration, promoting a bone formation only on the surface [203].

The mechanical properties of scaffolds used for bone regeneration, are affected by the degree of porosity, the pore size and, of course, the interconnectivity. Although increased porosity and pore size result in a promotion of bone ingrowth, the drawback is a reduction in mechanical properties, since the large volume of void compromises the structural integrity of the scaffold.

The degradation rate of the material used to produce the scaffold, should be taken into account, because if the material has a fast degradation rate, the porosity should not be high, since the rapid erosion of the trabeculae and the bridge of the material could compromise mechanical and structural integrity, before substitution by newly formed bone. On the other side, if the degradation rate of the biomaterial is low, it is possible to reach high porosity, since the presence of channels and interconnected pores can accelerate degradation due to macrophages via oxidation and/or hydrolysis.

There is not an exact porosity and pore size suggested to achieve an optimal bone regeneration, since the multitude of studies *in vitro* and *in vivo* presents different results, depending on the biomaterial used, from the cells and cytokines used and from the bone features *in vivo*.

A balance between micro- and macro-porosity is recommended, in fact it is necessary that the material possesses both pore <100μm which allow protein adsorption and promote cells aggregation and large pore >300 μm, which allow vascularization and new bone ingrowth[208]. Furthermore, a balance between large and small pore allows controlling the mechanical properties and the degradation rate. In order to increase and customize the mechanical properties and the degradation behavior, ceramic or hard materials are coupled with a natural or synthetic polymer[209]. Furthermore, coating the bone graft ceramic with, for example, natural materials such as collagen protein, gives to the material such biomimetic properties and could enhance the osteointegration of the scaffold[116].

Ceramic materials used as bone graft materials, could be formed in different ways, such as particles, three-dimensional scaffolds, injectable pastes[210]. On the base of the application, it is possible to choose a different type of bone graft material. In large bone defects, dental technician prefers to use three-dimensional scaffolds to fill the void and sustain the surrounding tissues. Particles as bone

graft are widely used, since they could be mixed with blood and could also fill irregular or small shape around titanium dental implants. Injectable bone grafts are usually a mix of natural or synthetic polymers with ceramic micro-particles; their use is increased in the last years, since they are easy to use and could fill irregular void and defects, otherwise difficult to reach.
Bone grafts are usually coupled with membranes, in order to achieve an excellent osteointegration which excludes gingival epithelial cells from the bone socket [41].

2.4 GUIDED TISSUE REGENERATION MEMBRANES (GTR)

Melcher's hypothesis assumes that certain cell populations will have the potential to generate new cementum, alveolar bone and periodontal ligament, if they have the opportunity to populate the periodontal wound[23]. This condition is obtained when epithelial cells or fibroblast cells from gingiva front and connective tissue are excluded from the wound space. This goal is arised through development of a membrane barrier, which guides soft tissue regeneration without downgrowth in the defect site[31]. Characteristics for guided tissue regeneration membranes should include biocompatibility, cell exclusion, space maintenance, tissue integration and ease of use. Membrane should separate the gingiva flap from the coagulum in the wound space, furthermore it should withstand the masticatory stress, the flap tension and avoid the collapse of the soft tissue, maintaining the space for the regeneration of new alveolar bone[41]. An important property is the tissue integration ability, since it could ensure the stabilization of the wound and guide fibroblast cells to regenerate soft tissue without a downgrowth in the periodontal defect space. Finally, it is really important that the developed membrane for GTR is manageable and easy to use by the dental technician, without any additional difficult during the surgical procedures. The first material used as barrier membrane was cellulose acetate filter and, for the first time, the guided tissue process was histological verified. From that point of view, different types of barrier materials have been developed, resorbable and non-resorbable, natural and synthetic

2.4.1 Non-resorbable

The mechanical stability, the ability to retain their shape, besides the proved capacity to exclude cell migration from the gingiva in the wound defect, are the principle advantages of nonresorbable membranes[41]. Despite the encouraging results obtained with this type of GTR, the second surgical procedure and the possibility to interfere with the healing process are the principle drawbacks and, thus, further efforts in the development of resorbable membranes are necessary. Non-resorbable membranes include expanded polytetrafluorethylene (ePTFE) and titanium reinforced PTFE meshes.

The ePTFE membrane was the first membrane approved for clinical use[211]. ePTFE is chemically indentical to PTFE, but while PTFE is non-porous, the high thermal tensile stress process in ePTFE allows formation of a porous microstructure and fibrils, which allow connective tissue ingrowth. ePTFE, also called commercially Goretex®, is formed by two layers: the first is an open structure which promotes cell ingrowth, the second layer is an occlusive membrane serving as a space provider for regeneration, inhibiting the cell downgrowth and giving structural stability[212,213]. The first layer is 1 mm thick and has 90% of porosity, instead the second layer is around 0.15 mm thick and 30% porous. Goretex® non-resorbable membrane, has an exceptional inertness, biocompatibility, and does not elicit foreign body reaction. Many clinical trials have been done, and histological analysis revealed that ePTFE membrane can lead to significant periodontal regeneration after 3 months healing period[212,214]. Although many studies demonstrated the efficacy of ePTFE membranes, some studies did not find any significant difference between the use of ePTFE membrane and the conventional debridement therapy with open flap, furthermore the insertion of ePTFE GTR membrane has been seen to cause additional complication such as pain, purulence and additional costs for the second surgery procedure[215].

Another non-resorbable membrane with ePTFE reinforced with a titanium mesh between the two layers was made, increasing the mechanical strength and the maintenance of a better space[41,216]. The addition of a titanium mesh allows a better positioning under the flap by the dental practice and increases the stability of the membrane, avoiding collapse. Furthermore, the microporosity of the membrane avoids cell ingrowth, but allows fluid infiltrations. Animal studies demonstrated relevant cementum and bone regeneration[217,218], although in clinical trials no difference was detected between ePTFE membrane and titanium mesh GTR[216,219]. Furthermore, the main disadvantage is the increased exposure, due to its stiffness and also to a more complex secondary surgery to remove it[220].

The future of periodonatal tissue regeneration, and of tissue engineering in general is to develop and use materials which could degrade during tissue formation without any further surgical procedure, in order to decrease patient's pain and hospitalization costs. Furthermore, the use of natural resorbable membranes allows bioactivity to be promoted during the regeneration process. For these reasons, in the last decades many efforts have been spent, in order to study and develop degradable membranes made with both natural or synthetic materials[31,41].

2.4.2 Resorbable

Natural. Due to the need of a second surgery to retrieve non-resorbable membranes, in the last decades the demand for bio-absorbable membranes with comparable, and even better clinical

outcome, became real. Furthermore, the resorbable membrane reduces the patient discomfort, as well as costs and eliminates any possible surgical complication[41]. A disadvantage of resorbable membranes is the unpredictable resorption, due to the different degradation process which could take place, enzymatically or by hydrolysis. In addition, it is mandatory that the degradation products do not interfere with the regeneration process and do not induce any inflammatory response[31]. Resorbable membranes could be derived from natural sources or made by a synthetic process. Natural membranes have the advantage to be biocompatible, to be no cytotoxic and to be potentially bioactive, but they lack of degradation rate and mechanical properties[41,221]. On the other hand, synthetic materials are more predictable since it is possible to tune the degradability and mechanical properties, but are less biocompatible and could generate foreign–body reaction.

Type I collagen is the major component of periodontal connective tissue, and has been used in medicine so far, as biocompatibility, low immunogenicity, hemostatic properties and ability to attract and activate periodontal ligament and gingival fibroblast cells have been proven[141]. The bioactivity of collagen and its potential to augment tissue thickness is demonstrated by the interaction with various cell types during the healing process. Several commercially available collagen membranes have been developed, such as Geistlich BioOss®, Tecnoss Osteobiol®, Zimmer BioMend® and BioMend Extended®, Braun Lyoplant®, etc.. The behavior of the collagen GTR depends on the source of the collagen used and from the process technique[222]. Collagen is usually extracted from skin, tendons, or pericardium of animals, usually bovine and porcine[141]. Collagen extracted from animal sources need to be purified and, according to the EU guidelines, chemical elimination of viral and bacterial contamination must be performed, then the collagen antigenicity is eliminated by removing lipid and non-collagen protein remnants. A sequential segment analysis, biocompatibility and sterility are checked step by step[31]. The most common chemical modification process, after purification, is the chemical crosslinking, usually performed using aldehyde treatment, which increases the mechanical strength, reduces the degradation rate and the water uptake capacity, but increases the risk of cytotoxicity, mostly due to the possibility of crosslinking traces inside the collagen network[143]. Degradation of collagen is due to collagenase enzymes, which cut the collagen chain and transform it in gelatin, which is then degraded via aminoacids gelatinases[31,141]. The resorption time of collagen GTR varies from 4 weeks to 6 months, depending on the source and on the crosslinking processes[223,224]. Several research groups compared non-resorbable ePTFE membranes with collagen membranes and the results show that collagen membranes stimulate proliferation of gingival fibroblast cells, promoting extracellular matrix synthesis in a significant high amount. *Wang et al*. showed higher osteoblast adhesion on collagen surfaces and collagen membrane to stay intact since the epithelial proliferation is critical, preventing

apical proliferation[225]. Animal studies conducted on BioOss Collagen® membrane showed that the collagen membrane led to periodontal regeneration, that the material resorbs in 8 weeks and that, during resorption, a slightly inflammatory zone, completely disappeared after total resorption, is visible around the implant[79,223]. A huge amount of studies on collagen membranes has been conducted, and all of them reveal excellent biocompatibility and promotion of periodontal regeneration, but collagen membrane show limited toughness and low space maintenance[31,226]. Therefore, there is still the opportunity and the need to develop a material which could swell and fill the irregular shape of the gingiva flap, in order to stabilize the membrane, coupled with antiadhesive properties which guides epithelial regeneration without generating inflammatory reactions and to prevent cell in-growth in the wound defect.

Synthetic. Synthetic materials used in GTR are usually based on organic aliphatic thermoplastic polymers, such as poly-lactic, poly-glycolic and co-polymers of them. The advantage of using synthetic materials, is that it is possible to customize some properties, such as degradation kinetic and mechanical properties, by varying the length of the chain and the amount of lactides or glycols[227]. The most important drawback associated with this materials is that, during the hydrolysis, by-products increase the local pH and usually cause cytotoxicity[228]. Furthermore, these materials typically degrade trough a bulk degradation, compromising the mechanical stability and interfering with periodontal regeneration. There are some commercially available synthetic products, the first of which to appear on the market was Guidor®, a double-layer membrane, made of polylactic acid and citric acid ester acetyl tributyl citrate. This membrane is the example of how it is possible to produce a particular morphology in a precise manner. The external layer of Guidor® has rectangular perforations to promote tissue integration, while the internal layer has smaller circular perforations and an outer space to maintain the space between the membrane and the root surface[41,229]. Between the two layers, an internal spacer promotes tissue ingrowth. Animal studies showed complete resorption of the membrane within 12 weeks, even if it was detected as a foreign-body. Several other products have been developed, such as Resolute®, made of poly-lactic-co-glycolic acid co-polymer, reinforced with polyglycolide fiber[230], Vycril Periodontal Mesh® , made of a co-polymer of lactide and glycolide[231], Atrisorb®, which is produced in a flowable form of polylactic and is formed after exposure in 0.9% saline solution for 6 minutes, by the dental practice[232]. All these membranes have been tested *in vitro* and *in vivo* and show good periodontal regeneration, but many of them provoke foreign-body reaction, mostly due to degradation of by-products[41]. Besides the already mentioned polyester membranes, some research groups have been trying to produce polyurethane membranes, which degrade via enzymatic degradation[233]. Animal

studies showed that these membranes cause inflammation and that recession was more pronounced than in polylactic membranes[41,234].

2.5 GROWTH FACTORS IN PERIODONTAL TISSUE ENGINEERING

Bone grafts and membrane materials promote periodontal regeneration, usually in an osteoconductive manner[112]. In order to enhance healing and to promote periodontal regeneration via induction of osteoblast differentiation, incorporation of bioactive molecules into the scaffold and their local release for a period of time is a potential approach[235]. Two different procedures to incorporate growth factors are available, one of which is during the preparation of the material[236], the second one is after the fabrication[237,238]. The bioactive molecules which are demonstrated to exert strong effects in promoting periodontal wound repair in preclinical and clinical studies, include Platelet-rich growth factor (PDGF), Bone morphogenetic proteins (BMPs) and enamel matrix derivative (EMD)[235,239–241]. The high potential of growth factors derives from their ability to stimulate the interaction between mesenchymal stem cells and epithelial stem cells during tooth formation, with all the consequent processes, such as collagen formation, mineralized matrix deposition and fibroblast proliferation, etc[235,242]. Bone grafts or membranes alone, have the potential to conduct new periodontal tissue formation, thus by adding these kinds of active molecules it is possible to produce osteoinductive materials. The morphology of the scaffold, in which signaling molecules are incorporated, is fundamental in order to achieve a prolonged and effective release. In particular, the incorporated molecules are released in a diffusion mechanism which depends on porosity and interconnectivity of the material. Furthermore, the degradation properties of the graft or of the membrane could affect the release of growth factors, that would be fast because of the degradation rate, and also the way in which the material degrades, for example by surface or bulk degradation, resulting in a controlled or burst release, respectively[184,243–245]. The research and development process, aimed at obtaining a commercially available product containing growth factors, is difficult in terms of cost and regulatory steps, but of course growth factors have a great potential in tissue engineering, hence it is important to know which are their potential and possible applications.

Platelet-rich growth factor (PDGF): is one of the principal growth factors related to wound healing, since several *in vitro* and *in vivo* studies showed enhanced proliferation and migration of periodontal ligament cells. In nature, PDGF is formed by the conjugation of two polypeptides, growth factor-BB and growth factor-AA, encoded by two different genes. Several investigators have demonstrated that all isoforms have effect on proliferative activity *in vitro* [246,247]. PDGF is a

chemotactic growth factor, which promotes collagen synthesis, stimulates gingival fibroblast hyaluronate synthesis and fibroblast proliferation. Furthermore, in culture with osteoblast-like cells, PDGF regulates the expression of alkaline phosphatase and osteocalcin[248]. PDGF was applied, by *Lynch et al*.[249], in conjunction with another growth factor, the insulin-like growth factor-1, in dog experiments, and the results demonstrated an important effectiveness on periodontal regeneration. Furthermore, human clinical trials showed that the combination of these two growth factors stimulate significant bone regeneration in periodontal defects[250]. Several studies were performed, in order to compare the effect of PDGF and IGF-1, and the results indicate that PDGF alone could significant stimulate formation of new cementum and collagen production[251]. Molecular cloning and large scale purification has allowed production of recombinant human platelet-derived growth factor and a combination of this molecule with β-TCP is commercially available (GEM 21®, Osteohealth)[252].

Bone morphogenetic proteins (BMPs): BMPs are members of the superfamily of transforming growth factor-beta (TGF-β)[235,252]. BMPs are abundant proteins in bone and are produced by several cell types, including osteoblasts. BMPs are retained in bone allografts, in particular BMP-2, -4 and -7; due to this characteristics, allograft materials are defined osteoinductive and could influence cell behavior *in vivo*. Several animal and human studies demonstrated the potential of BMPs growth factors in periodontal regeneration, showing a significant increase in alveolar bone healing[253–255]. BMPs influence cells, in particular could encourage division and chemotaxis of undifferentiated mesenchymal cells and osteoblast precursors, inducing expression of genes involved in bone formation, like osteocalcin and alkaline phosphatase[256].

Enamel matrix derivative (EMD): in order to achieve periodontal regeneration, mimicking the process that takes place during tooth formation is a fascinating approach. During the development of a nascent root and periodontal tissue, Hertwig's cells secrete enamel matrix proteins, which are deposited onto root surface, providing an initial and essential step in which the surrounding cells are influenced to migrate and form cementum, periodontal ligament and alveolar bone[257,258]. The presence of this layer between the peripheral dentin and the developing cementum, have led to the development of Enamel matrix derivatives in the form of purified acid extracts of proteins from pig enamel matrix (Emdogain®, Strauman AG, Basel, Switzerland)[259,260]. The principal component of EMD is Amelogenin, which is composed by highly conserved extracellular matrix proteins codified by one gene[241]. Amelogenin, under physiological condition, is assembled into nanospheres which form an extracellular matrix, that during degradation by enzyme metalloproteinases, release in a controlled process bioactive peptides for weeks[42,242]. This process promotes wound healing, bone

formation and root resorption. EMD could mimic odontogenesis by the recruitment and stimulation of cementoblasts to form root-cementum in the root-surface[261]. Then, the new root-cementum will lead to regeneration of periodontal fibers and alveolar bone. The first application of Emdogian® was in 1997 and nowadays it is the only product on the market that has the potential for triggering clinically significant regenerative responses in periodontal ligament cells[259].

2.6 GENE THERAPY APPROACH IN PERIODONTAL TISSUE ENGINEERING

The major drawback associated with the use of local delivery of growth factors is their short biological half-life *in vivo*[240,262]. Furthermore, a high dosage of bioactive molecules is typically required to promote tissue regeneration; hence, an alternative approach to growth factor delivery is the use of gene therapy for periodontal regeneration[262]. Gene therapy consists in the insertion of genes into an individual's cells in order to promote the expression of a specific growth factor, and consequently a biological effect[37,239,263]. Two main strategies have been developed to induce cells to produce specific growth factors: 1) *in vivo* technique, in which the gene vector could be introduced directly into the target site[262,264]; 2) *ex vivo* technique, in which selected cells can be harvested, expanded and genetically transduced and then re-implanted[265].

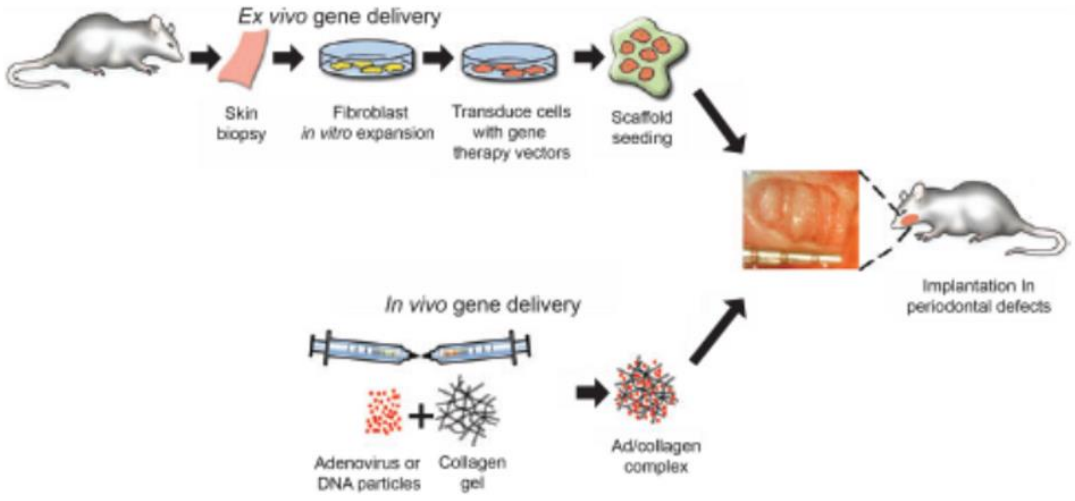

Figure 5. Gene delivery approaches in periodontal tissue engineering[240].

Gene therapy has been applied to induce the expression of the most used growth factors, such as PDGF[266–268] and BMPs[269]. In the *in vivo* technique the gene of interest is directly delivered in the body, altering the normal expression of the target cells. In the *ex vivo* technique, instead, the target cells are taken with a biopsy and then, by usually using an adenovirus vector, the genetic material is

incorporated into cells and transfected cells are subsequently re-implanted in the periodontal defect[240,270]. Despite the great potential of these technique, there are several issues, in particular related to the safety of the adenovirus vector, that mean gene therapy could be a potent approach for the future, but many studies have still to be done to prove the safety[263,271].

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CHAPTER III

PERIODONTAL DISEASES

Periodontium is defined as a complex system of tissues supporting and casing the tooth[1]. Periodontium tissue includes root cementum, periodontal ligament, dentogingival junction and alveolar bone[1,2]. Each of the periodontal tissues has its specialized structure, which defines its function, that is only achieved through structural integrity and interaction between components. The National Institute of Dental and Craniofacial Research reports that 86% of adults over 70 years has a moderate periodontitis and, over 22% has lost its own teeth which involve several health, psychological and economical issues[3]. Any disorder that affects the surrounding tissue which supports the teeth, related to inflammation, trauma, genetic, metabolic or neoplastic development is considered as a periodontal disease[4]. The most common procedure to replace tooth loss is by using titanium implants, but practice is successful if there is sufficient bone which could sustain the implant and the future crown[5–7]. Periodontal diseases are usually referred to as common inflammatory disorders of gingivitis, which could evolve in periodontitis, caused by pathogenic microflora in the biofilm or dental plaque which forms around the teeth[8–10]. Many studies have been conducted to understand the cause of these diseases, prevention procedures and many solutions have been developed to control inflammation and infection, to achieve a complete periodontal regeneration. The most effective prevention and treatment involves controlling of the bacterial biofilm and other risk factors, in order to arrest the progressive disease and to restore the lost surrounding tissue[11,12].

3.1 GINGIVITIS AND PERIODONTITIS

By its nature, the masticatory apparatus provides contiguity of a rich of bacteria environment (oral cavity) with soft and skeletal tissue. The mildest form of periodontal disease, known as gingivitis, is caused by the bacterial biofilm that accumulates on teeth adjacent to the gingiva[13–16]. Gingivitis affects around 90% of adults worldwide, in particular periodontal diseases, in developing country, are very common, although no correlation is present between gingivitis and the indigenous populations[17,18], in fact this is just the result of a low oral hygiene, being, by the way, a readily reversible disease through a simple, effective oral care[19–21]. Gingivitis does not affect the tissue that supports tooth, however if the gingivitis is not controlled, inflammation and bacterial contamination

could deeply extend into tissues and cause loss of supporting connective tissue and alveolar bone, causing the so called periodontitis. Once a periodontal pocket is formed and has been filled with bacteria, the situation becomes largely irreversible with a high probability of tooth loss[22]. Periodontitis is the major cause of tooth loss, as the result of bone resorption around the tooth. The bacterial contact continuously stimulates immune system cells, which react activating defensive mechanism that result in an inflammatory response[23]. Inflammation is the result of a cascade of correlated events: the immune system responses after an external stimuli, like bacterial infection, is triggered, through production of cytokines and chemokines (such as interleukin 1β); these cytokines promote cells to differentiate into osteoclasts, causing loss of physiological homeostasis between osteoblasts and osteoclasts, that means an imbalance between resorption and production of new mineralized bone, which causes tooth loss, pain, discomfort, impaired mastication and, furthermore, many expensive procedures and psychological consequences[4,23].

Gingivitis and successive periodontitis are the result of accumulation of pathogenic microorganisms in the biofilm, which could be enhanced by genetic and environmental factor, for example tobacco use. Furthermore, many studies demonstrated the correlation between periodontitis and other pathologies, for example periodontal disease have been associated with cardiovascular disease, stroke, pulmonary disease, and diabetes[24,25].

3.1.1 Causes of gingivitis and periodontitis

3.1.1.1 Oral microorganisms

Enormous effort has been devoted to the study of periodontal disease associated to the microflora. Cultural studies revealed that more than 500 distinct microbial species can be found in dental plaque, furthermore even more of this well-studied and familiar microbial environment remains uncharacterized[26]. The oral cavity, like all the surfaces of body in contact with the external environment, has a substantial microflora living in symbiosis with a healthy host. Hundreds of different species of aerobic and anaerobic bacteria, grow on tooth surface as complex, colonies and form the so called biofilm[27,28]. First, bacteria adhere on the tooth surface and, following accumulation, they produce an extracellular polymeric substance forming a complex multilayer structure called biofilm, which protects cells from the action of immune system and external agents. Bacterial counts in the supragingival zone, on one tooth surface can exceed 1×10^9 bacteria, below the gum, the number of bacteria for a healthy shallow crevice could reach 1×10^3, and increase until 1×10^8 in the periodontal pocket[29]. During maturation of dental plaque and until the state that is associated with periodontal disease, the number of gram-negative and gram-positive bacteria increases. A constant and frequent cleaning of the teeth, (every 48 h) can maintain the biofilm mass

at an amount compatible with gingival health[30,31]. Research efforts have identified specific clusters of bacteria species that are commonly present in the subgingival sites and are associated with periodontal disease. These pathogens include *Porphyromonas gingivalis, Tannerella forsythensis, Trepnema denticola,* and *Actinobacillus actinomycetemcomitans*[32–34]. These and other bacteria and fungi as *Candida albicans,* are associated with periodontal disease, in particular in young adults[35]. Infections of periodontal tissues caused by these and other organisms are accompanied by the release of bacterial leucotoxin, proteolytic enzymes such as collagenases, fibrinolysin and others, which degrade the connective tissue and modify the bone homeostasis. In addition to biofilm formation, and consequently penetration of bacteria in periodontal pocket from gingival sites, other co-factors could enhance and promote periodontal disease, such as genetics, smoke, stress, concomitant pathologies[36].

3.1.1.2 Tobacco and alcohol use

Tobacco use is a major risk factor for periodontal disease[37]. The rate of periodontal disease progression is increased in smokers and decreased to the same rate as non-smokers after tobacco cessation[38]. Tobacco enhances biofilm formation and could promote gingivitis, loss of tooth support and precancerous gingival leukoplakia. Furthermore, treatments after diagnosis of periodontitis are less effective in smokers and regeneration of periodontal tissue after surgical procedures is compromised by tobacco, which could promote new biofilm formation in the periodontal socket and cause the so called peri-implantitis, the resorption of bone around the titanium implant[38–41]. In the US, about half the risk of periodontitis can be attributable to smoking[42].

Alcohol consumption has also a significant correlation with loss of periodontal support[43]. Many studies on the negative effects of alcohol consumption on degeneration of periodontal disease[43] have been conducted. Researchers demonstrated that consumption of alcohol can occasionally cause inflammation of the gingiva and, if it falls in the abuse, the alcohol's drying effect on mouth can contribute to the formation of plaque, a sticky bacterial layer that can trigger an inflammatory response in gums. Alcohol slows production of saliva, which helps to neutralize the acids produced by plaque, so an accumulation of these acids can lead to the development of the early stages of periodontal disease[44]. The effects of alcohol or tobacco on periodontal disease have been explained by poor oral hygiene of chronic alcoholic consumers or smokers. However, biologic plausibility of this relationship exists, in fact both alcohol and tobacco have adverse effect on the neutrophil's function, on the clotting mechanisms, causing complement deficiency, increased resorption and decreased formation of bone, reducing the vitamin B-complex production and having direct toxic effects on periodontal tissues[45–47].

3.1.1.3 Presence of other pathologies

Several studies conducted on patients with osteoporosis, showed a possible correlation between the presence of osteopenia and the increased progression of periodontal attachment loss[48,49].

People affected by type I and type 2 diabetes, have more widespread or severe periodontal disease than individual without diabetes[50]. However, people with a controlled diabetes do not show any increased risk of periodontal disease than people without diabetes[51]. The correlation between diabetes and periodontitis is not unexpected, since diabetes is associated with impaired wound healing, increased monocyte response to dental plaque antigens and impaired neutrophil chemotactic responses and all of these events can lead to increased local tissue destruction[52].

Finally, people with HIV disease have a relation with oral necrotizing at gingiva and periodontal level[53]. Before the advent of highly active antiretroviral therapies (HAART), the oral disorder in patient with HIV virus were more common[54]. The disorder was characterized by pain, bleeding gums, halitosis, low-grade fever and malaise. The severity of oral symptoms of HIV has generally reduced in population with access to HAART[54].

3.1.1.4 Nutrition and stress

Nutrition and stress are two causes which have poor demonstration in correlation with periodontal disease[55]. It is demonstrated that a Vitamin C deficiency causes a decrease in the formation and maintenance of collagen, an increase in periodontal inflammation, hemorrhages and tooth loss[564]. However, in developed countries studies trying to demonstrate correlation between hypovitaminosis and periodontal disease failed, and also in the developing country the relation is poorly quantified. For sure, malnutrition is an important factor which may promote gingivitis and future periodontitis, in particular in populations which could acquire unusual species of oral bacteria from living near cattle[57]. Physio-social disease, as many other diseases, may have an effect in the progression of periodontal disease, but their precise role in the pathogenesis is unknown[58,59].

3.1.1.5 Genetics

During the last decades, the scientific literature has seen an exponential increase in the number of reports claiming links for genetic polymorphisms with a variety of medical diseases, particularly chronic immune and inflammatory conditions[60]. Periodontal diseases are multifactorial in nature, while microbial and other environmental factors are believed to initiate and modulate periodontal disease progression, there now exist strong supporting data that genetic and environmental risk factors play a role in the predisposition to and progression of periodontal diseases[61,62]. Several features like cytokines, cell-surface receptors, chemokines, enzymes and others that are related to antigen recognition, the immune system, host response, among others, are determined by genetic

components, polymorphisms of which may increase the susceptibility of an individual to periodontal disease[63]. The application of genetic information, identifying genes and their polymorphisms, can result in a novel diagnostic method for risk assessment, early detection, and selection of treatment approaches. The relation between microbial cause and periodontal disease was demonstrated and well established[22]. However, if periodontitis was simply caused by a combination of specific periodontal pathogens, the disease should have developed in the majority of subjects infected by these organisms, which happens just in the subject with gingivitis and minor periodontitis. In subjects in whom the disease quickly progressed from chronic gingivitis to destructive periodontitis, the cause could not be explained by the microbiology alone. The scientific literature, during the last decades, has been seeing an exponential increase in the number of reports claiming links for genetic polymorphisms with a variety of medical diseases, particularly chronic immune and inflammatory conditions[64,65]. Most of genetic research in periodontitis has now focused on gene polymorphisms that play a role in immunoregulation or metabolism, such as cytokines, cell-surface receptors, chemokines, enzymes and others that are related to antigen recognition. Future strategies for utilization of genetic polymorphisms in periodontics should consider two factors. The first, is to perform large-scale genetic analysis in different populations using many target genes; the second factor is the need to develop a statistical analysis which combines genetic aspects with environmental factors[60,66,67].

3.2 PATHOGENESIS AND DIAGNOSIS

Pathogenesis is the mode of origin or development of diseases. Gingivitis and chronic periodontitis are due to a combination of multiple factors; in particular, the bacteria biofilm is the principal responsible of gingivitis and, if not treated, it could transform into periodontitis. Although bacteria are necessary for periodontal disease to take place, a susceptible host is however needed. Chronic marginal gingivitis is clinically characterized by gingival redness, edema, bleeding, changes in contour, loss of tissue adaption to the teeth and increased flow of gingival crevicular fluid[23]. The inflammatory and immune system response, which is stimulated in the gingival and periodontal tissues in response to the chronic presence of plaque bacteria, results in a destruction of the periodontal tissues leading to clinical signs of periodontitis. During an infection, the host response is essentially protective, but both hypo-responsiveness and hyper-responsiveness of certain pathways could result in enhanced tissue destruction[68]. Both the host and bacteria in the periodontal biofilm release proteolytic enzymes that damage tissue. The initial stage of gingivitis involves an initial lesion which appears as an acute inflammatory response with characteristic neutrophilic infiltration[69]. This first stage is due to chemotactic attraction of neutrophils by bacterial constituents

and direct vasodilatory effects of bacterial products[23,70]. Progression of lesion is dominated by the expression of cytokines which are responsible for recruitment, differentiation and growth of the characteristic cell types with progressive chronicity of the lesion[23]. At this time point, an accurate removal of plaque results in a restoration of healthy tissue without residual tissue destruction[71]. If not treated, chronic lesion could necrotize as acute ulcerative gingivitis, which is an acute infection of the gingiva characterized by interdental soft tissue necrosis, ulceration, pain and bleeding[72]. It is histologically characterized by a bacterial infiltration in the connective tissue. A common feature of nearly all cases is a very poor oral hygiene and nearly all cases can be managed with local debridement, improved plaque control, and judicious use of antibiotics[22,23].

Periodontitis is clinically different from gingivitis, since it is characterized by loss of the connective tissue attachment to the teeth in presence of gingival inflammation[8,73]. Many studies demonstrated the similarity in the histopathology between gingivitis and periodontitis, and seems that periodontal inflammation is a consequence of gingiva inflammation[74], but the mechanisms of this progression are not clear, and the factors that lead to the initiation of periodontitis lesions are unknown[75]. The infiltration of bacteria in the periodontal pocket is the major cause of progression of gingivitis in periodontitis, and results in a loss of periodontal ligament and disruption of its attachment to the cementum, as well as to the alveolar bone resorption[23]. Bone resorption occurs when inflammatory mediators reach a critical concentration, which depends on the expression of pro-inflammatory cytokines, such as interleukin 1β (IL-1β) [76]. On the opposite site, pro-inflammatory cytokines expression levels are controlled by the expression of anti-inflammatory cytokines, like interleukin 10 (IL-10)[77]. In normal physiological conditions, there is a balance between bone formation and bone resorption. As it happens in certain inflammatory conditions, this balance can be altered and, in the case of bone tissue, bone formation could be enhanced, or diminished. This equilibrium is regulated by the relative expression of proteins, such as receptor activator of nuclear factor kappa-B (RANKL) and the soluble decoy receptor osteoprotegerin (OPG). During an inflammation response, RANKL/OPG ratio is altered by the action of pro-inflammatory cytokines, such as IL-1β, that induces osteoclastogenesis increasing the expression of RANKL, while counteraction by anti-inflammatory mediators, such as IL-10, decreases RANKL or increases OPG, in order to inhibit osteoclastogenesis. During periodontitis, this equilibrium is loss and osteoclastogenesis is promoted, resulting in alveolar bone resorption; without an adequate treatment, active periodontitis leads to tooth loss[71].

Diagnosis of gingivitis and further periodontitis is important to find a possible treatment strategy and to avoid tooth loss. Chronic gingivitis usually results in bleeding from the gum during tooth brushing, or in the most critical case could be present an underlying blood dyscrasias or bleeding

disorders. Gingivitis could be avoided by an accurate oral hygiene, and systematic controls by specialists. Otherwise, chronic periodontitis is usually asymptomatic, until the disease is so severe that teeth shift, loosen, or are lost. Furthermore, patients with advanced periodontitis may also have recurrent periodontal abscesses and halitosis. The clinical diagnosis of periodontal disease is based on visual and radiographic assessment of the periodontal tissues and on measurement of the space between gum and tooth, which is usually around 1 to 3 mm[78]. During a clinical examination, pocket depth and tissue support are measured at four to six locations around every tooth and the amount of supragingival periodontal biofilm, dental calculus, gingival bleeding and exudate are recorded[79,80]. These methods are used to diagnose an existing disease, to determine the prognosis and to monitor the disease progression. Emerging diagnostic methods include the use of biomarker assays, intraoral computer tomography (CT) and saliva-based diagnostic methods. The first emerging diagnostic method is based on the fact that the inflammatory exudate adjacent to the teeth contains several biomarkers of periodontal inflammation that might be useful in the prediction of future disease risk[81]. CT could be used in future to obtain a three-dimensional reconstruction of alveolar bone and to calculate important parameter such us height, porosity, density and the possibility to compare data from different site and from different time points[82]. Finally, saliva in the future will replace blood for many diagnostic assays, and is already used to monitor the concentration of drug and viral load, for example[83]. For periodontal disease, saliva-based diagnostic methods are promising because periodontal pathogens are readily detectable in it[84].

3.3 PREVENTION AND TREATMENT OF GINGIVITIS AND PERIODONTITIS

Controlling risk factors that cause gingivitis and periodontitis is the common approach to prevent degeneration of periodontal disease. The widely accepted causes related to the gingivitis and periodontitis is the formation of bacteria plaque on teeth, in the absence of proper oral hygiene[15,16,31]. Furthermore, several ancillary factors such as smoking, diabetes, poor education, infrequent dental attendance and stress could enhance the progression of periodontal disease[4,8]. Tooth brushing and the use of dental floss and other devices to remove bacterial plaque from the teeth are the most common ways of disrupting or removing the periodontal biofilm from teeth, however these procedures are effective if used every days[31]. In fact, after oral hygiene procedures are ceased, the biofilm begins to form on the teeth within 24 hours and could cause gingivitis in 10 days[85]. However, through tooth cleaning, the gingiva could return in healthy conditions in about 1 week, that means that, by accurate tooth cleaning, gingivitis could be controlled and periodontitis could be avoided for many years[31,85]. In the last years, many mouthwashes and dentifrices with

antimicrobial effect have become commercially available, and could be helpful in the disruption of biofilm. In addition, mechanical cleaning methods, performed by practitioners, can reduce gingivitis[86,87]. The role of these prevention methods, have not been established in the prevention of periodontitis, but there are evidences that antimicrobial compounds, and a proper cleaning of teeth, can reduce recurrence of periodontal disease after non-surgical periodontal treatment[86,88]. The major ancillary risk associated with the progression of periodontal disease, is the use of tobacco[39]. The rate of periodontal disease progression increases in smokers than in non-smokers, furthermore treatments of periodontitis are less efficacy in smokers people[89–92]. These approaches need an adequate health education and promotion program, in particular in the developing country, where poor general health, restricted access to dental care and inadequate oral hygiene usually translate into a high occurrence of gingivitis and periodontitis. Gingivitis could be prevented and treated, usually with dental care and, in some case with the use of antimicrobial compounds, on the other hand, periodontitis treatments would involve both non-surgical and surgical approach in the most chronic case[93–95]. Dental plaque and calculus can be removed from tooth and root surface manually or with specific instruments, in particular it is important to mechanically act and with attention to the periodontal pocket. Once a periodontal pocket becomes filled with bacteria, the situation becomes largely irreversible and, in this case, a surgical procedure might be necessary[8,10]. For patients with advanced disease, a variety of types of periodontal surgery to reduce the depth of periodontal pocket is used, gaining access for debridement of residual dental calculus and plaque, and to stimulate regeneration of lost periodontal tissue support by using bone grafting materials, guided tissue regeneration membranes and biological substances[96,97]. Coupled with surgical or non-surgical treatment, a supplemental use of systemic antibiotics is usually provided[98] or local antiseptic drugs[99], which provide additional benefit compared with debridement alone[100,101].

In addition to mechanical therapy, a novel approach using high and low level energy laser lights has attracted considerable attention in periodontics[102,103]. Recent evidences have demonstrated that the application of laser energy could be useful to kill bacteria and to control inflammation, furthermore it could stimulate tissue regeneration in periodontal and peri-implant disease[104,105]. High-level laser therapy is nowadays used to debride diseased tissues and to render aseptic and nontoxic the treated sites[106]. When a high-level laser light is applied, a low level of energy penetrates or scatters into the surrounding tissues and this low level energy is used to stimulate tissues and cells without substantially changing the tissue, and recent studies showed the effect in the increase of periodontal wound healing[107]. If high- and low- level laser therapy are properly used, the combined effects would result in improved pocket healing, in combination with the regeneration of soft and bone tissues[106].

3.4 PERI-IMPLANTITIS AND PERI-PROSTHETIC INFECTION

In order to replace the function of a tooth loss, the most common procedure is the use of a screw dental implant[5]. Implants could be divided in three categories: endosteal (implant within the bone), subperiosteal (framework placed on bone) or transosteal (implants placed through the bone from the superior to the inferior aspect)[108]. The most used dental implant is endosteal and could be placed inside the bone with the external cap covered by soft tissue, before to place the crown, or the coronal aspect could be placed at the margin of gingiva and not covered with soft tissue. The most commonly used metal for dental implant is Titanium and Titanium-Aluminum-Vanadium alloy (Ti_6Al_4V)[109]. The osteointegration of Titanium implant is related to the design of the screw, the surface roughness, the presence of bioactive molecules on the surface, and the status of the surrounding tissue[110,111]. Nowadays, surface treatment of dental implant is a well-known technique which allows obtaining a micro-roughness surface that directly interacts with osteoblast cells and promote a strong fixation between bone and implant[112,113]. New concepts about the surface of the implant are the use of biological molecules, such as collagen and hyaluronic acid on the surface not only to conduct new bone formation, but also to stimulate it[114–116].

As in natural teeth, also for implants many external factors could influence the success of the surgical procedures. The main problem is the bacterial contamination of the implant surface, which results in an early inflammation of the soft tissue and could progress in a resorption of bone and connective tissues around the implant and a consequent failure of it[117,118]. Peri-implantitis is a site-specific infectious disease that causes an inflammatory process in soft tissues and bone loss around an osteointegrated implant in function.

Although high success rates for endosteal implants have been reported, failures occur, and same implants are lost or removed. Around 1% to 5% of implants at 15 years of follow up fail, and in the 10% of failure the cause could be related to the peri-implantitis[119–121]. One of the major causes of peri-implantitits is the bacterial colonization of implant surfaces, but ancillary risk factors are involved, such as previous periodontitis, poor oral hygiene, tobacco and alcohol consumption, pre- and post-operative therapies and genetic susceptibility. The microflora present in mouth has a fundamental impact on the biofilm formation on the surface of the implant, furthermore periodontal compromised patients have a higher risk of peri-implantitits than unaffected patients, a transmission of periodontal pathogens from periodontal sites to implant is possible[122]. Several researchers studied the possible associations between a previous periodontitis and peri-implantitis, and found that subjects with a history of periodontitis have a higher risk of re-infection of the implant site[123–125]. The peri-implant disease refers to the pathological inflammatory situation that affects the tissue

surrounding the implant, and could be classified in peri-implantitis mucositis and peri-implantitis[11,126]. Mucositis, as gingivitis, is defined as a reversible inflammation of the soft tissue surrounding the implant, and could be controlled after the surgical procedures with a proper oral hygiene, and by the use of antimicrobial compounds and antibiotic drugs, via systemic or local administration[127]. Peri-implantitis is an inflammatory reaction at the interface between implant and bone, due to an infiltration of bacteria, which causes a loss of bone and consequent mobility and failure of the implant[11]. Most of the bacteria found in the plaque during periodontitis is also found in the biofilm formation on the failed implant, that means the histopathology of periodontitis is similar to that of peri-implantitis[128–130]. Peri-implantitis could be also caused by the biomechanical issue, due to an overloading at the bone implant surface, that could provoke loss of osteointegration and losing of the artificial support, causing infection of the surrounding tissues and consequently inflammatory process.[131]

Diagnosis of peri-implantitis is based on color changes of soft tissue, radiology evidence of a vertical resorption of alveolar bone, bleeding, suppuration, swelling of the peri-implant tissues and formation of a peri-implant pocket[132]. Treatments of peri-implantitis are quite similar to those adopted for periodontitis. There are surgical and non-surgical approaches, which involve debridement of the infected site and administration of local or systemic antibiotic drugs[11]. However, the reasons why peri-implantitis occurs are poorly understood, even if oral microflora seems to be a defining factor for success or failure of a dental implant. Techniques to prevent peri-implantitis involve the prevention of bacteria biofilm formation on the implant surface and the removal of all microorganisms adhered to the sites of implantation[133,134]. When an implant is exposed to the oral cavity, its surface becomes immediately covered by a salivary pellicle and a protein layer, forming a substrate further colonized by oral microorganisms, which start forming the biofilm[27,135].

A biofilm is an assembling of microbial cells that is irreversible associated with a surface and enclosed in a matrix of primarily polysaccharide material. Biofilms are primarily composed of microbial cells and extracellular polymeric substances (EPS). EPS may account for 50 % to 90 % of total organic carbon of biofilms and could be considered the primary matrix material of biofilm[136]. EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. *Van Leeuwenhoek*, first observed microorganisms on tooth surface by using a simple optical microscope, and can be credited with the first discovery of a microbial biofilm[27,137,138].

Periprosthetic infection (PPI) is a consequence of implant insertion procedures and could be due to a previously periodontitis, to an infection of the prosthesis during insertion, or during the healing process due to a poor oral hygiene[139,140]. In order to avoid contamination of the dental implant surface, there are three different approaches: local debridement, surface decontamination and

antibiotic therapy[132], coupled with regenerative procedures using bone graft substitutes in combination with membranes[97]. The decontamination of dental implants prior to surgical insertion, could be done using different techniques, such as air-powder abrasive techniques followed by citric acid application, air-powder abrasive technique alone, gauze soaked in saline followed by citric acid or in chlorhexidine[141,142]. These approaches could reduce the nosocomial infection, but do not reduce the risk of infection if the microorganisms are still present in the insertion site. Local debridement of the infected site, coupled with a regenerative procedure using bone graft and membrane, is the most promising and used technique. In addition, it is possible to administrate a systemic or local antibiotic therapy[143–146]. Conventional systemic delivery of a high dose antibiotic drug causes systemic toxicity with associated renal and liver complications, resulting in the need for hospitalization for monitoring[147,148]. For this reason, in the last decades, many studies have been conducted aimed at developing bone graft which have the ability to release antibiotics *in situ*[99,149].

3.5 ANTIBACTERIAL MATERIALS

Coupling bone grafts and titanium implant is still one of the best solutions to replace bone and dental tissue loss, in particular in large defects, where the present bone is not enough to ensure stability for the implant. It is necessary the insertion of a so called bone filler material. This kind of surgical approach, promotes bone formation and gives stability, guiding bone regeneration around the implant. Despite the high success rate, in 1-5% of procedures, the implant fails and must be removed[121,139]. Many studies have been done on the side of titanium and titanium alloy implants, in order to achieve a good implant bone interface anchorage, in particular surface treatments of implants are nowadays well developed and could ensure their stability[112–114,116], if combined with a biomaterial that guides bone regeneration and controls the infection, to avoid the devastating consequences of periprosthetic infection (PPI) that are hospitalization, high dosage antibiotic therapies, implant removal, limitation in oral function and more surgical procedures, all culminating in financial and psychological burden for both patients and healthcare team.

Current strategies to PPI prevention involve either increasing the rate of new bone formation or the release of antibiotics, in most of the cases with a conventional systemic delivery of a high dose that is not effective in the control of PPI, due to a specific adhesion of bacteria on the biomaterials, and to the very low penetration of the antibiotic into the osseous defect[27,101,149]. Scaffolding properties alone is not enough, large bone defects, zones with acute infections, where bacterial growth is a high possibility and re-infection of native bone are often the result of necrotic tissue removal. Furthermore, if we take in consideration dental defects, bacterial infection risk increases, mostly

due to the natural function of the mouth and teeth, at the interface with a lot of bacteria from different sources.

Obviously, it is necessary to find strategies to prevent PPI, hence the most suitable solution is to release an antibiotic drug *in situ*, using a complex release system to reduce infection and, at the same time, to promote bone formation avoiding fibroblast infiltration from the surrounding soft tissues.

Antibiotics used in drug release complexes should be effective against bacteria, as well as biocompatible, and not to damage the surrounding native tissues.

Staphylococcus epidermidis was inserted in the list of National Nosocomial Surveillance System Report as one of the most often isolated bacterial pathogens in hospitals, and as the most important pathogen involved in nosocomial and periprosthetic infections [135,150,151]. *S. epidermidis* colonizes the skin and the mucous membranes of the human body and is the most abundant bacterium present at the interface between body and external environment[150]. Apart chronic diseases due to previous periodontal pathologies, another important factor which could affect the fate of a dental implant, is the possibility of a contamination of the implant before insertion. Concerning this possibility the most important group of infections caused by *S. epidermidis* are infections caused by foreign bodies, such as implanted devices. This possibility, is one of the most usually cause of PPI, which involves the failure of the surgical procedure. On these devices, bacteria and, in particular, *S. epidermidis,* form the so called biofilm[152]. Due to the poor penetration of antibiotics inside the polysaccharide matrix, the infection results difficult to be eradicated, in particular in submerged implants[11,153]. The infection related to bone defects are quite common not only in periodontal tissue engineering, but in all the surgical procedures which involve open bone fractures and trauma, in particular many studies related to the infection of the hip joint replacement have been conducted[143,145]. Conventional treatments which involve debridement of the pathological site, can only control but not treat the local infection, however synchronous bone regeneration and control of the infection are necessary in clinical treatment. Recently, vancomycin hydrochloride (VCA.HCL or VCA), as one of the most commonly used antibiotic drugs for the treatment of serious, life-threatening infections, has been proved effective against Gram-positive bacteria, and specifically against *Staphiloccocus epidermidis,* which is related to the infection of bone defects, in particular in the nosocomial infections[154,155]. Vancomycin is a widely used antibiotic that provides bactericidal activity and biocompatibility[155,156]. In the clinical practice, vancomycin is normally administered intravenously for systemic therapy, and the dose levels should be monitored in an effort to reduce adverse effects, such as renal disease and toxicity. However, in order to reach effective local dosage, it is necessary to give a high systemic dosage; furthermore, penetration of antibiotics in the

biofilm matrix is difficult if the antibiotic dose provided intravenously is low. For these reasons, local drug delivery systems have aroused great interest in the treatment of PPI and many different systems have been developed or are under developing. The most critical part during the developing of a system for bone regeneration drug delivery is the selection of bone graft materials, since they should have drug-sustained release ability but also an effective ossification bioactivity. The first material used in general bone tissue engineering as drug delivery system, was poly(methylmethacrylate) (PMMA) cement, which is impregnated with antibiotic during the synthesis[157,158]. However, although PMMA cement combined with antibiotic is still considered the gold standard, it has many disadvantages, such as exothermic reaction which avoids the use of sensitive antibiotic drugs, the slow rate of degradation or the non–degradable characteristic make the kinetic release and period of antibiotic deliver difficult to be accurately controlled. In order to overcome the drawbacks of PMMA cement and other non-resorbable materials (i.e. hydroxyapatite cement, allograft), several biodegradable and absorbable bone materials have been developed as antibiotic carriers to prevent or control PPI[11,159–161]. One possible solution, aimed at controlling the release *in situ* and at providing a sustained release for a long time, is to covalently bond antibiotic to the surface of bone graft, or directly on the surface of the titanium implant[12,162–164]. The second way, is to combine biopolymers with bioceramic materials to create a complex system which is able to regenerate new bone and release, in a controlled manner, antibiotic drugs over time. *Antoci et al*. covalently bonded vancomycin on the surface of titanium alloy[162], and tested it against *Staphiloccocus aureus* and *Staphiloccocus epidermidis*. Attachment of bacteria to the implant surface is the first step in the development of PPI and, for these reasons, preventing this adhesion could be a solution to prevent bacterial colonization and infection of the wound site. They tested bacterial adhesion and biofilm formation with different assays and demonstrated that vancomycin bonded on the titanium surface is able to inhibit bacterial growth within 4 weeks. This system has a great potential in the prevention of the devastating bone PPI, however the main drawback is that the use of titanium dental implant is dependent on the amount of bone which is present in the defect site. Periodontal disease usually involves the extraction of the tooth and the loss of consistent amount of bone; so, it is usually mandatory to use a previous bone grafting material to achieve bone regeneration, which could sustain the future implant fixation[97]. Usually, tissues affected by gingivitis or periodontitis have the highest probability to be re-infected after surgical procedures, so it is necessary to use a material able to prevent and control bacterial growth during bone regeneration. For example, *Lian et al*.[159] developed a complex system, made of nano-hydroxyapatite coated with collagen and poly(lactic acid) loaded with vancomycin. The aim of this work, was to develop a bone graft with the ability to regenerate new bone and, in parallel, to prevent bacterial

proliferation through a sustained release for long time. They demonstrated that the addition of vancomycin to the system did not affect the in vitro and in vivo biocompatibility of the composite, which showed excellent cell adhesion, spreading and proliferation. Furthermore, no specific inflammation response was detected in subcutaneous implantation. Finally, a long-term release was assessed, with effective and safe antibacterial ability. However, polyester material made with lactic acid or glycolic acid, go through a bulk degradation, which make the system unpredictable, furthermore many studies showed that the degradation products of this kind of polyester increase the local acidity and stimulate a foreign response[165]. Hence, an approach more predictable is to be preferred, thus using natural materials which could mimic the extracellular environment and could release the drug in a sustained way for the first week, the time-point in which the highest possibility of re-infection exists.

As it is explained in the following paragraphs, the objective of this research work was to design, develop and characterize a set of materials which could prevent PPI, promoting new bone formation and achieving an excellent periodontal regeneration.

3.6 OBJECTIVE OF THE PhD THESIS

Periodontium is a complex system of different tissues, such as connective tissue, cartilage and bone, which work together to sustain the tooth[2]. Gingivitis and periodontitis are devastating diseases that could affect the structure and function of the periodontal tissue[4]. When the gingivitis are not treated and controlled with a correct oral hygiene, they could evolve in periodontitis, which could seriously damage the tissue surrounding the tooth and lead tooth loss[8,22]. The main objective of periodontal tissue engineering is to regenerate the tooth's supporting tissues. Periodontal tissue regeneration involves formation of new connective tissue (cementum and periodontal ligament) and new alveolar bone. The restoration of tooth by using a titanium dental implant is nowadays a quite common procedure[5]. However, the positive fate of a surgical procedure that involves an insertion of titanium screw depends on the quality and quantity of alveolar bone which is present in the extraction site[6]. Conventional procedures involve the debridement of the infected site and the use of a bone graft material and GTR membranes aimed at promoting new alveolar bone formation and soft tissue restoration[97]. Autogenous grafts are considered the current gold standard bone replacement graft materials[166]. However, the main complication is that they require a second surgery and it is important to consider the possible donor site complications such as infection and pain. On the other side, allografts from a donor of the same species could be a source of disease transmission and could stimulate a foreign body reaction[167]. In the last decades, the use of synthetic materials has

aroused great interest, in particular synthetic ceramic materials such as tricalcium phosphate and hydroxyapatite have been used for their good reproducibility, biocompatibility, and non-immunogenicity, but especially because of their similarity to the components of the native bone mineral phase[168]. However, the commercially available products work well as bone graft materials in order to promote bone regeneration but they have not effect against possible re-infections. Periodontal defects are usually due to periodontitis and the probability of a new re-infection, around the titanium implant or before placing it, is high. Hence, it is important to not only promote new bone regeneration, but also to control the bacterial contamination and the possible peri-implantitis or periprosthetic infection (PPI). Whereas peri-implantitis are usually due to an infection which follows a previous periodontitis, PPI could may be also due to a nosocomial infection or to the natural function of the oral cavity which is full of bacteria. Titanium implant surface is a perfect field on which the bacteria could grow fast and form a biofilm which is hard to eliminate and leads to the implant failure[117]. Conventional therapy to control infection of the wound site involves the use of an antibiotic treatment via systemic delivery during the regeneration time[100]. Classical antibiotic therapy is not effective in the control of the infection, mainly due to a specific adhesion of bacteria on the biomaterials and to the very low penetration of the antibiotic into the osseous defect. Furthermore, in order to achieve an effective dosage in the defect site, a prolonged and high delivery dosage via systemic administration, which could generate renal disease, is necessary.

The main objective of this doctoral thesis is to develop a set of novel biomaterials, designed to improve periodontal bone regeneration in patients and to control or prevent the bacterial infection in the wound site, via a sustained *in situ* drug release. The PhD thesis was conducted in the R&D department of Nobil Bio Ricerche srl (NBR), a biomedical company founded in 1994 and located in Portacomaro, in the Italian region of Piemonte. NBR is involved in the bone-contacting medical devices area and its core business is the surface treatment and packaging of titanium dental implants. Costumers are small to medium-size companies that produce dental implants and operate in the market of dental devices. NBR treated, last year, about 400.000 dental implants, serving more than 30 different producers from Italy and other West European countries. Beside acting as a provider to dental implant producers, the company developed and market a bone filler for oral surgery and it is developing several other biomaterials, in particular focusing on biomimetic materials in order to achieve an excellent bone regeneration and a prevention/control of infections.

Materials developed are based on a combination of synthetic and natural materials.

Materials developed during the Doctoral research period are based on β-tricalcium phosphate and hydroxyapatite, used as osteoconductive ceramic materials to produce porous scaffolds or particles, since they are widely used as material in bone regeneration and many works have assessed they

efficacy in bone regeneration during *in vivo* studies[169–172]. These hard materials were coupled with natural polysaccharides, pectin and chitosan. Pectin and chitosan are nontoxic natural polysaccharides that have aroused great interest in the last years in tissue engineering, for their ability to crosslink by means of intermolecular interactions, and to form stable and biocompatible complexes which may simulate the extracellular matrix and interact with cells from the surrounding tissues[173,174]. Pectin is already widely used in food industry but, in the last decades, it has found such application in bone tissue engineering, in particular as a drug carrier. Chitosan is a widely used material and, owing to its biocompatibility, its intrinsic antibacterial nature, its ability to not induce a foreign body reaction and to promote cell adhesion, proliferation and differentiation, it finds many applications, especially in bone tissue engineering.
Three different materials have been developed and characterized:

1. *Three-dimensional porous scaffold coated with a polyelectrolyte complex for periprosthetic infection prevention*
2. *Bioceramic-reinforced hydrogel for alveolar bone regeneration*
3. *Antiadhesive guided tissue regeneration membrane*

The first project was aimed to develop a complex system in order to prevent periprosthetic infection (PPI) in large bone defects. PPI is a consequence of implant insertion procedures and strategies for its prevention involve either an increase in the rate of new bone formation or the release of antibiotics such as vancomycin. Modern surface-engineering approaches allow combining these strategies: in this work, a novel three-dimensional porous scaffold produced using HA and β-TCP, coupled with pectin (PEC)-chitosan (CHIT) polyelectrolyte (PEI), and loaded with vancomycin (VCA) was developed and characterized.
The osteointegration of dental implants and the consequent long-term success is guaranteed by the presence, in the extraction site, of a healthy and sufficient alveolar bone[6,7]. Placement of titanium implants is contraindicated until a vertical bone augmentation is obtained. This goal is achieved using bone graft materials, which should simulate extracellular matrix (ECM), in order to promote osteoblast proliferation and fill the void, maintaining the space without collapsing until the new bone is formed. The goal of the second project was to design, develop and characterize a novel chitosan-pectin hydrogel reinforced with biphasic calcium phosphate particles within 100-300 μm. The polysaccharide nature of hydrogels simulates the ECM of natural bone, and the ceramic particles promote high osteoblast proliferation. Furthermore, the mouldability of the scaffold allows its easy use in highly irregular defects, too.

Melcher's hypothesis[175] assesses that an excellent periodontal regeneration could be achieved if the epithelial and fibroblast cells were excluded from the root site, where stimulating the migration of pre-osteoblast cells is very important. On the basis of this hypothesis, many guided tissue membranes, resorbable and non-resorbable, have been developed in the last years. The gold standard ePTFE membrane has the great disadvantage that it makes a second surgery to be necessary, while on the other side the resorbable membranes currently available showed an unpredictable resorption time and consequent unpredictable periodontal regeneration[176,177]. In the third project, we developed a biomimetic GTR membrane, using a polyelectrolyte complex bulk material and coated with an antiadhesive hyaluronic acid. In this way, we hypothesized to control the regeneration of soft tissue on the membrane surface avoiding the cells down growth, and the stability of the PEI complex could sustain the tissue and could make the regeneration of the tissues predictable. Furthermore, the swelling behavior of the materials allows the membrane to act as a bearing and reduces the possible inflammation response of the surrounding tissues.

The main results about these three projects are shown in the following sections. Since the research work was conducted in the R&D department of NBR company, several other projects have been developed and are under developing, in the field of biomaterials for periodontal regeneration, however the results will not be shown in this thesis.

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CHAPTER IV

RESEARCH AND DEVELOPMENT

4.1 MATERIALS

4.1.1 Treatment of periprosthetic infection using an engineered porous scaffold

4.1.1.1 Materials

In this work we designed, developed and characterized a macroporous ceramic scaffold, functionalized with a polyelectrolyte coating loaded with vancomycin. β-tricalcium phosphate (β-TCP), chitosan (CHIT) with medium molecular weight (Mw = 400 kDa) from crab shell, pectin (PEC) from citrus peel, vancomycin (VCA) and all other chemicals were purchased from Sigma-Aldrich. Hydroxyapatite (HA) was purchased from Fluidinova (Portugal) and the dispersing agent Dolapix CE 64 was purchased from Zschimmer&Schwarz (Germany).

4.1.1.2 Three-dimensional porous scaffold preparation

HA was used because of its ability to enhance the mechanical strength of the scaffold, while β-TCP because of its degradability; they were mixed in a percentage of 25 wt.% and 75 wt.%, respectively, to reach an optimum compromise between the two properties. Briefly, the preparation of the ceramic scaffolds involved the mixing of the HA and β-TCP powders (45 wt.%) with a binding agent (poly(vinyl alcohol), 8 wt.%), and ultrapure water (47 wt.%) to obtain a ceramic slurry. Dolapix CE 64 was added as a dispersing agent (0.5 wt.% of the solid load). Polyurethane (PU) sponge impregnation method was used to obtain macroporous ceramic scaffolds[1,2]. A commercial PU sponge cube (45 ppi) of 11x11x11 mm^3 was soaked into the ceramic slurry for 90 s, followed by compression along the three spatial directions (20 kPa), until 30% of height and left at room temperature for 5 min before repeating the cycle. Impregnation/compression cycles were repeated for 3 times. The ceramic-coated PU sponge was left to dry overnight at 37 °C and then sintered in a furnace at 1150 °C for 12 h in air (heating rate 5 °C/min), in order to obtain a porous HA/βTCP scaffold of 10x10x10 mm^3 (a volumetric retention of 24% was calculated).

4.1.1.3 Engineered coating preparation

Pectin powder was dissolved in a concentration of 1% in acetate buffer (pH 5.5); then, in the same solution, 1% of vancomycin was dissolved. Separately, chitosan 0.5% powder was dissolved in acetate buffer (pH 5.5). The coating process involves two steps of immersion. In the first step, the sintered ceramic scaffold was immersed in the pectin-vancomycin (PEC-VCA) solution for a defined time (60 s). In this step, pectin polyanionic polysaccharides were crosslinked on the surface of the ceramic scaffold due to the Ca^{2+} ions released from the scaffold; then, the clad material was freeze-dried overnight and a HA/βTCP_PEC/VCA scaffold was obtained. The first step allows obtaining a pectin-vancomycin coating with a mass of 7.24 ± 1.77 mg, of which 5.04 ± 0.31 mg of vancomycin (calculated from HPLC release studies). In the second step, the HA/βTCP_PEC/VCA scaffold was immersed for 30 minutes in a chitosan solution: a polyelectrolyte was generated from polycationic chitosan and polyanionic pectin. The construct was then freeze-dried overnight and a final material called HA/βTCP_PEC/VCA_CHIT was obtained. The coating process was evaluated on 20 samples and showed to be repeatable with a final mass value of 11.07 ± 1.7 mg.

4.1.2 Novel bioceramic-reinforced hydrogel for alveolar bone regeneration

4.1.2.1 Materials

In this work, a chitosan/pectin hydrogel reinforced with biphasic calcium phosphate particles was designed, developed and characterized. β-tricalcium phosphate (βTCP), chitosan with medium molecular weight from crab shell (Mw = 400 kDa), pectin from citrus peel and all other chemicals were purchased from Sigma-Aldrich. Hydroxyapatite (HA) was purchased from Fluidinova.

4.1.2.2 Preparation of calcium phosphate particles

HA and β-TCP were mixed in a percentage of 25 wt.% and 75 wt.% respectively, on the basis of a previous study by Morra et al.[3]. Briefly, the preparation of the ceramic slurry involved the mixing of the HA and β-TCP powders (31 wt.%) in ultrapure water (69%). Guar gum was previously dissolved in ultrapure water (3% wt.% of the water content), acting as a pore forming. The slurry was then desiccated in oven at 80 °C for 8 h and then shaped in circular disks using a hydraulic press (Mignon EA/SSN) with a pressure of 17.5 MPa for 10 s. The ceramic disks were then sintered in a furnace at 1100 °C for 1 h in air (heating rate 1 °C/min)[3]. Sintered disks were ground in particles using a grinder machine (GM200, Retsch) and the resulting ceramic powder was sieved in order to obtain a range of particles between 100 and 300 μm. The biphasic calcium phosphate

particles obtained were washed in ultrapure water and desiccated overnight at room temperature under laminar flow.

4.1.2.3 Preparation of composite hydrogel

Pectin powder (P) was dissolved in a concentration of 7% wt. in acetate buffer (pH 5.5); in the same solution chitosan powder (C) was added to obtain a chitosan solution of 4% wt. and a pectin/chitosan rate of 20/80. The solution was kept under stirring for 6 h, then it was centrifuged and the polyelectrolyte (PEI) precipitated was collected. PEI complex (10 % wt.) was mixed using a Thinky Mixer (Retsch) with HA/βTCP particles (90% wt.). The resulting slurry was poured in a circular shape polyester mold and freeze-dried with Lyo5P for 12 h, at 0.06 mbar and -56 °C in order to obtain pectin/chitosan_ceramic particles (PCC) composite hydrogel. For the pectin_chitosan based hydrogel (PC), we poured the PEI solution in a circular polyester mold, and freeze-dried with Lyo5P for 12 h, at 0.06 mbar and -56 °C**.**

4.1.3 Novel pectin/chitosan guided tissue regeneration membrane coated with hyaluronic acid

4.1.3.1 Materials

Chitosan (C) with medium molecular weight (Mw = 400 kDa) from crab shell and pectin (P) from citrus peel, were purchased from Sigma-Aldrich. Hyaluronic acid (HyA) with medium molecular weight were purchased from HTL Biotechnology.

4.1.3.2 Preparation of pectin/chitosan-hyaluronic acid membrane

Pectin powder was dissolved in a concentration of 7% wt. in acetate buffer (pH 5.5); in the same solution, chitosan powder was added in order to obtain a chitosan solution of 4% wt. and a pectin/chitosan rate of 20/80. The solution was kept under stirring for 6 h, then it was centrifuged and the polyelectrolyte (PEI) precipitated was collected. The resulting slurry was poured in a rectangular polystyrene mold and freeze-dried with Lyo5P for 12 h, at 0.06 mbar and -56 °C in order to obtain pectin/chitosan membrane (PCm). Pectin/chitosan membranes were soaked in a solution of hyaluronic acid 0.1% w/w for 2 h and crosslinked using ionic interaction between carboxylic group of hyaluronic acid and amine group of chitosan. The resulting material was re-lyophilized for 12 h, at 0.06 mbar and -56 °C, in order to obtain an antiadhesive surface (PCmHyA).

4.2 METHODS

The following paragraphs describe the procedures and protocols used to characterize the materials developed. At the end of the section, a table summarizes which test has been performed for each research work.

4.2.1 Chemical characterization

ATR-IR

Attenuated total reflectance infrared spectroscopy analysis were performed using a Nicolet iS 10 ATR-IR spectrometer, produced by Thermo Scientific and equipped with a diamond crystal. Samples to be analyzed were placed on the crystal and kept in place by the specific crimping tool. Experimental setup was conducted by acquisition of 32 scans in the range of 500 – 4000 cm^{-1}, with a resolution of 4 cm^{-1}

XPS

X-Ray photoelectron spectra were obtained using a Perkin Elmer PHI 5400 ESCA system, equipped with a Mg anode operating at 10 kV and 200 W. Base pressure was 10^{-8} Pa and the diameter of the analyzed spot 2 mm. A wide range survey spectra was acquired from 0 to 1000 eV of binding energy, and detailed high resolution peaks of relevant elements were also collected. Quantification of elements was performed using the software and sensitivity factors were supplied by the manufacturer. Samples were fixed to the instruments sample holder on one side, while measurements were performed on the other side.

XRD

Phase analysis was conducted using X-ray diffraction (XRD, X'Pert Philips, Cu Kα radiation) during a preliminary study to detect phase composition and phase transformation of the HA/βTCP mix after the sintering process at two different temperatures, in order to decide which processing temperature was the optimal. The test was carried out on the material powder at room temperature.

4.2.2 Structural characterization

SEM

Scanning Electron Microscopy (SEM) analysis was performed to analyze the morphology of porous scaffolds. The samples were mounted on the aluminum stubs and sputtered with gold at 15 mA for 2 min using Agar Sputter Coater. The morphology of samples was captured using a scanning

electron microscope using EVO MA10 system (Zeiss) equipped with a micro-analysis system AZTec (Oxford University UK). The quantitative investigation of the organic surface layer on the ceramic scaffold, was performed with the Energy Dispersive X-ray spectroscopy (EDX) system.

µCT

The microstructure of the scaffold was studied in a nondestructive manner by micro-computed tomography (µ-CT), with a desktop µ-CT scanner (SkyScan 1174, Aartselaar, Belgium). The scanner was set at a voltage of 50 kV and a current of 800 µA, and the sample was scanned at 9.23 µm pixel resolution.

For the ceramic porous scaffold the exposure time per projection was 10000 ms and an aluminum filter of 720 µm was used. The samples scanned had a size of 10x10x10 mm^3.

For the composite hydrogel, the exposure time per projection was 2300 ms and no filter was used. The sample had a cylindrical size of 10mm of diameter and 10mm of height. Imaging analysis were conducted using the CT-Analyzer software, reconstructing 2D tomographic raw images. No contrasting agent was used. Imaging analysis were conducted using the CT Analyzer software, reconstructing 2D tomographic raw images. The threshold levels of the grayscale images were equally adjusted for all the samples to allow the measurement of the porosity.

4.2.3 Physical characterization

Swelling study

For investigating the hydration kinetic, the samples (10mm diameter, 2 mm thickness, three for each case studied) were allowed to swell in different solutions (pH of 2.5, 5.5 and 7.4 for composite hydrogel and pH of 7.4 phosphate buffered saline solution (PBS) for GTR membrane), after recording the initial weight (W_0) at 37 °C. The swollen disks were taken out of the solution at regular time intervals, blotted with filter paper to remove excess surface water, and their swollen weights (W_s) were noted. The water uptake by the network was determined by the following equation:

Hydration Degree (%) = [(Ws – W0)/Ws] x 100 (1)

Degradation study

The stability of the samples was analyzed at different pH (2.5, 5.5 and 7.4 for composite hydrogel, 3 and 7.4 for the porous ceramic scaffold and 7.4 PBS for GTR membrane) at 37 °C. The samples, three for each type, were cut in a cylindrical or cubic shape (10 mm of diameter and 2 mm of height; or 1x1x1 cm^3) and immersed in different solutions, after recording their initial weight (W_0).

During the degradation study, samples were taken out at specific time intervals, freeze-dried and weighted (W_f). The mass loss percentage was calculated using the following equation:

Mass Loss (%) = $[(W_0 – W_f)/W_0] \times 100$ (2)

Solution at pH 2.5: 100 g of potassium dihydrogen phosphate were dissolved in 800 ml of ultrapure water; the pH was adjusted to 2.5 with hydrochloric acid and sufficient water to produce 1000 ml.

Buffer Solution at pH 3: was prepared following the instruction reported in ISO 10993-14:2001 ("Biological evaluation of medical devices: Identification and quantification of degradation products from ceramics"). 21 g of citric acid monohydrate were dissolved in 500 ml of ultrapure water in a 1000 ml volumetric flask, then 200 ml of sodium hydroxide 1 M was added and the solution diluted to the mark with water; 40.4 ml of this solution were mixed with 59.6 ml of 0.1 M hydrochloric acid.

Solution at pH 5.5: 0.49 g of sodium phosphate dihydrate, 13.8 g of potassium phosphate and 0.49 g of sodium chloride were dissolved in 1000 ml of ultrapure water.

Solution at pH 7.4: 0.78 g of sodium phosphate dihydrate, 0.097 g of potassium phosphate and 4 g of sodium chloride were dissolved in 500 ml of ultrapure water.

Buffer solution at pH 7.4: was prepared following the instruction reported in ISO 10993-14:2001 ("Biological evaluation of medical devices: Identification and quantification of degradation products from ceramics"). TRIS-HCl solution was prepared by dissolving 13.25 g of tris(hydroxymethyl) aminomethane in 500 ml of water; pH 7.4 was reached adding an appropriate amount of hydrochloric acid 1 M.

Dulbecco's Phosphate Buffered Saline solution (PBS): was purchased from Gibco® by Life Technologies (Life Technologies Corporation, Paisley, UK).

High performance liquid chromatography

A release study was performed using High Performance Liquid Chromatography (Shimatdzu LC 2010 AHT equipped with Diode array Shimadzu SPD-M10AVP) technique. Three specimens (10x10x10 mm size) for each sample were immersed in PBS solution at 37 °C for 1 week. At each time point, samples were taken out and immersed in fresh PBS solution. The releasing solution was

then filtered with 0.2μm cellulose acetate filters and analyzed using a low pressure gradient method, with a mobile phase made-up by 5 mM ammonium acetate in 0.5 % glacial acetic acid in water and 5 mM ammonium acetate in 0.5% glacial acetic acid in acetonitrile. We used a C18 Kinetex reverse phase column (Phenomenex, Torrance, USA) with a flow rate of 1 ml/min and an injection volume of 20 µl. Vancomycin peak was analyzed at 280 nm.

The amount of vancomycin released was calculated from a linear regression curve (from 0.025 mg/ml to 2.5 mg/ml) with R^2>0.999.

4.2.4 Mechanical characterization

Mechanical characterization was performed using a Bose ElectroForce 5500 equipped with 100 N load cell.

Compressive test

For porous ceramic scaffolds, samples were tested in a cubic shape of 10 mm gauge length, 10 mm of width and 10 mm of thickness, and tested until failure, with a crosshead speed of 1 mm/min.

For composite hydrogel, samples were cut in a circular shape with 10 mm of diameter, and 10 mm of gauge length, with a constant crosshead speed of 1 mm/min. The mechanical properties were performed in both dry and hydrated conditions (soaked in PBS at 37 °C for 24 h). For the composite hydrogel, during preliminary tests no sample could reach a failure, so we decided to perform the compressive test until 40% of the height of the sample which was considered a sufficient value to see elastic and plastic deformation of the samples.

Force-displacement curves obtained from the software-machine, were converted to stress-strain curves. Stress (σ, MPa) was obtained by dividing the applied force (N) with the cross section area (mm^2), while strain was obtained from the displacement using $((L-L_0)x100/(L_0))$, where L0 was initial gauge length and L was instantaneous gauge length.

For porous ceramic scaffolds the 5-15% of strain region was used to measure the modulus of samples and instantaneous drop in more than 20% stress was considered as a fracture point.

For composite hydrogels, the elastic modulus was calculated in the linear region between 0.5% to 5 % of strain, and the stress at 40% of strain was related as maximum elastic stress. The area under the stress-strain curve was calculated and related to the toughness of the sample.

Cyclic compressive stress

For cycling testing, 100 loading and unloading cycles were performed. A first load until 16% of initial height was performed and then a sinus cyclic curve with a frequency of 0.5 Hz and a constant crosshead speed of 1.2 mm/min was performed between 16 and 41% of initial height. Stress-strain

curve was reported and the amount of energy adsorbed and percentage recovered during the deformation cycle were calculated every 10 cycles.

Tensile test

Soft membranes were cut in a rectangular shape with 20 mm gauge length, 2 mm width and a thickness around 2 mm. The mechanical properties were tested in both as-prepared (dry) and hydrated conditions (soaked in PBS for 10 min). For the uniaxial tensile test, samples were stretched until failure (or until the maximum displacement of the testing machine) at the crosshead of 0.2 mm/s. Force-displacement curves, obtained from the machine, were converted to stress-strain curves. The stress (σ, MPa) was obtained by dividing the applied force (N) with cross section area (mm^2) and strain was obtained from the displacement using ((L-L0) x 100/(L0)), where L0 was the initial gauge length and L was instantaneous gauge length. Young's Modulus was calculated in the linear stress-strain region by fitting a straight line.

Suture retention test

A suture retention test was performed on soft membranes and the protocol setup of ANSI/AAMI/ISO 7198:1998/2001/(R) 2004 "Cardiovascular implants-tubular vascular prostheses" was followed. In accordance with it, the suture was carried out at 2 mm from the side of the sample, the crosshead speed is fixed at 1 mm/s, and the amplitude of analysis was fixed at the maximum of the machine, 12 mm. Force-displacement curves obtained from the machine were converted to stress-strain curves. The stress (σ, MPa) was obtained by dividing the applied force (N) with cross section area (mm^2) and strain was obtained from the displacement using ((L-L0) x 100/(L0)), where L0 was the initial gauge length and L was instantaneous gauge length. Suture retention strength was defined as fracture strength during the process test.

4.2.5 Biological characterization

Antibacterial properties

The strain *S. epidermidis* RP62A (ATCC 35984) used in this study is a slime and capsular polysaccharide producer. It was routinely maintained on both tryptic soy agar (TSA, Sigma) plates and monthly transferred to new plates and as frozen suspension in liquid culture medium supplemented with 10% glycerol. The bacterial suspension was obtained by inoculating 100 ml tryptic soy broth (TSB, Sigma) and incubating overnight at 37 °C. After overnight incubation, the suspension was washed three times with PBS, and finally re-suspended in PBS. PBS used for this

experiment has the following composition: 0.2gl^{-1} KCL, 0.2 gl^{-1} KH_2PO_4, 8.0 gl^{-1} NaCl, 1.15 gl^{-1} Na_2HPO_4 with pH at room temperature = 7.3 ± 0.3 and osmolality (mOsm kg^{-1} H_2O) = 290 ± 5%. The bacterial suspension, according to previous experiments, was spectrophotometrically adjusted to the optical density required to obtain $1x10^9$ colony forming units [(CFU)ml^{-1}].

Serial bacterial dilution

For the serial dilution test, 5 ml of bacterial suspension were poured in 6-wells plate (Greiner) containing a total of two replicates for each sample, for each time point (0 h, 24 h and 1week). The bacterial suspension was then incubated with samples for 2 h at 37 °C. The number of viable CFU in the inoculum was determined by spreading 0.1 ml portions from the serial tenfold dilutions of bacterial suspension on TSA. CFU were counted after 48 h of incubation at 37 °C. At the end of the incubation time, samples were carefully rinsed with sterile PBS in order to remove loosely adherent bacteria, and were then sonicated for 10 min in 3 ml of PBS. Previous experiments have shown that, by this way, it is possible to achieve a complete detachment of all bacterial cells from the specimens. The number of viable CFU was evaluated by spreading portions of 0.1 ml from the serial tenfold dilutions (four dilutions and three replicates each) of suspension on TSA plates. Also in this case, CFU counting was performed after 48 h of incubation at 37 °C.

Bacteria adhesion test

For adhesion experiments, 5 ml of bacterial suspension were poured in 6-wells plates (Greiner) containing a total of two replicates for each sample, for each time point (0 h, 24 h and 1week). The bacterial suspension was incubated with the samples for 2 h at 37 °C. After incubation time, samples were carefully rinsed with PBS in order to remove non-adherent bacterial cells, then were fixed in 5% glutaraldehyde – PBS solution and dehydrated using increasing concentration of ethanol in water–ethanol solutions up to 100% ethanol. Dehydrated samples were gold sputter-coated (AGAR Sputter Coater) and observed with an EVO MA10 (Zeiss) scanning electron microscope (SEM) equipped with a micro analysis system AZTec (Oxford University UK). Adherent bacteria on each replicate were counted in five different fields and reported as percentage of analyzed area.

Inflammatory response: TaqMan *Real-Time* PCR

The murine macrophage cell line J774.2 (European Collection of Cell Cultures) was maintained in Dulbecco's modified Eagle's medium (Gibco Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 4 mM l-

glutamine. Cells were grown in a 100% humidified incubator at 37 °C with 10% CO_2 and passaged 2–3 days before use. The J774.A1 cells (2×10^4 ml^{-1}) were seeded into 6-wells tissue culture polystyrene plates (9.6 cm^2 of growth area; Falcon™) containing the samples.

After 4 h, RNA was isolated from J774.A1 cell line using the MagMax-96 Total RNA Isolation Kit (Life Technologies) and then reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. Total RNA concentration was then measured by using a Spectrophotometer (UV-1700, Shimadzu) and RNA quality was assessed by evaluating A_{260}/A_{280} ratio ranging from 1.8 to 2.1. Real-time PCR was performed with the TaqMan Gene Expression Master Mix (Applied Biosystems) with the Applied Biosystems StepOne Plus instrument (Applied Biosystems). The primer sets for the Real-time PCR of mouse *interleukin-1β* (IL-1β), *interleukin-6 (IL-6), interleukin-10* (IL-10), *monocyte chemotactic protein-1* (MCP-1) and *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) (Applied Biosystems Assay's ID: Mm01336189_m1, Mm99999056_m1, Mm99999062_m1, Mm99999056_m1, Mm03302249_g1 respectively) were chosen from the collection of the TaqMan Gene Expression Assays (Applied Biosystems). The analysis was conducted using the method of Comparative C_T ($\Delta\Delta C_T$), which was designed following the manufacturer's instructions.

Osteoblast – like cell culture

Osteoblast-like SaOS-2 cells were used for the cell growth experiments. Experimental cell culture medium (BIOCHROM KG, Berlin) consisted of Minimum Eagle's Medium without l-glutamine, 10% fetal bovine serum, streptomycin (100 μg/l), penicillin (100 U/ml), and 2 mmoles/l l-glutamine in 250-ml plastic culture flask (Corning™). Cells were cultured at 37°C in a humidified incubator equilibrated with 5% CO_2. Cells were harvested prior to confluence by means of a sterile trypsin-EDTA solution (0.5 trypsin g/l, 0.2 g/l EDTA in normal phosphate buffered saline, pH 7.4), re-suspended in the experimental cell culture medium, and diluted to 1×10^5 cells/ml. For experiments, 5 ml of the cell suspension were seeded into 6-well tissue culture polystyrene plates (9.6 cm^2 of growth area; Falcon™), containing the samples. Experiments were performed in triplicate. For SEM analysis, at the selected time point, samples were carefully rinsed with PBS and fixed in 5% glutaraldehyde-PBS. Samples were dehydrated using increasing concentration of ethanol in water-ethanol solutions up to 100% ethanol. Dehydrated samples were gold sputter-coated (AGAR Sputter Coater). Scanning electron microscopy (SEM) was performed using an EVO MA10 (Zeiss), equipped with a micro-analysis system AZTec (Oxford Instruments, UK).

The expression of *Collagen* (COLL1A1); *Osteopontin* (OPN); *Osteocalcin* (OCN); *Runt-related transcription factor 2* (RUNX2); *Alkaline Phosphatase* (ALP) and *Osteonectin* (SPARC) genes as cell proliferation, differentiation and mineralization markers, was assessed using the real time reverse transcription polymerase chain reaction (qRT-PCR). Osteoblast –like SaOS-2 cells were cultured as previously described and the total RNA was extracted using MagMax Total RNA Isolation Kit (Applied Biosystems, Milan, Italy) following the manufacturer's instruction. RNA quality was assessed by checking the A260/A280 ratio (1.6 – 2.0). Then, total RNA was used as a template for cDNA synthesis using random hexamers as primers and the MultiScribe® Reverse Transcriptase (high capacity cDNA RT Kit from Applied Biosystems). cDNA amplification and relative gene quantification were performed using TaqMan probe and primers from Applied Biosystems (Hs 00164004_m1, COLL1A1; Hs 00960641_m1, OPN; Hs 00609452_m1, OCN; Hs 01047976_m1, RUNX2; Hs 01029144_m1, ALP; Hs 00234160_m1, SPARC). Real time PCR was performed in duplicate for all samples and targets on a Step-One instrument (Applied Biosystems). PCRs were carried out in a total volume of 20 µl and the amplification was performed as follows: after an initial denaturation at 95 °C for 10 min, the PCR was run for 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. To normalize the content of cDNA samples, the comparative threshold (Ct) cycle method, consisting on the normalization of the number of target gene copies versus the endogenous reference gene GAPDH, was used. The Ct is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold baseline when amplification of the PCR product is first detected. For comparative analysis of gene expression, data were obtained using ΔC_T method.

Fibroblast cell culture and adhesion test

Fibroblast L929 was used in the cell adhesion experiments. Experimental cell culture medium (BIOCHROM KG, Berlin) consisted of Minimum Eagle's Medium without L-glutamine, 10 % fetal bovine serum, streptomycin (100 g/l), penicillin 100 U/ml, and 2 mmol/l L-glutamine in 250 ml plastic culture flask (Corning TM). Cells were cultured at 37 °C in a humidified incubator equilibrated with 5 % CO_2. Cells were harvested prior to confluence by means of a sterile trypsin-EDTA solution (0.5 g/l trypsin, 0.2 g/l EDTA in normal phosphate buffered saline, pH 7.4), re-suspended in the experimental cell culture medium, and diluted to 1 x 10^5 cells per ml. For experiments, 5 ml of the cell suspension were seeded into six-well tissue culture polystyrene plates, containing the samples (GTR membranes). Experiments were performed in triplicate, at two different time points (24 h and 1 week). To evaluate cell adhesion, cells were seeded at a given density on test samples. At given time intervals, 24 h and 1 week, DNA quantification was

performed, by removing samples from the wells, rinsing with PBS and placing them in another multiwall plate. The total DNA was extracted after 1 week using MagMax Total DNA Isolation Kit (Applied Biosystems, Italy) following the manufacturer's instruction, and the absorbance at 260 nm proper of DNA material was detected.

Cytotoxicity test was performed using neutral red assay. Briefly, it is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The samples after 24 h and 1 week of fibroblast cells culture, were incubated for 2 h with a medium containing neutral red, and subsequently washed with ultrapure water. The stained cells were visualized using an inverted microscope in a standard mode (Leica DMI 4000 B, Germany).

Statistical analysis

Experimental data were presented as mean ± standard deviation. Statistical differences between samples were analyzed using two-way ANOVA using Tukey's multiple comparison test, one-way ANOVA using Tukey's analysis and Student's t-test. Statistical significance was represented as *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

4.3 SUMMARY OF THE TESTS PERFORMED

SAMPLE	ATR-IR	XPS	EDX	XRD
Engineered scaffold	x	x	x	x
Composite hydrogel	x		x	x
GTR Membrane	x	x		

SAMPLE	µCT	SEM
Engineered scaffold	x	x
Composite hydrogel	x	x
GTR Membrane		x

SAMPLE	Release study	Swelling study	Degradation study
Engineered scaffold	x		x
Composite hydrogel		x	x
GTR Membrane		x	x

SAMPLE	Compressive test	Tensile test	Compressive cycle test	Suture retention stress test
Engineered scaffold	x			
Composite hydrogel	x		x	
GTR Membrane		x		x

SAMPLE	Antibacterial properties	Inflammation gene expression	Osteoblast-like cell culture	Fibroblast cell culture
Engineered scaffold	x	x	x	
Composite hydrogel		x	x	
GTR Membrane				x

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4.4 TREATMENT OF PERIPROSTHETIC INFECTION USING AN ENGINEERED POROUS SCAFFOLD

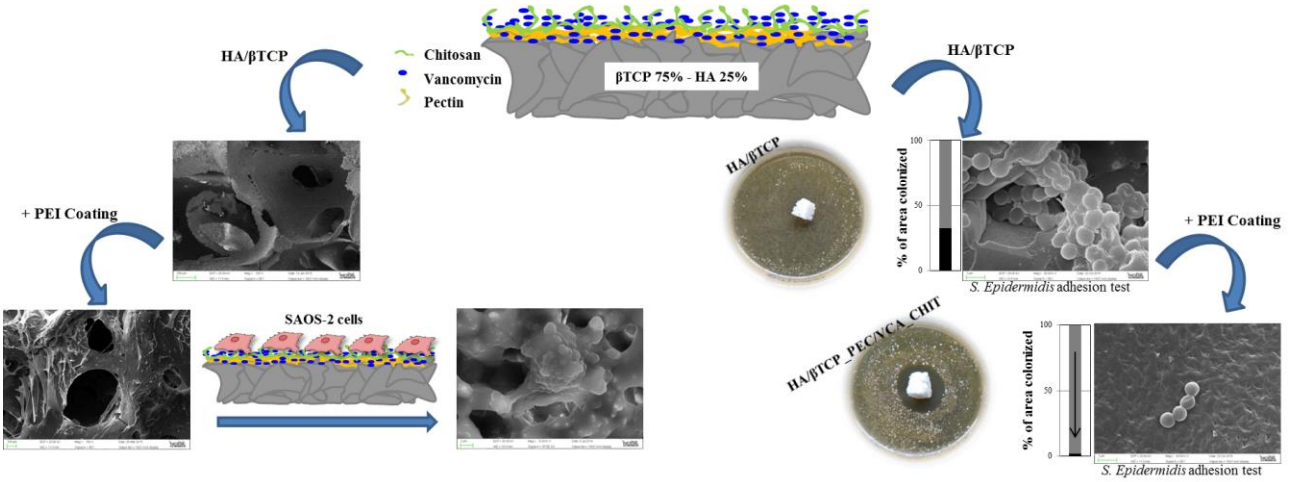

Figure 6. Graphical abstract of engineered scaffold developed.

4.4.1 Introduction

Coupling bone graft and titanium implants is still one of the best available solutions to replace bone and dental tissue loss. In particular, in large defects, where the residual bone is not enough to ensure stability for the implant, the insertion of a so-called bone filler material is often necessary. This kind of surgical approach promotes bone formation and gives stability, guiding bone regeneration around the implant. Many of these procedures are successful, with an implant survival rate greater than 90% at 10-15 years of follow-up[1–4]. Despite this success rate, in 1-5% of the procedures, the implant fails and must be removed[5,6]. The reason for this could be explained by the biomechanical issue on one side, due to an overloading at the bone implant surface[7–9], and by the biological failure on the other side, associated with microbial plaque accumulation and bacterial contamination[10–12]. Current strategies for the prevention of PPI involve either an increase in the rate of new bone formation by graft material and systemic administration of antibiotics. In the last decades, the development of grafting materials has aroused great interest[13–15]. In particular, synthetic ceramic materials as tricalcium phosphate and hydroxyapatite have been used for their good reproducibility, biocompatibility, and non-immunogenicity, but especially because of their similarity to the components of the native bone mineral phase[16–18]. In large bone defects, completely filling the void and giving a sufficient mechanical resistance to the implant are the principal issues as they are a key factor for the induction of cell migration and proliferation inside the scaffold, in order to achieve a satisfactory osteointegration. The hydroxyapatite/β-tricalcium phosphate mix is one of the most

used biphasic materials for its great biocompatibility, good degradation rate and mechanical support[19]. Morra et al., in a recent work[20], developed a new bone filler in particles, named Synergoss®, made with HA/βTCP in percentage of 25 wt.% and 75 wt.% respectively. *In vivo* studies demonstrated that this filler allows new bone formation, coupled with a compatible degradation rate[20]. The balance between HA and βTCP is a key point to obtain both mechanical strength and degradation, and to stimulate excellent osteointegration[21,22]. However, dentistry practices for large bone defects are expected to use three–dimensional (3D) porous scaffold, in order to fill large voids, to stimulate cell infiltration through interconnected pores and to increase mechanical stability for the immediate implant loading. The scaffold properties, alone, are not enough, in fact large bone defects are often the result of the removal of necrotic tissue or of a zone with an acute infection; furthermore, considering the natural function of the oral cavity which is at the interface with a lot of bacteria, bacterial growth and re-infection of native bone are a strong possibility[23–25]. The traditional antibiotic therapy is not effective in the control of PPI, because of the bacteria specific adhesion on the biomaterials, and to the very low antibiotic's penetration into the osseous defect[10,26,27]. Furthermore, systemic delivery of a high dose can causes systemic toxicity with associated renal and liver complications, resulting in the need for hospitalization for monitoring[3,28–30]. Development of a complex release system aimed at reducing infection and, at the same time, at promoting bone formation, could be a solution. Antibiotics used in drug release complexes should be effective against bacteria, as well as biocompatible and they should not damage the surrounding native tissues. Vancomycin is a widely used antibiotic that provides bactericidal activity against the most relevant germs and shows efficacy and biocompatibility in clinical use[31–33]. One solution is the combination of the ceramic material with a polymeric coating, which allows encapsulation of drugs and increases the biocompatibility, thus generating a biomimetic surface[13,34]. In particular, collagenous materials were used, since collagen is a key component of bone, is biocompatible and its degradation products are nontoxic[20]. Collagen is a very expensive component and, in order to obtain a stable surface functionalization without a faster degradation *in vivo*, a chemical crosslinking is needed, which implies the use of chemical compounds such as glutaraldehyde, which may cause cytotoxic effect on the surrounding tissues[35]. Pectin and chitosan are nontoxic natural polysaccharides that have aroused great interest in the last years in tissue engineering, due to their ability to crosslink by means of intermolecular interactions and to form stable and biocompatible complexes which may simulate the extracellular matrix and interact with cells from surrounding tissues[36–41]. Pectin is a natural anionic polysaccharide, a major component of citrus cell walls or apple peel by-product, consisting in a poly D - galacturonic chain with carboxyl groups, which could be ionically crosslinked by calcium ions (Ca^{2+}) forming the so

called “egg box” structure. Furthermore, ionic interactions occur with polycationic polysaccharides, in order to form a well-known polyelectrolyte structure (PEI)[36]. Pectin is already widely used in the food industry and, in the last decades, it has found application in bone tissue engineering, in particular as a drug carrier[42–45]. The limit of pectin is its great water-solubility, which causes fast dissolution and, when used as a drug carrier, a burst release of the therapeutic molecules occurs. To overcome this problem, many research groups have been trying to combine pectin with other materials[46–51]. In particular, chitosan is a natural polycationic material that could form a stable polyelectrolyte composite in acidic environment when mixed with pectin. Chitosan derives from chitin, which can be extracted from crustaceous exoskeleton and is composed by β–(1,4)–glucosamine and N-acetyl-D-glucosamine. Owing to its biocompatibility, its intrinsic antibacterial nature, its ability to not induce any foreign body reaction and to promote cell adhesion, proliferation and differentiation, chitosan has aroused great interest in tissue engineering and pharmaceuticals, founding many applications, especially in bone tissue engineering[39,40,52–54]. In this work, we developed and produced an engineered scaffold coupling inorganic and organic phases loaded with vancomycin, as a novel system to prevent and control periprosthetic infection in dental large bone defects. As inorganic phase, we used a highly porous 3D biphasic ceramic (25 wt.% of hydroxyapatite and 75 wt.% of β-tricalcium phosphate). We hypothesized and demonstrated that the functionalization of the ceramic scaffold with a pectin-chitosan PEI coating allows the control of vancomycin release, inhibits bacterial proliferation and biofilm formation, stabilizes the degradation rate in physiological and acidic environment and promotes osteoblast proliferation without compromising the mechanical properties; moreover, gene expression results demonstrated that PEI treatment elicits anti-inflammatory responses. We therefore successfully manufactured a three dimensional construct that could prevent the generation of periprosthetic infection and promote new bone formation in large dental bone defects.

4.4.2 Results and Discussion

Calcium phosphate materials are widely used in bone and dental tissue engineering, since they are the principal compound of inorganic phase in native bone[15,20,55]. In particular, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is the most stable form of calcium phosphate and the most abundant component in bone, around 65%[56,57]. Many studies show osteoconduction stimulation coupled with good mechanical properties; therefore, the degradation rate of hydroxyapatite is very slow[58,59]. In this work, we decided to combine hydroxyapatite with β-tricalcium phosphate, in the percentage of 25 % - 75 % respectively, in order to manage the degradation properties of the scaffold and to achieve a replacement by the host bone during implantation with a physiological rate. Preparation of the

three dimensional scaffold involved a three step process. In the first one, a polyurethane sponge was impregnated with a ceramic slurry in a properly manner and, after one night in oven to allow water to evaporate, it was sintered. In the second step, the ceramic porous scaffold was impregnated with a solution of pectin and vancomycin, lyophilized and, in the third step, it was soaked in a solution of chitosan to form a polyelectrolyte (PEI) on the surface of the material; the resulting material is called HA/βTCP_PEC/VCA_CHIT scaffold. In this work, we compared this material with a ceramic porous scaffold (HA/βTCP), a ceramic porous scaffold impregnated with vancomycin (HA/βTCP_VCA) and a ceramic porous scaffold impregnated with a solution of pectin and vancomycin (HA/βTCP_PEC/VCA).

4.4.2.1 Chemical characterization

ATR-IR

In order to control the preparation process of the ceramic scaffold and, in particular, the formation of the PEI complex on the surface, we performed different surface analysis. ATR-IR spectra reported in **Figure 7 a** confirm that all peaks belong to the inorganic material. In particular, spectrum of HA/βTCP sintered materials contained peaks from both hydroxyapatite and tricalcium phosphate. Typically, a peak at 1125 cm^{-1} belongs to tricalcium phosphate, while peaks at lower wavenumbers are typically associated with hydroxyapatite. **Figure 8 a** is a focus on triply degenerated asymmetric stretching mode (ν3) of the P-O bond of the phosphate group (1125 cm^{-1} and 1025 cm^{-1}-1010 cm^{-1})[60,61]. **Figure 7 b** shows spectra of pectin powder, chitosan powder, pectin/chitosan physical mixture, a pectin/chitosan polyelectrolyte complex and a HA/βTCP scaffold with a PEI functionalization. Pectin and chitosan show typical spectra of polysaccharides; in particular, the region between 3700 cm^{-1} and 3000 cm^{-1} for pectin and chitosan is assigned to the O–H stretching vibration (νOH), while the region between 3000–2800 cm^{-1} belongs to C-H stretching vibration (νCH) (**Figure 8 b**). Deeper analysis on pectin spectra show two bands associated with the stretching vibration at 1740 cm^{-1} of carbonyl group, corresponding to the methyl ester group ($COOCH_3$) and carboxyl acid (COOH), while the band at 1606 cm^{-1} belongs to the stretching vibration of the carbonyl group of the carboxylate ion (COO^-). Concerning chitosan spectra, the band at 1647 cm^{-1} is due to the C=O stretching vibration of amide I, whilst the band at 1580 cm^{-1} is due to the NH bending amide II, maybe overlapped to the N-H vibration of the amine groups. (**Figure 8 b)**.

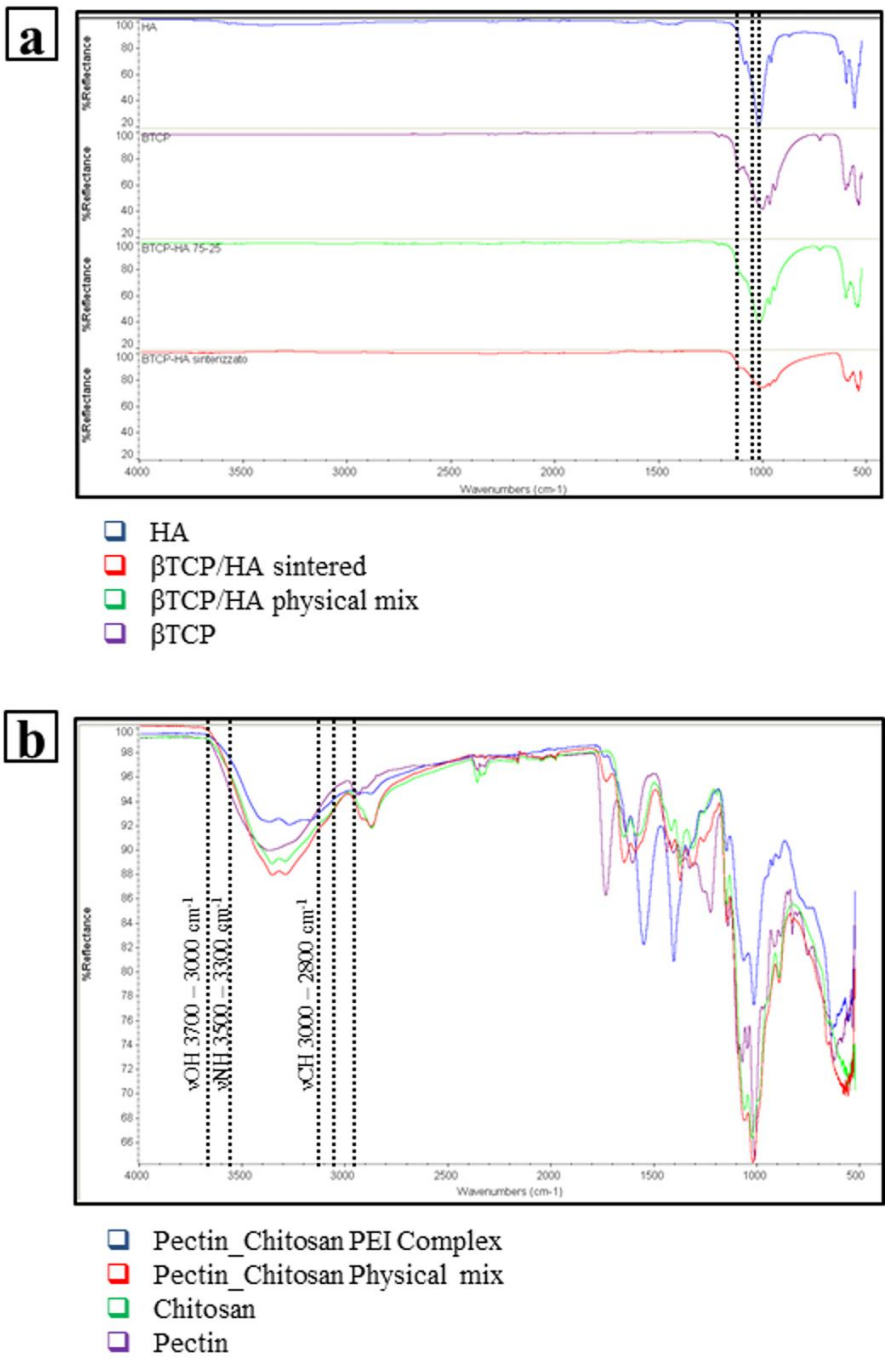

Figure 7. ATR-IR spectra of raw materials and HA/βTCP scaffold (a) and of HA/βTCP_PEC/CHIT scaffold (b).

The polyelectrolyte complex is formed and stabilized by the electrostatic interactions that occur between the positive charge of chitosan, NH_3^+, and the negative one of pectin, COO^- **(Figure 8 b)**; amine band shift to 1557 cm^{-1} confirms the formation of a pectin-chitosan complex. A series of overlapping are also present in the PEI spectra, due to H-bonding interactions between COOH groups of pectin or NH_2 groups of chitosan and OH or $COOCH_3$ groups within the complex. Band assignment is consistent with available literature[36,38]. The most important observation is underlined by the spectra of the HA/βTCP_PEC/CHIT scaffold, where both bands, the one assigned to the PEI

complex and the other one belonging to the inorganic phase, are presents, in particular the band at 1557 cm^{-1} and those between 950 cm^{-1} and 1140 cm^{-1}, respectively **(Figure 8 b)**. This analysis confirms the successful preparation of the materials, with a ceramic material functionalized with a polyelectrolyte on the surface. Furthermore, XRD analysis show that no phase transformation occurs at the sintering temperature (**Figure 9 a, b, c)**.

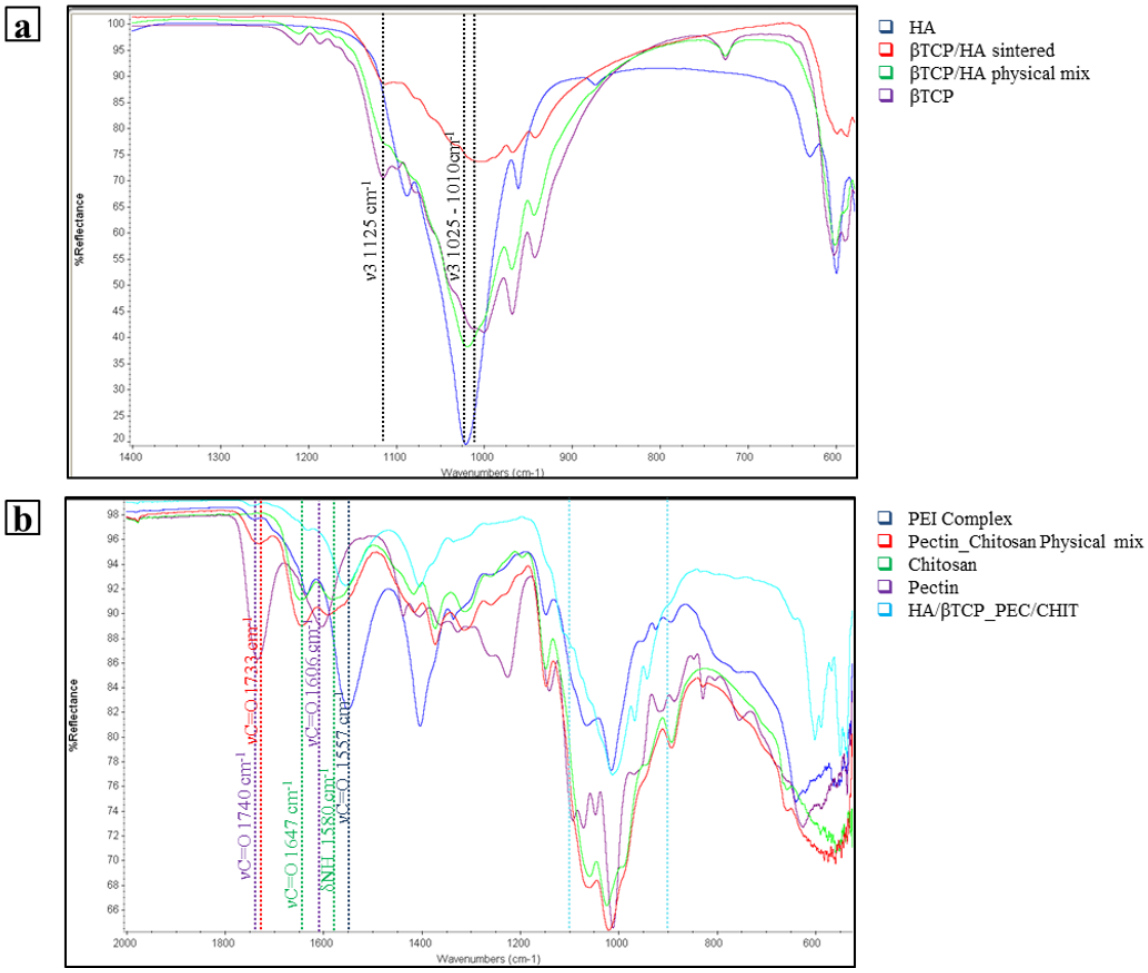

Figure 8. a) IR spectra of HA, βTCP powder, and HA/βTCP (25/75 wt.%) biphasic composite before and after sintering process, the peak at 1125 cm^{-1} its belongs tricalcium phosphate, while the peak at the lower wavenumber are typically associated to hydroxyapatite. b) IR spectra of pectin powder, chitosan powder, pectin-chitosan as physical mixture, pectin-chitosan as polyelectrolyte complex, and the engineered scaffold HA/βTCP_PEC/CHIT, (it is possible to observe the shift of the amine band to 1557 cm^{-1}, due to the PEI complex, and the bands belonging inorganic phase, in particular between 950 cm^{-1} and 1140 cm^{-1}).

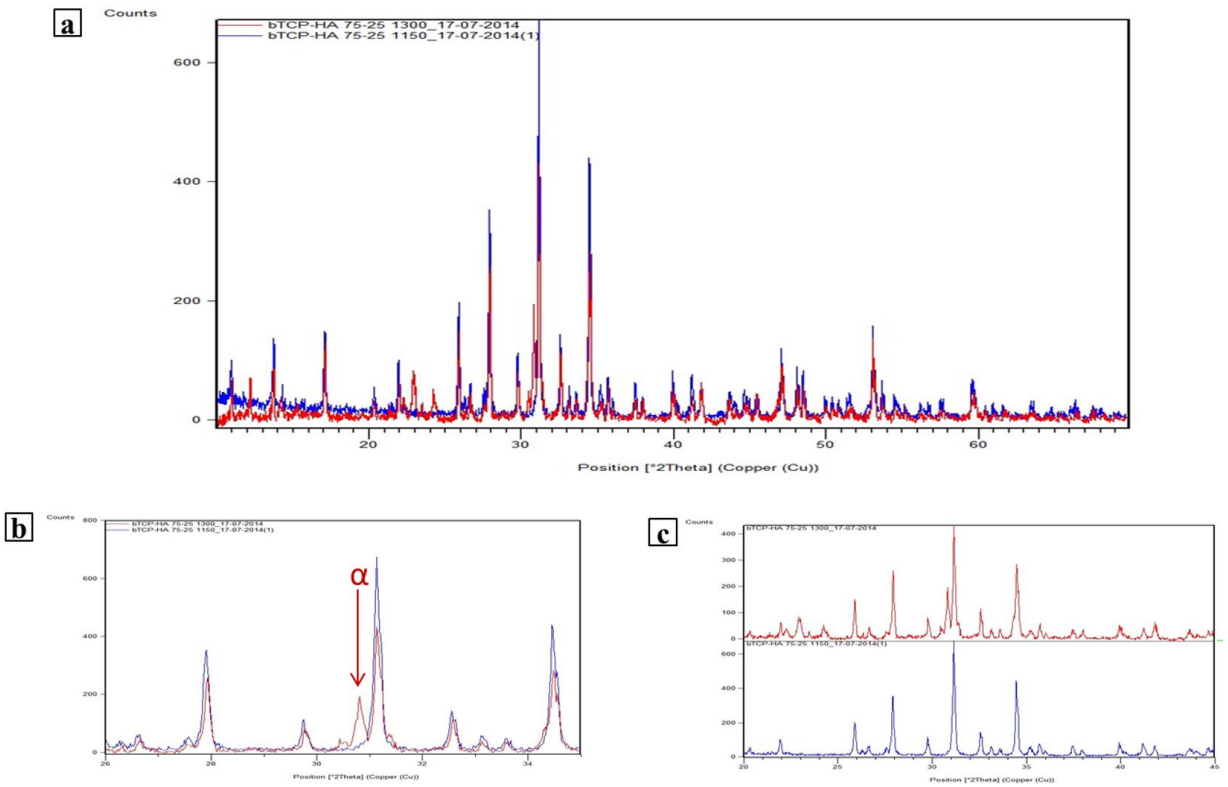

Figure 9. XRD analysis on HA/βTCP scaffold sintered at 1150 °C (blue line) and 1300 °C (red line). Sintering process performed at 1300 °C leads the transformation of βTCP in αTCP. After these analysis the sintering temperature of 1150 °C was chosen, in order to preserve the βTCP structure.

XPS Analysis and EDX analysis

Chemical composition of the surface of four different ceramic disks functionalized with pectin, chitosan and the pectin-chitosan polyelectrolyte complex, has been detected by XPS analysis, as it is shown in **Figure 10 a.** As expected, the percentage of Ca and P is high on the surface of HA/βTCP, while a significant reduction of Ca and P and an increase of N and C were detected on HA/βTCP_PEC/CHIT samples. Spectra in **Figure 10 b** show a reduction of the peaks associated to Ca 2p3 and P 2p3 and the appearance of N 1s peak for the sample functionalized with PEI complex. Samples with pectin have a greater reduction for Ca and P compared to the one functionalized with chitosan, showing that the procedure to functionalize with pectin first, is an optimal way to obtain a stable and uniform coating available for chitosan, in order to generate a stable and uniform polyelectrolyte complex on the surface of ceramic **(Table 1)**.

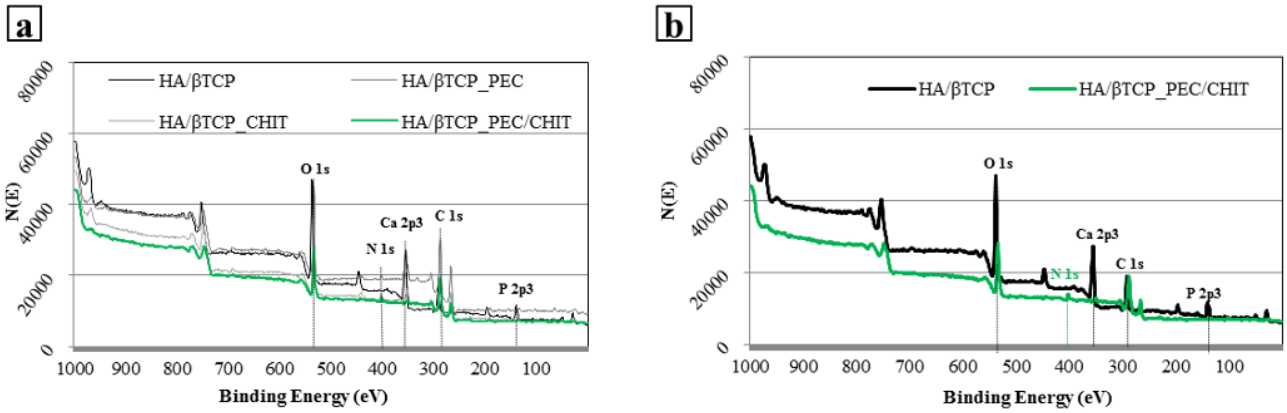

Figure 10. a, b) XPS spectra of HA/βTCP, HA/βTCP_PEC, HA/βTCP_CHIT and HA/βTCP_PEC/CHIT, a reduction of the peak associated at Ca 2p3 and P 2p3 and the appearance of N 1s peak for HA/βTCP_PEC/CHIT sample was detected.

	O	C	Ca	P	N
HA/βTCP	*30*	*34,3*	*19,5*	*16,2*	*0*
HA/βTCP_PEC	*26,1*	*64,2*	*4,3*	*3,4*	*2*
HA/βTCP_CHIT	*32*	*52*	*6,9*	*5,7*	*3,4*
HA/βTCP_PEC/CHIT	*25,6*	*65*	*2,4*	*1,9*	*5,1*

Table 1. Chemical composition (at.%) of HA/βTCP, HA/βTCP_PEC, HA/βTCP_CHIT and HA/βTCP_PEC/CHIT.

Furthermore, EDX analysis made on the surface of HA/βTCP porous scaffold and HA/βTCP_PEC/CHIT scaffold confirm the presence of organic material through the appearance of Carbon (C) peaks in the spectra (**Figure 11**).

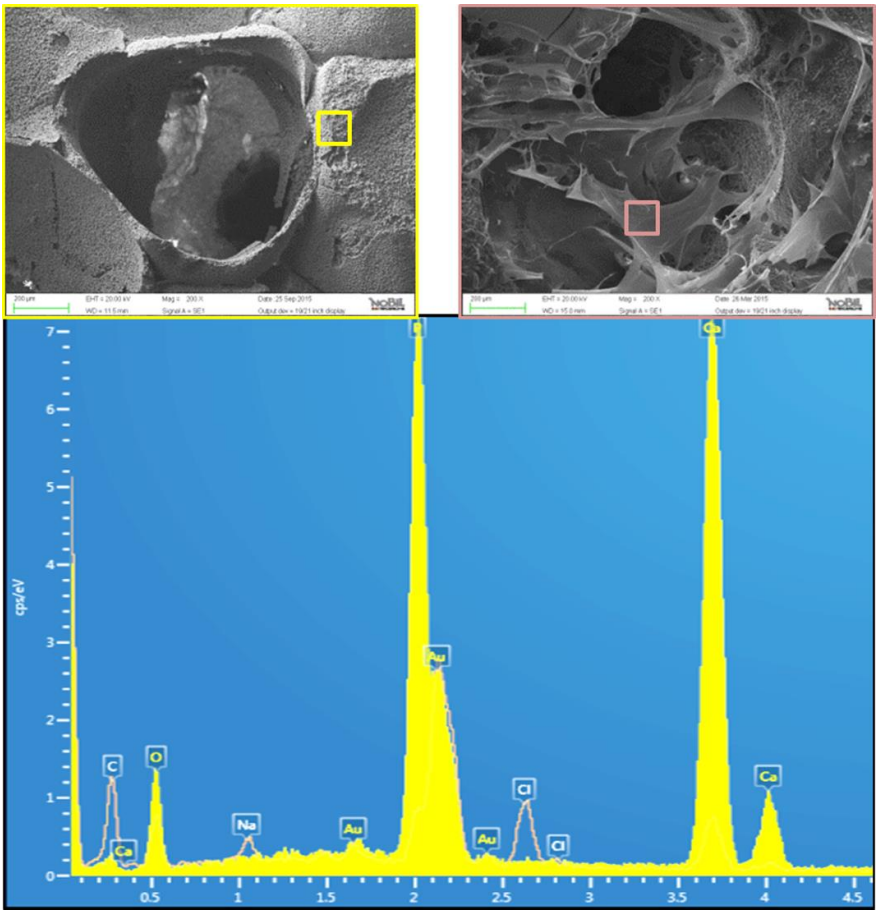

Figure 11. EDX analysis on HA/βTCP and HA/βTCP_PEC/VCA_CHIT porous scaffold confirm the presence of organic materials, through the appearance of C peak. ***Scale bar: 200 µm.***

4.4.2.2 Structural characterization

µCT analysis

High porosity and interconnectivity of our ceramic scaffold HA/βTCP were qualitatively demonstrated by a capillarity test using red ink (**Figure 12 a).** In order to confirm this hypothesis, we studied the morphological properties of our materials using the µCT and SEM techniques, on HA/βTCP and HA/βTCP_PEC/VCA_CHIT scaffolds. A 3-D representation of HA/βTCP ceramic scaffolds morphology is shown in **Figure 12 b**. The results demonstrated that the ceramic scaffolds resulting from the impregnation process of the PU open cells sponge, is characterized by open and interconnected macro-pores. The calculated porosity for both scaffolds is about 45 - 50 vol.%, in particular macropore size ranged within 100 –1100 µm for HA/ βTCP and HA/βTCP_PEC/CHIT scaffolds.

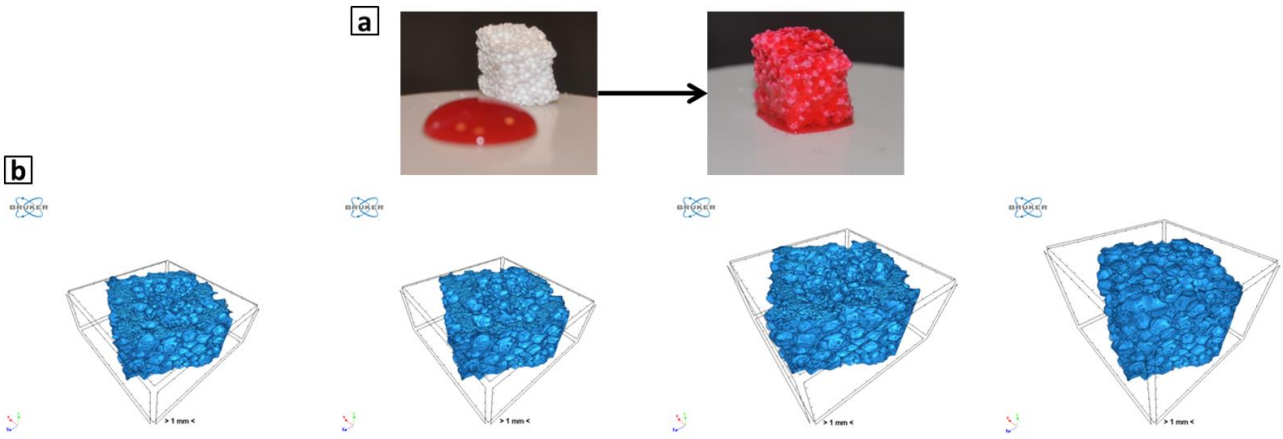

Figure 12. a) capillarity test performed on HA/βTCP scaffold. b) Three dimensional reconstruction of HA/βTCP_PEC/VCA_CHIT scaffold.

The qualitative interconnection and open porosity, demonstrated through capillarity test, were also confirmed by the morphometric parameters calculated from 3-D image analysis. The value of total porosity (P), structural thickness (St.Th.), structural separation (St. Sp.), Total Porosity (P) and Open porosity (OP) have been compared between HA/βTCP and HA/βTCP _PEC/CHIT [62] **(Table 2)**.

Sample	St. Sp. [µm]	St. Th. [µm]	P [%]	OP [%]
HA/βTCP	839.98	414.46	49.57	98.02
HA/βTCP_PEC/VCA_CHIT	807.68	441.59	45.98	98.34

Table 2. Microstructural parameters calculated from µCT analysis. HA/βTCP_PEC/CHIT scaffold shows a similar morphology to the natural bone.

As expected, the St.Sp. decreases with the addition of PEI complex, from 839.9 µm to 807.7 µm; as well as the total porosity (P) decreases from 49.6 % for HA/βTCP to 45.9 % for HA/βTCP_PEC/CHIT. We hypothesize that the addition of pectin_chitosan, as a coating complex, increases the thickness of the trabeculae, reinforcing the mechanical structure of the scaffold. This assumption was assessed through the calculation of St.Th. parameters, which increases from 414.46 µm for HA/ βTCP to 441.6 µm for HA/ βTCP_PEC/CHIT scaffold. PEI functionalization slightly decreases the porosity, decreases the structure separation and increases the structure thickness. However, the open porosity is maintained around 98% of the total porosity, which is an important characteristic to promote cell infiltration into the scaffold. This distribution was also assessed by SEM investigation **(Figure 13)**, where interconnected macropores with their struts are shown. SEM

investigation shows a particular and explanatory case where a huge macropore is divided and the trabeculae are reinforced by PEI polymer **(Figure 13)**[63]. Comparing the value found through the calculation of the morphometric parameter with the reference parameter from a study conducted on 46 bone implant sites of the maxilla and mandible from 32 volunteers, it was found that the values of the engineered porous scaffold were in the range or in the same order of magnitude of the native mandibular bone [62].

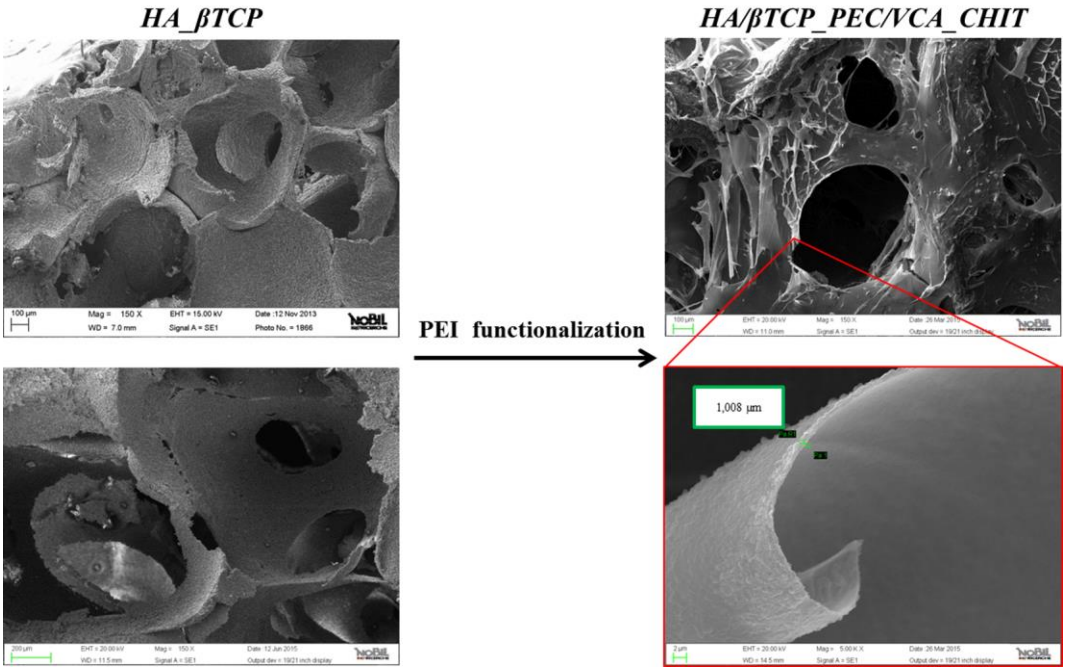

Figure 13. SEM investigation before (HA/βTCP) and after coating with PEI (HA/βTCP_PEC/VCA_CHIT). PEI polymer reinforced the structure and maintained the interconnected porosity.

Morphologic analysis showed that PEI functionalization allows achieving a scaffold with a porosity, a pore size distribution, a trabecular internal arrangement and a surface area that could potentially direct vascularization and nutrients and promote cellular infiltration and bone growth inside the pores. A critical role in bone formation is played by porosity, pore size and interconnectivity of biomaterials. Pores in biomaterials for bone regeneration are necessary for tissue formation, since they allow migration and proliferation of osteoblasts and mesenchymal cells, promote vascularization and improve mechanical strength, by promoting anchoring between natural tissues and biomaterials at the interface[64]. Pores could be divided in two classes: micropores (< 5 um) and macropores (> 100 um), both of them important for bioresorption of materials, however macroporosities are fundamental to the promotion of new bone growth [64,65] .

Osteogenesis *in vitro* and *in vivo* is affected by the morphology of the materials, as well as the mechanical properties. Lower and micro-porosity show high osteogenesis *in vitro*, because cells are forced to aggregation; instead, *in vivo* higher and interconnected porosity results in an increase of bone growth, however higher porosity coupled with high pore size translates in poor mechanical

properties, too[64]. Micro-porosity creates a hypoxia environment that promotes osteochondral differentiation before osteogenesis, instead macropores promote vascularization and facilitate the oxygen transport inside the scaffold. Many researchers have studied optimum pore sizes for bone and have shown that alkaline phosphatase activity, osteocalcin content and new bone formation are higher in sample with a high percentage of pore, namely between 300 and 400 μm [66]. However, many studies show that even microporosity contributes to induct protein adsorption and bone-like apatite formation with its larger surface area. Another important property is the interconnectivity of pores, because a spatial continuous connection promotes bone ingrowth. In addition to new bone formation, even morphology influences the mechanical stability of the scaffold, in fact high porosity, high pore size and high interconnection are translated in a loss of mechanical properties [67,68]. One possible strategy to achieve high porosity and good mechanical properties, is to combine a highly porous ceramic scaffold with a polymeric phase, that could reinforce the structure and in parallel maintain the porosity in a range that allows new bone infiltration [63].

4.4.2.3 *Mechanical characterization*

Compression Test

Compressive stress is the principal load to which a scaffold developed for bone and dental tissue regeneration is subjected. A biomaterial should not only resist to stress, but it is also quite important that a complete disintegration does not happen; this is because some debris could migrate and cause an inflammatory response **(Figure 14 a).** In this study, we compared the mechanical properties of HA/βTCP, HA/βTCP_VCA, HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT scaffolds, in order to assess how PEI functionalization can affect the resistance of the materials. In **Figure 14 b,d** an example of stress-strain curve for HA/βTCP scaffold and for HA/βTCP_PEC/VCA_CHIT scaffold is reported. For both samples, the stress-strain curve shows a so called "pop-in behavior", already reported in other works, related to the crack propagation inside the material trabeculae[69]. After an initial increase of stress, the trabecular structure starts to drop, but the scaffold is still able to withstand the load, so stress rises again until a maximum is reached, where the struts cracking occur. The HA/βTCP_PEC/VCA_CHIT scaffold shows a compressive strength of 1.12 ± 0.14 MPa, compared to 0.51 ± 0.14 MPa for the HA/βTCP one, 0.47 ± 0.03 MPa for HA/βTCP_VCA, and 0.73 ± 0.11 MPa for HA/βTCP_PEC/VCA **(Figure 14 e)**. These results suggest that the addition of a polysaccharide in the structure of the scaffold could help to increase the mechanical strength. The addition of pectin or a pectin-chitosan complex produces a reinforced composite scaffold, with the result of an increase in the mechanical properties. Elastic modulus calculated from the linear part of the stress-strain curve confirms the trend already assessed for the compressive strength; 46.7 ± 3.48

MPa for HA/βTCP, 24.34 ± 4.01 MPa for HA/βTCP_VCA, 59.78 ± 4.67 MPa for HA/βTCP_PEC/VCA and 77.61 ± 13.14 MPa for HA/βTCP_PEC/VCA_CHIT **(Figure 14 f)**. The ceramic scaffold treated with vancomycin (HA/βTCP_VCA) alone shows a mechanical strength slightly lower than the control HA/βTCP; a reason for this could be vancomycin treatment's procedure, which involves the scaffold immersion in an acidic environment that could start degrading the trabeculae and the sintering necks, thus making the scaffold less resistant. The PEI complex yields a more cohesive scaffold, also after a critical stress was reached, making the structure not subjected to collapse, differently from the HA/βTCP scaffold which undergoes a catastrophic failure, with debris production that could cause some problems *in vivo* **(Figure 14 a, c**).

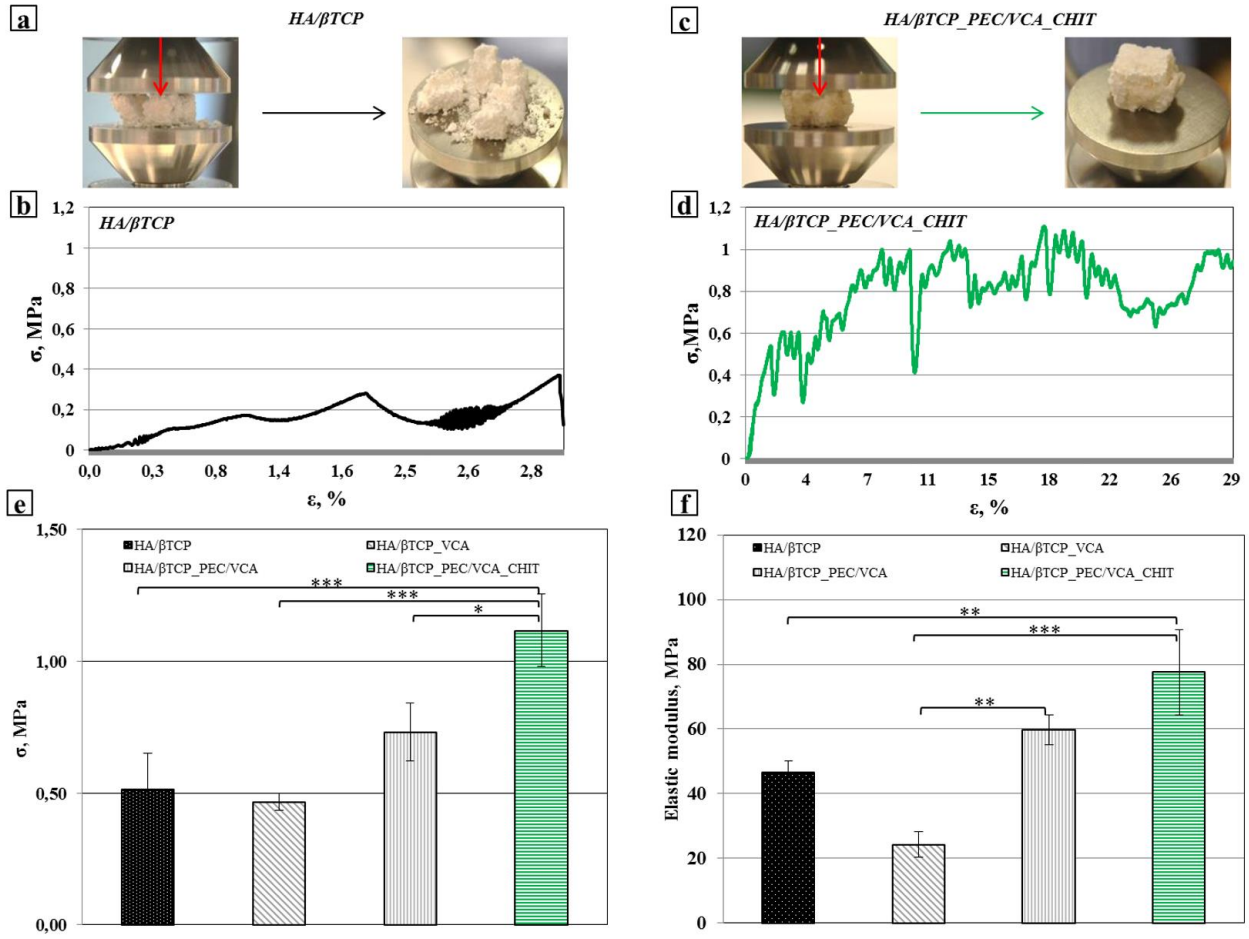

Figure 14. a,b) An example of compressive test and stress-strain curve for HA/βTCP and for HA/βTCP_PEC/VCA_CHIT (c,d) are reported. The PEI functionalization makes the material more cohesive and resistant, since the compressive strength and compressive modulus increase with the coating process (e, f).The data are represented as mean ± standard deviation (n=3). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA with Tukey analysis test).

4.4.2.4 *Physical characterization*

Degradation properties of the developed material have been tested in neutral conditions (buffer solution at pH 7.4) and in acidic environment (buffer solution at pH 3). We compared HA/βTCP_PEC/VCA_CHIT scaffolds with the HA/βTCP, HA/βTCP_VCA, HA/βTCP_PEC/VCA one, three samples for each were analyzed. Initial weight was recorded and, at each time point, samples were taken out from solution, lyophilized and weighted. The percentage of mass loss was calculated from the initial weight and from the weight after soaking by Eq. (1).

Degradation at pH 7.4

Results of the degradation test in physiological condition for HA/βTCP, HA/βTCP_VCA, HA/βTCP_PEC/VCA, and HA/βTCP_PEC/VCA_CHIT samples show a linear low degradation for all samples; in particular, pH 7.4 does not affect the ceramic structure within 1 week, in fact 1.33 ± 0.05 % and 5.83 ± 0.19 % of mass loss was reached for HA/βTCP and HA/βTCP _VCA scaffolds, respectively **(Figure 15 a)**. In the case of scaffolds functionalized with pectin or pectin_chitosan, pH is an important factor that could change the behavior of the coating.

In particular, in HA/βTCP_PEC/VCA_CHIT scaffolds weight loss mainly occurs for a ionic interaction break, established between pectin and chitosan chains, with the subsequent free chain solubilization. Ionized amino groups of chitosan (NH^{3+}) transformation in non-ionized amino groups (NH_2) and preservation of ionized state of COO^-, occur at pH 7.4. With non-ionized NH_2 groups, chitosan results insoluble in water, while ionized pectin is completely solubilized through water uptake. In the case of HA/βTCP_PEC/VCA scaffolds, the presence of a ionized pectin (COO^-) allows water uptake and consequent solubilization of chain. Moreover, the possible interaction between COO^- and Ca^{2+} could be neutralized from counterions present in the water uptake, inside the network.

For this reason, mass loss for HA/βTCP_PEC/VCA, 22.08 ± 1.25 mg and HA/βTCP_PEC/VCA/CHIT, 11.79 ± 3.28 mg, is mainly ascribed to pectin polymer solubilization, which dissolution probably carries away some CaP material. This hypothesis could be confirmed by SEM investigations after 1 week **(Figure 16)**, when pectin is almost totally absent on the HA/βTCP_PEC/VCA scaffold surface, while a network is still preserved on HA/βTCP_PEC/VCA_CHIT scaffolds. The pH trend in solution was monitored during the degradation test, in order to see if the degradation products could affect the surrounding environment **(Figure 15 b)**. No significant changing in the pH was recorded, but a slight decrease between 48 and 96 h for HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT scaffolds,

probably due to a maximum degradation of pectin polymers dissolved in the solution; however, at 168 h, pH for HA/βTCP_PEC/VCA_CHIT scaffolds was 7.45.

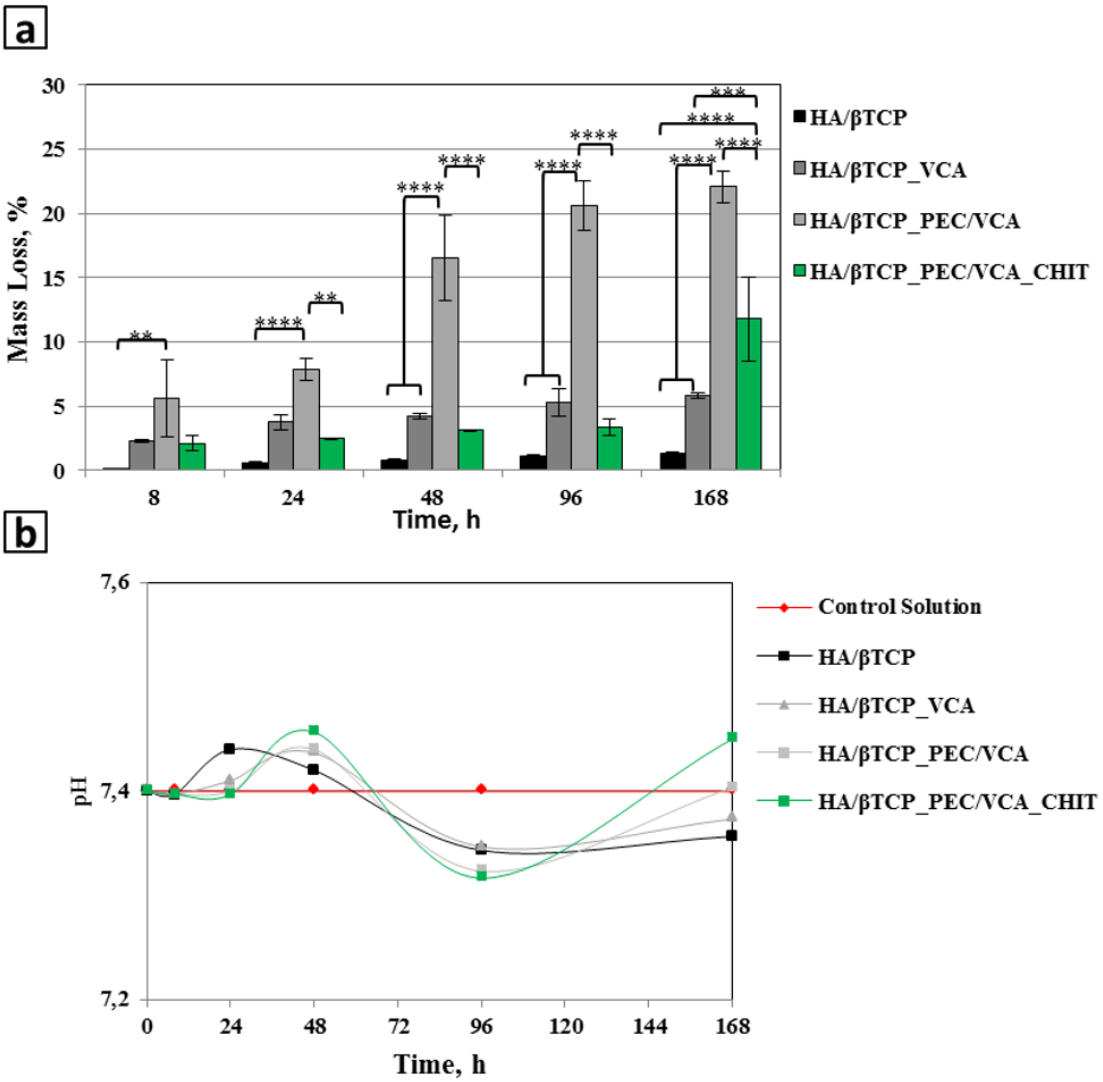

Figure 15. Degradation stability in physiological condition was evaluated for HA/βTCP, HA/βTCP_VCA, HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT scaffolds. a) The mass loss of engineered scaffolds was monitored over a period of 1 week (168 h). In neutral physiological solution, the neutralization of chitosan occurs, and the solubility of pectin increase. b) pH trend of the solution was monitored over the degradation test. Degradation products do not affect significantly the pH of the solution. The data represented as mean ± standard deviation (n=3). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA with Tukey analysis test).

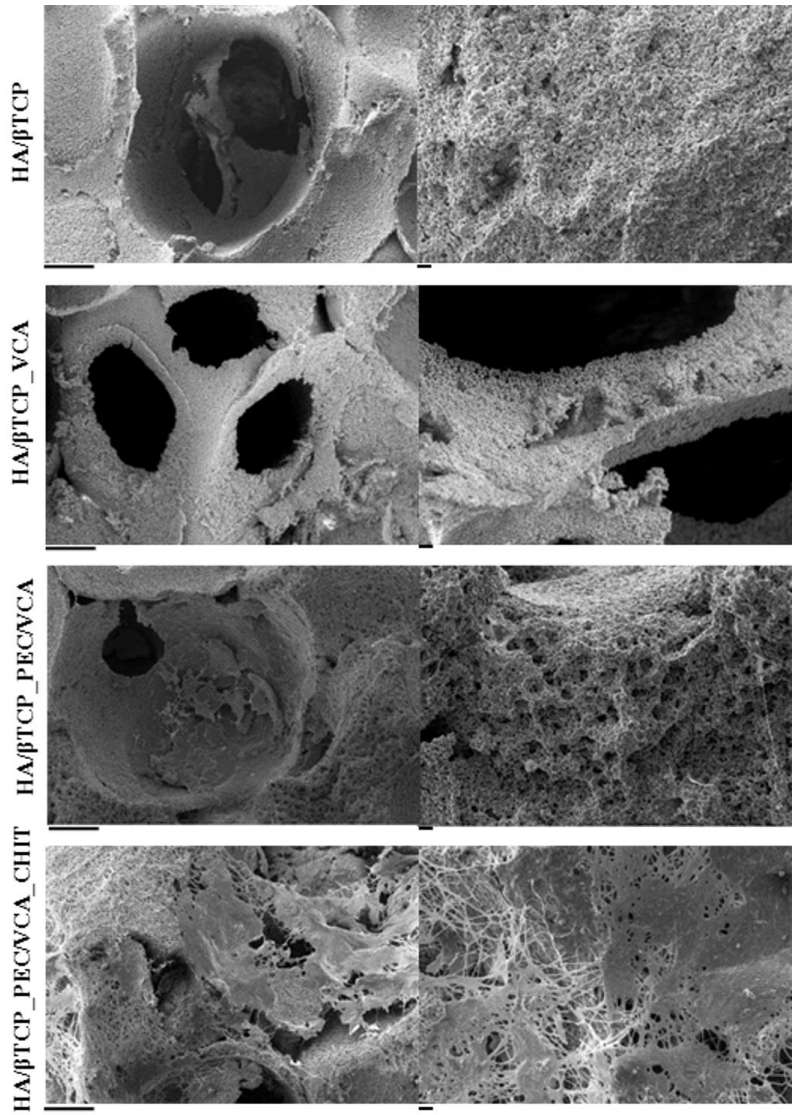

Figure 16. SEM investigation for each sample at two different magnification (200x and 500x), after 1 week of soaking at pH 7.4, confirm dissolution of pectin on HA/βTCP_PEC/VCA scaffold, and the partial dissolution on HA/βTCP_PEC/VCA_CHIT scaffold. ***Scale bar: 200 µm and 2 µm.***

Degradation at pH 3

Acidic condition represents an extreme working condition for calcium phosphate bone substitute. During PPI, it is quite normal that pH is less than the physiological one (7.4), since PPIs are usually the consequence of a bacterial infection. Presence of bacteria in a bone wound may decrease the level of tissue pH below its normal value, because bacteria subtract nutrients and oxygen to cells, creating ischemic conditions which result in an hypoxia, making metabolism to become more anaerobic, which is more acidic. Hence, it is important to evaluate the material behavior in an acidic environment. Biphasic calcium phosphate scaffold degradation depends on materials composition, for example βTCP dissolves to a greater extent and, therefore, faster than HA[40,58,59]. Moreover, acidic conditions cause a faster dissolution of calcium phosphate materials and an increase in the

tendency to fractures. HA/βTCP_PEC/VCA scaffolds are stable till 48 h, until pectin coating resists in the acidic environment in which pectin is soluble, and at 72 h, HA/βTCP_PEC/VCA scaffolds are completely dissolved. Pectin-chitosan complex coating on HA/βTCP scaffolds protects the calcium phosphate material from degradation in acidic environment and, after 168 h. HA/βTCP_PEC/VCA_CHIT scaffolds loss 9.06 ± 2.01 % of initial mass **(Figure 17)**.

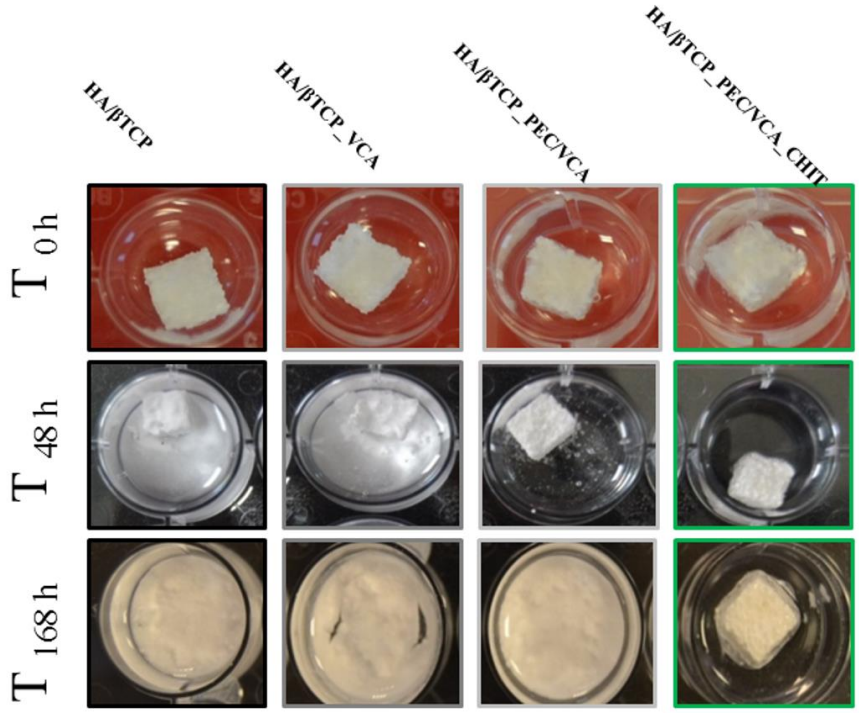

Figure 17. Optical images at time zero, after 24h and 168h of soaking at pH 3. The integrity of HA/βTCP_PEC/VCA_CHIT scaffold is clearly visible after 1 week.

HA/βTCP_PEC/VCA scaffolds are stable till 48 h, until pectin coating resists in the acidic environment in which pectin is soluble, furthermore pectin neutralization in the acidic environment could separate the interaction between Ca ions and COO- neutralized, and at 72 h, HA/βTCP_PEC/VCA scaffolds are completely dissolved. Pectin_chitosan complex coating on HA/βTCP scaffolds protects the calcium phosphate one, from degradation in acidic environment and, after 168 h, HA/βTCP_PEC/VCA_CHIT scaffolds loss 9.06 ± 2.01 mg of initial mass **(Figure 18 a)**.

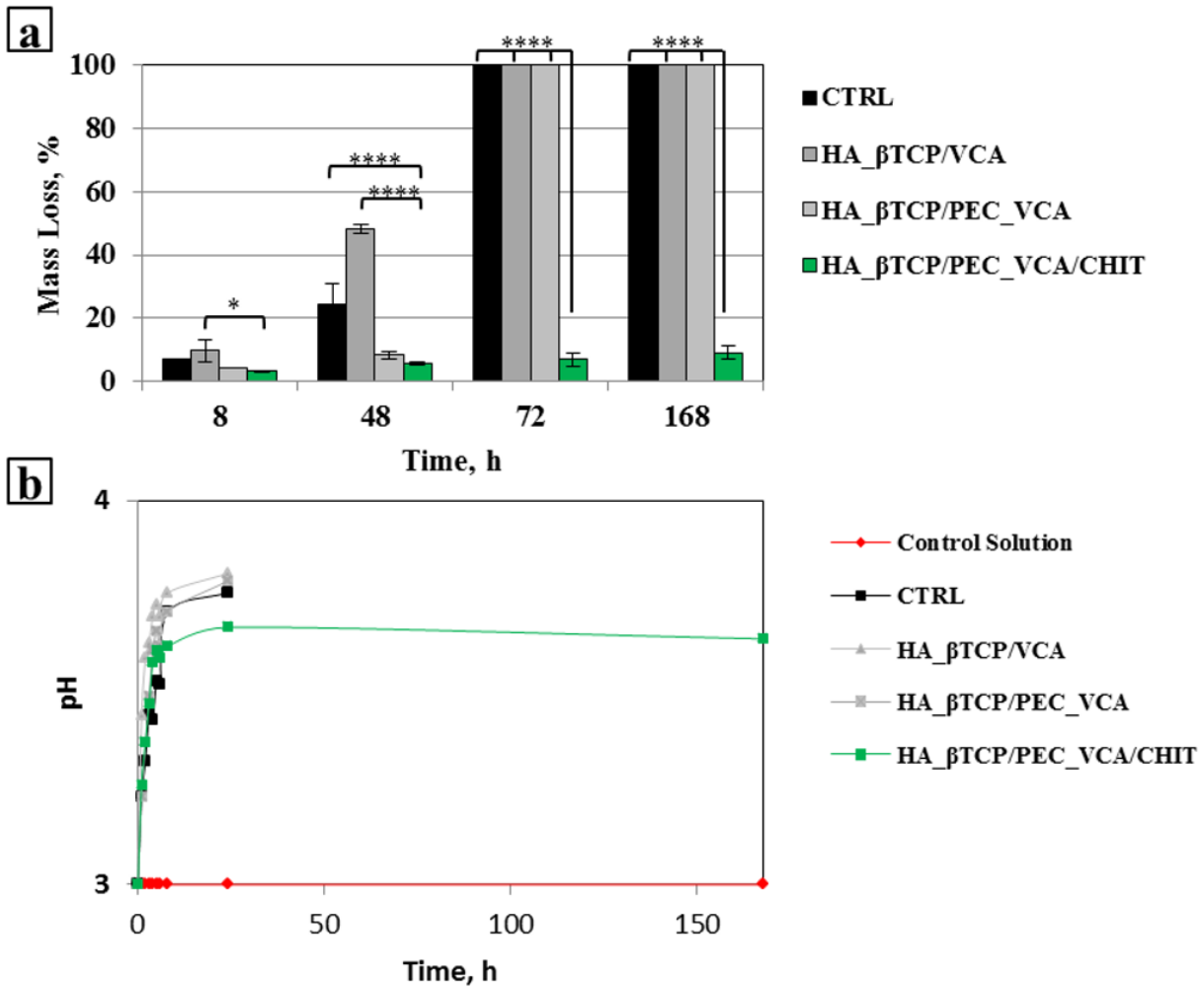

Figure 18. The stability of the engineered HA/βTCP_PEC/VCA_CHIT scaffold was monitored in acidic environment over a time of 168 h (1 week). a) mass loss of ceramic and ceramic coated scaffold was reported. HA/βTCP_PEC/VCA_CHIT scaffold shows a stable behavior during degradation in a pH 3 solution, due to the interaction between pectin and chitosan which protect ceramic materials from the acidic attack. b) pH trend was monitored every hour, within the first 8 h and after 24 h and 168 h. A slight increase in the pH value was detected for all sample tested, due to the dissolution of phosphate. The data are represented as mean ± standard deviation (n=3). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA with Tukey analysis test).

The pH measurement was recorded every hour upon the first 8 h and, at 24 and 168 h: a slight increase in pH trend was recorded compared to control solution, from 3 to 3.64 for HA/βTCP_PEC/VCA_CHIT, to 3.81 for HA/βTCP_PEC/VCA, to 3.79 for HA/βTCP_VCA and to 3.16 for HA/BTCP **(Figure 18 b)**.

SEM investigations show that, after 1 week in acidic environment, PEI surface coating, is degraded but still present, able to protect ceramic from a massive degradation **(Figure 19)**. Furthermore, since scaffold microarchitecture could influence the degradation rate, the reduced pore size resulting from PEI functionalization protects also grain and trabeculae from the acidic solution's

attack inside the scaffold. These results support the choice of HA/βTCP_PEC/VCA _CHIT materials as bone substitutes for preventing PPI, since structure is also conserved in acidic environment, allowing vancomycin release *in situ,* bacterial growth and adhesion reduction and new bone formation support.

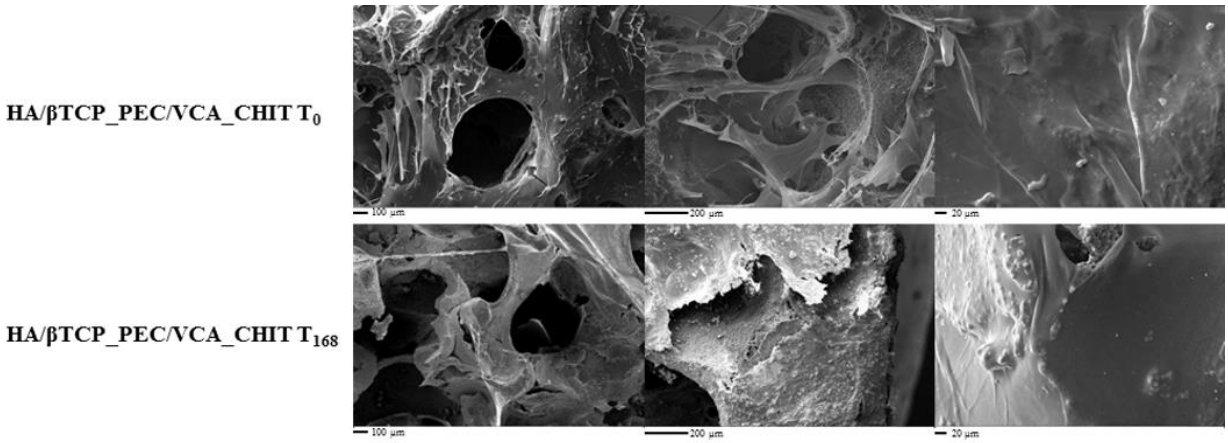

Figure 19. d) SEM investigation at different magnification (100x, 200x and 1000x) of the surface before (T0) and after 168 h of soaking in acidic solution (pH 3).

Release study and Agar Germ test

Infection of dental and orthopedic implants is a devastating consequence of bacterial presence in the bone wound site that necessitates a complete removal and antibiotic therapy via systemic administration[32,33]. A strategy to target bone healing and to avoid PPI is to provide a bone substitute which could fill the void and, in parallel, could release an antibiotic drug *in situ,* in order to reduce bacterial growth and proliferation. Further systemic administration of antibiotics reaches a poor infiltration in the bone substitute and makes bacterial infections difficult to treat[70]. Vancomycin is a glycopeptidic bactericidal antibiotic marked as a hydrochloride salt and it is in worldwide clinical use; results of vancomycin release for HA/βTCP_VCA, HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT scaffolds during 30 min, 2 h, 4 h, 8 h, 24 h, 48 h, 168 h, and 336 h (2 week) performed in physiological solution at physiological pH are reported. For HA/βTCP_VCA scaffolds, a massive burst release was recorded within 4 and 8 h, since vancomycin is a high soluble drug and, without any kind of interaction or encapsulation, but simply by adsorption on the ceramic surface and after soaking in physiological solution, all antibiotic is released in a few hours. Addition of pectin (HA/βTCP_PEC/VCA scaffold) permitted a prolonged retention of vancomycin; however the high swelling rate of pectin at physiological conditions causes a burst release within 24 h (**Figure 20)**.

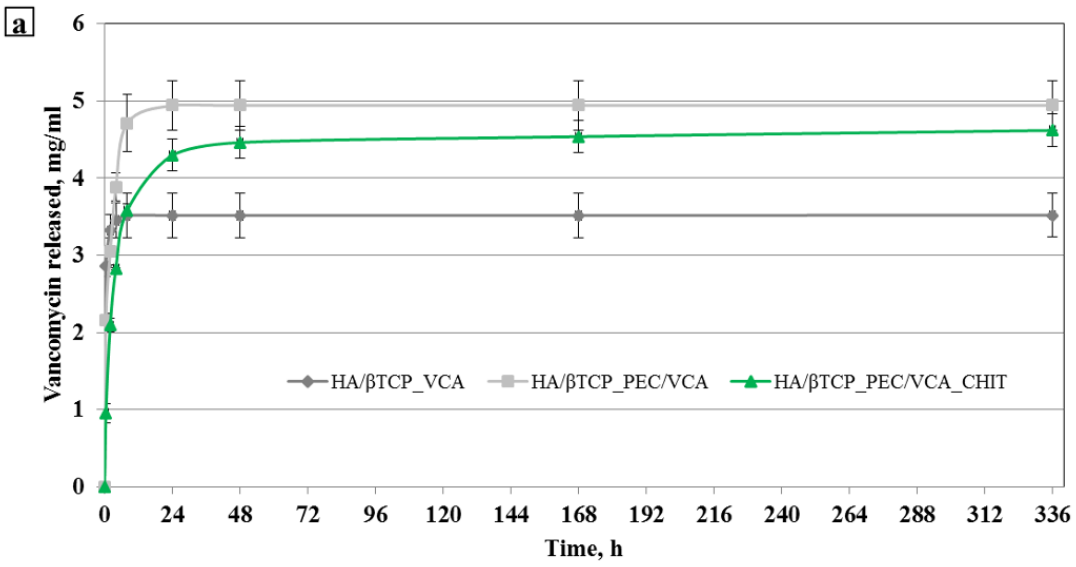

Figure 20. HA/βTCP_VCA, HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT scaffolds was soaked in physiological solution at 37 °C and at each time point, 30 min, 2 h, 4 h, 8 h, 24 h, 48 h, 168 h, and 336 h (2 week), the released solution was filtered and analyzed by High Performance Liquid Chromatography. (a) A burst release was reported for HA/βTCP_VCA and HA/βTCP_PEC/VCA scaffolds, within 8 h and 24 h respectively. HA/βTCP_PEC_VCA/CHIT scaffold shows a controlled and prolonged release until 2 week. The data are represented as mean ± standard deviation (n=3).

HA/βTCP_PEC/VCA_CHIT scaffolds show a controlled and prolonged release until 2 weeks, because the addition of chitosan and the formation of polyelectrolyte complex permit the creation of an engineered coating that controls vancomycin release (**Figure 20)**. The presence of the polyelectrolyte network allows vancomycin to be encapsulated and the PEI coating slow degradation allows a prolonged release to be reached, which is favorable in order to manage bacteria. The agar germ test confirms that the entrapped antibiotic is able to inhibit the growth and proliferation of *Staphylococcus epidermidis* **(Figure 21)**. *Staphylococcus epidermidis* is a microorganism usually present on skin and it is the main bacterium found in hospitals; for this reason, it has become the primary responsible of prosthetic infections, and since these infections are often indolent and clinically silent, diagnosis and consequent therapy are difficult[6,71]. The formation of a multilayer biofilm on the foreign body surface is difficult to be contrasted with a systemic antibiotic therapy; furthermore, it inhibits osteoblast proliferation up to implant failure[72]. In this work, we evaluated how bacteria (*Staphylococcus epidermidis)* adhere and proliferate and, eventually, form the biofilm on the scaffold surface; serial dilution test and SEM investigation were performed.

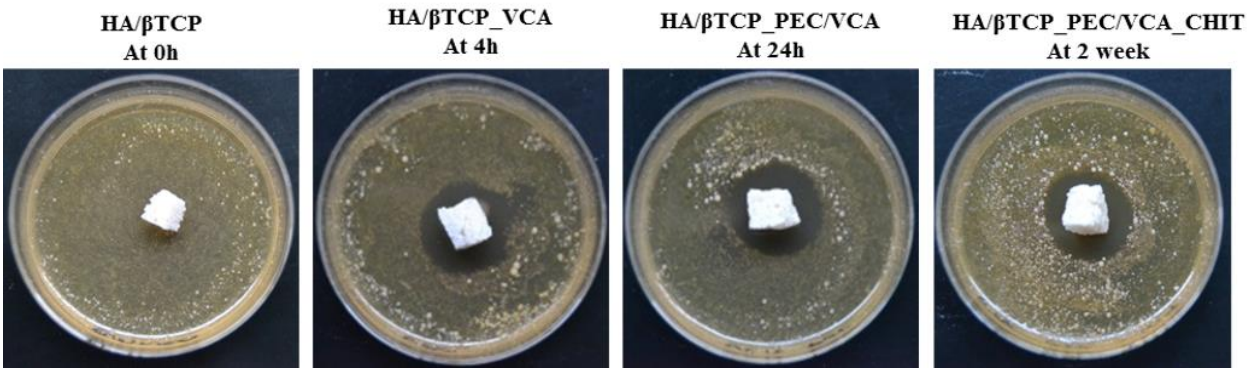

Figure 21. Agar germ test confirm that entrapped vancomycin scaffold is able to inhibit the growth and proliferation of *Staphylococcus epidermidis*. Inhibition zones are visible until the release of vancomycin persists, 4 h for HA/βTCP_VCA; 24 h for HA/βTCP_PEC/VCA and up to 2 weeks for HA/βTCP_PEC/VCA_CHIT. No inhibition zones are visible for HA/βTCP scaffold.

4.4.2.5 Biological characterization

Staphylococcus epidermidis is a microorganism usually present on skin and it is the main bacterium found in hospitals; for this reason, it has become the primary responsible of prosthetic infections, and since these infections are often indolent and clinically silent, diagnosis and consequent therapy are difficult to act[6,71].

During a periprosthetic infection, due to the presence of a foreign body, two different stages have been distinguished: the first step is the adhesion of bacteria on the implant surface and the second step is slower and involves bacteria proliferation, accumulation and cell-cell interactions that stimulate production of polysaccharides that result in a multilayer biofilm, which is difficult to contrast with a systemic antibiotic therapy, furthermore it inhibits osteoblast proliferation until implant failure occurs[72]. In this study, we evaluated how bacteria adhere and proliferate and, eventually, form the biofilm on the scaffold surface. We performed a serial dilution test on HA/βTCP, HA/βTCP_VCA, HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT scaffolds using a *Staphylococcus epidermidis* to quantify bacteria adhesion and proliferation through counting bacteria colonies forming units (CFU) (**Figure 22).** In order to evaluate the tendency of scaffolds to promote or to avoid a biofilm formation on the surface, SEM investigations were performed at different time points of vancomycin release.

Serial bacterial dilution test

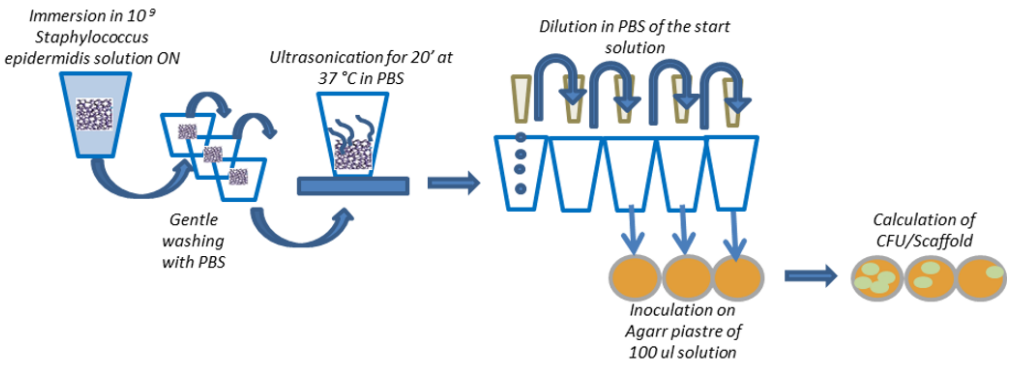

Figure 22. Schematic diagram of serial dilution test protocol

We tested the samples at three different time points of releasing, as prepared (T0), at 24 h and after 1 week. These data correlated with the HPLC vancomycin release test, giving a more precise quantification of bacteria growth on the surface. Bacteria adhesion depends on the vancomycin release but on the surface chemistry of the scaffold, too. In **Figure 23,** the reduction of CFU proliferated on the engineered HA/βTCP_PEC/VCA_CHIT, compared to the other scaffolds, at different time points, is clearly showed. HA/βTCP scaffold is highly colonized by bacteria within all-time points; obviously, surface micro-porosity, roughness, high macro-porosity and interconnectivity, as well as absence of a bactericidal action, leave the scaffold surface free for bacterial colonization; this is what could happen *in vivo* and, in this case, the most likely solution would be the implant to be removed, followed by intensive antibiotic therapies and a prolonged hospitalization period.

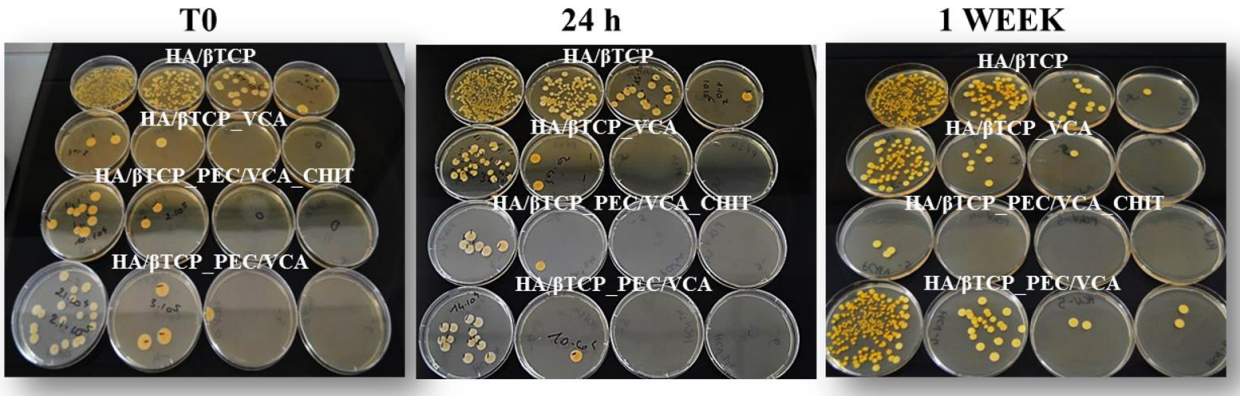

Figure 23. Images of Agar germ plate at time zero, after 24h and after 1 week. Photos shows the absence of CFU on Agar germ plate for HA/βTCP_PEC/VCA_CHIT scaffold at already at low dilution of the starting solution.

In **Figure 24,** the count of proliferated CFU on the scaffold surface at different time points is reported. HA/βTCP_VCA scaffold shows a CFU count of 1.28 x 10^6 at T0 and an increase of CFU at 1 week until 2.42 x 10^7; this behavior is confirmed by the release study, where after 8 h HA/βTCP_VCA all adsorbed vancomycin has been released in a burst. HA/βTCP_PEC/VCA at T0 released enough vancomycin to kill bacteria and to inhibit proliferation on scaffolds; CFU count of 1.88 x 10^5 increased at 24h 1 x 10^6 till at 1 week, reaching CFU 3.75 x 10^6. The engineered HA/βTCP_PEC/VCA_CHIT scaffold shows a similar CFU count compared to the HA/βTCP_PEC/VCA one, in the first time due to the slow release, but control provided by PEI coating allows bacterial adhesion and proliferation to be maintained low until 1 week, which is one order of magnitude low with respect to HA/βTCP_PEC/VCA and two orders of magnitude lower compared to HA/βTCP and HA/βTCP_VCA scaffolds. As it was demonstrated by HPLC release, a controlled and prolonged release allows inhibition of bacterial growth and proliferation on the biomaterial surface, which means that the material developed, HA/βTCP_PEC/VCA_CHIT, shows a behavior that confirms its potential use as bone filler to prevent periprosthetic infection, inhibiting the first step of infection: the bacterial adhesion.

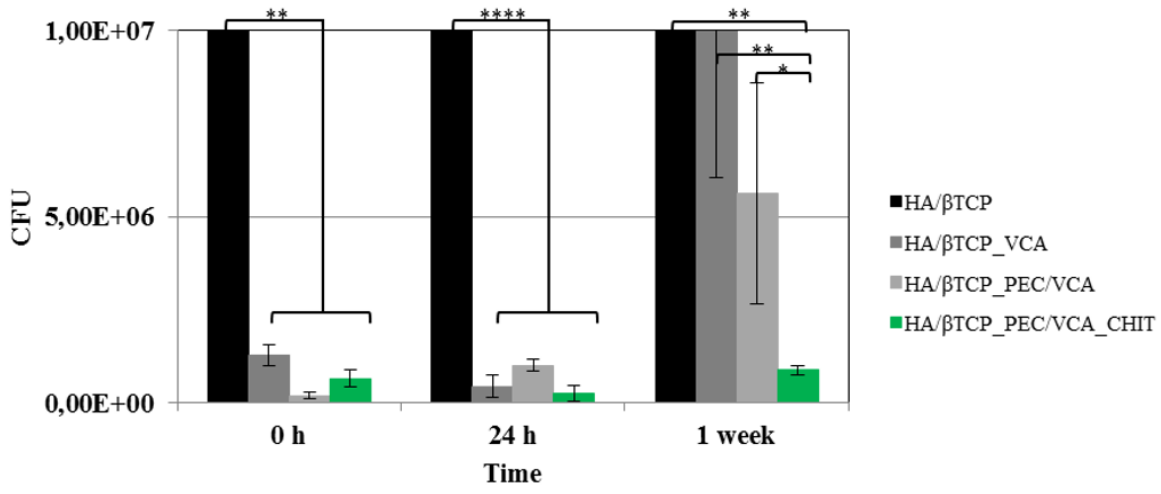

Figure 24. Graph of CFU counted on Agar germ plate at different dilution, the data confirm the low density of bacteria adhered on the surface of engineered HA/βTCP_PEC/VCA_CHIT material. The data are represented as mean ± standard deviation (n=4). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Two-way ANOVA with Tukey's multiple comparison test).

Bacterial adhesion test

Nosocomial infections are the most frequent type of infections caused by *Staphylococcus epidermidis*, which is the most frequently bacterium found in periprosthetic infection[71]. Biofilm formation is a two-step process, in which *Staphilococcus epidermidis,* first adheres on the material

surface and through an accumulation it produces an extracellular polymeric substance forming a multilayer structure complex called biofilm[73]. The ability of bacteria to adhere and grow in a biofilm structure inhibits the reaction of the immune system, furthermore the polysaccharides structure that envelops bacteria protects them from the antibiotic effects. Our goal was to design and develop a complex system which was able to inhibit the adhesion of bacteria and the consequent biofilm formation, coupling both the effect of a widely and successfully used antibiotic, and a layer-by-layer surface coating, exposing on the surface a chitosan layer, that has a well-known antimicrobial activity. In order to confirm that during the release process the proliferation and biofilm formation are controlled, SEM investigation was performed on the engineered scaffold HA/βTCP_PEC_VCA/CHIT material, in comparison with HA/βTCP, HA/βTCP_VCA and HA/βTCP_PEC/VCA samples **(Figure 25).**

All samples, two for each case, were cultured with *Staphylococcus epidermidis* in as prepared (T0) conditions and after a release in physiological solution for 24 h and 1 week. Then, 5 SEM images for each sample have been taken on the surface of scaffolds, at three different magnification 2000X, 5000X and 10000X. For each images at 10000X, *Staphylococcus epidermidis* were manually counted by ImageJ software and a percentage of area covered by bacteria respect the total area of the images was calculated. HA/βTCP sample, as already proven by the serial dilution test, shows high bacterial adhesion and growth at each time point. The microporosity on the surface and the interconnected macropores are perfect niches for bacterial proliferation. SEM images at 30000X show an initial accumulation of bacteria inside the pores and an early deposition of polysaccharides materials **(Figure 26).**

The addition of vancomycin, just adsorbed on the ceramic surface (HA/βTCP_VCA), partially inhibits bacterial adhesion and proliferation at the first point, in as prepared condition; but at 24h and even more after 1 week of release, the presence of *Staphylococcus epidermidis* on the surface was similar to the HA/βTCP sample, furthermore at 1 week SEM images taken at 30000x show a cell-cell interaction and an initial deposition of extracellular polysaccharides matrix **(Figure 26).**

Pectin_vancomycin complex (HA/βTCP_PEC/VCA) allows bacterial growth to be inhibited, in particular at time zero and after 24 h, then the coating's degradation makes ceramic surface to be available and a later colonization to start at 1 week.

A completely different situation was visualized for the engineered developed scaffold (HA/βTCP_PEC/VCA_CHIT), in which the presence of a bilayer on the surface, which entrapped a vancomycin drug active in a long-term release, inhibits the first adhesion step and, therefore, biofilm formation until 1 week of release **(Figure 25, Figure 26).**

Chitosan has been investigated for a long time as an antimicrobial material, in such a way that in last decades the tendency is to consider chitosan as a bacteriostatic material that hinders bacterial growth, rather than a bactericidal one[53]. Different mechanisms aimed at explaining how chitosan interacts with bacteria and inhibits growth are under investigations; the most probably hypothesis are as following: 1) wall cell leakage, due to ionic surface interactions, since chitosan is a polycationic material, 2) penetration of chitosan inside the nuclei of microorganisms which inhibits mRNA and protein synthesis and 3) suppression of nutrients necessary for microbial growth by formation of an external barrier[53,54]. Nowadays, it is not well understood which one of these mechanisms is the predominant one, probably a combination of effects is to take into account, but it is proven that chitosan has an antibacterial activity against gram positive and gram negative bacteria, particularly medium and low molecular weight chitosan.

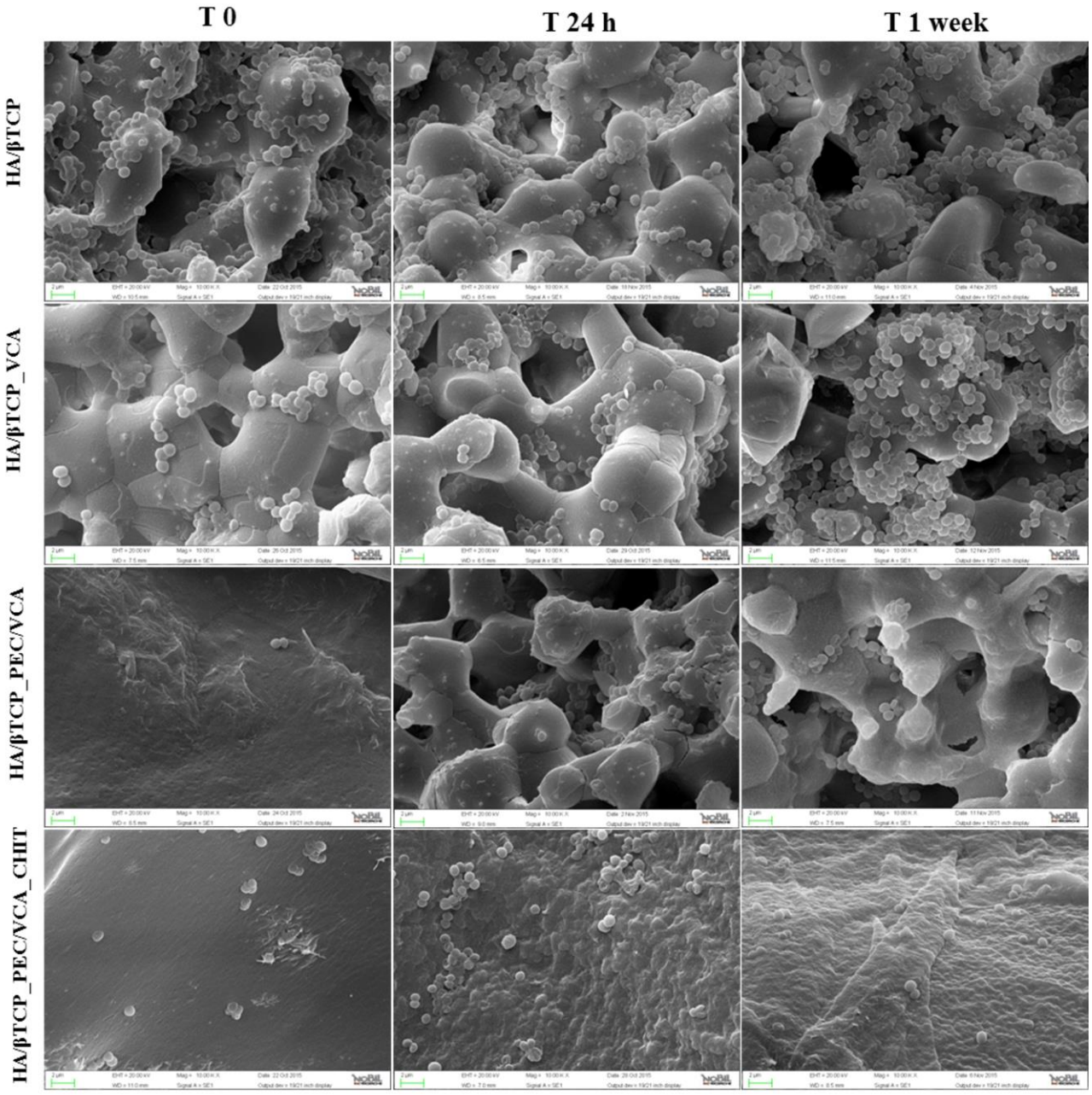

Figure 25. All samples was kept in physiological solution at 37 °C for 24 h and 1 week, then *Staphylococcus epidermidis* was grown on the surface for 2 h and then a fixation process was performed in order to investigate the surface by SEM analysis. SEM images of HA/βTCP, HA/βTCP_VCA, HA/βTCP_PEC/VCA, HA/βTCP_PEC/VCA_CHIT scaffolds in as prepared condition (T 0 h, no release), after 24 h of releasing and after 1 week of releasing. Images were taken at 10000x, scale bar 2 µm. Low bacterial adhesion on HA/βTCP_PEC/VCA_CHIT at each time point was detected. Images at different magnification as supplemental data.

We report the percentage of the reference material surface area (660 μm^2) covered by bacteria cells **(Figure 27)**. As mean value of bacteria diameter, we consider 0.95 ± 0.12 µm that means an area of 0.71 ± 0.18 μm^2 (calculated as mean value on 180 measures). All samples, except for HA/βTCP scaffold, show a percentage of area covered by bacteria around 5 % of total area at time zero

(without release step). However, the results show that the area covered by bacteria on HA/βTCP_PEC/VCA_CHIT biomaterials is almost 10 fold lower at each time point compared to HA/βTCP scaffold. HA/βTCP_PEC/VCA scaffold shows a low value at time zero, then an increase is observed at 24 h and a value around 10 % is reached at 1 week of release. At 24 h, HA/βTCP_PEC/VCA scaffold is characterized by a great standard deviation that is mainly due to the non-homogeneity of the degraded pectin coating, which allows an accumulation of bacteria in some areas. HA/βTCP_VCA scaffold shows a trend demonstrated with serial bacterial dilution tests; the percentage of area covered by bacteria amounts to 10 % of total area at 24 h and rises to the value of 30 % at 1 week, which means that any kind of antibacterial effect is still present at this time point.

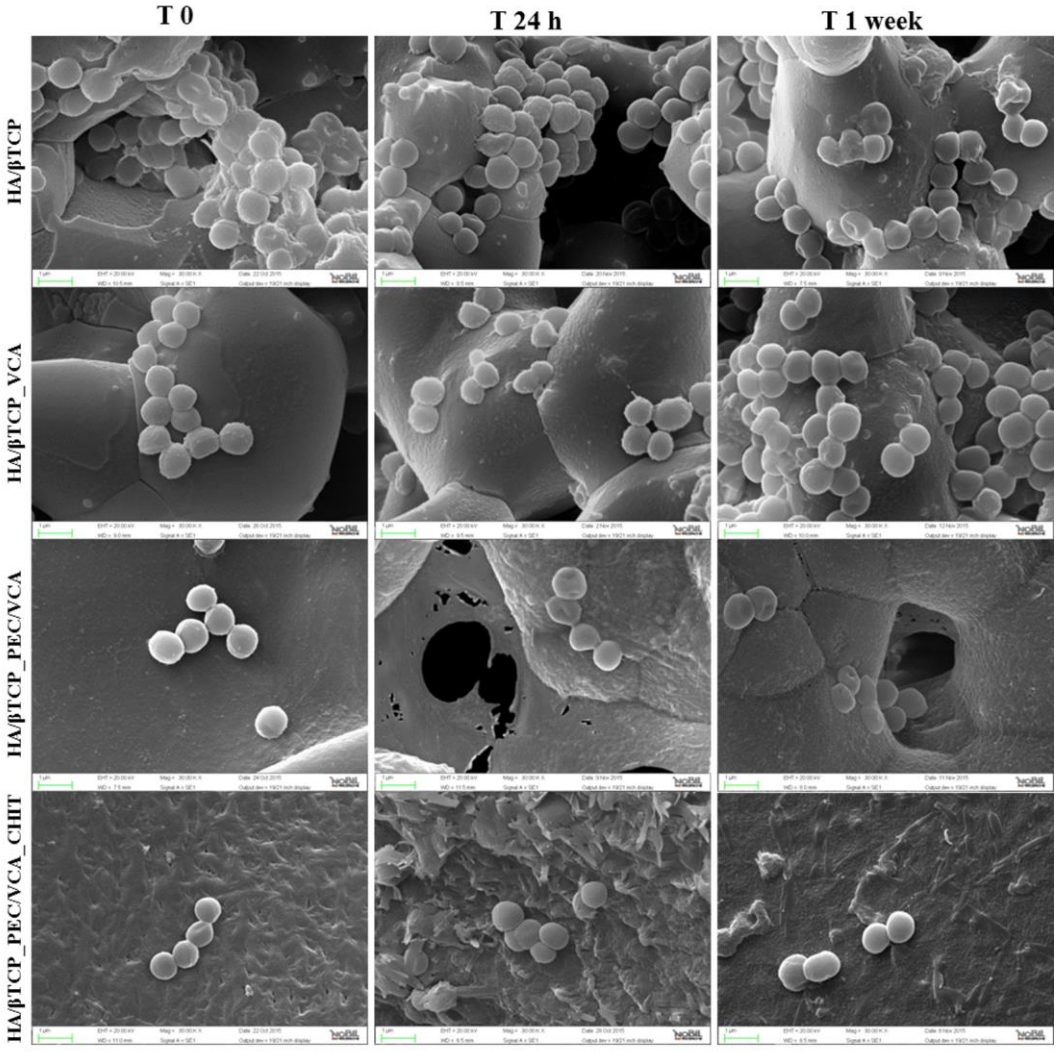

Figure 26. SEM images of HA/βTCP, HA/βTCP_VCA, HA/βTCP_PEC/VCA, HA/βTCP_PEC/VCA_CHIT scaffolds in as prepared condition (T 0 h, no release), after 24 h of releasing and after 1 week of releasing . Images were taken at 30000x, scale bar 1 µm. After 1 week SEM images show a cell-cell interaction and an initial deposition of extracellular polysaccharides matrix, in particular on the surface of HA/βTCP and HA/βTCP_VCA scaffolds.

These results show that a bilayer PEI between pectin and chitosan is necessary to encapsulate drugs for a long term release and to inhibit bacterial adhesion, since after 1 week of release less than 2 % of total area analyzed is covered by bacteria and, furthermore, most of the bacteria found on the surface are poor adhered, only blocked by the folding of the coating.

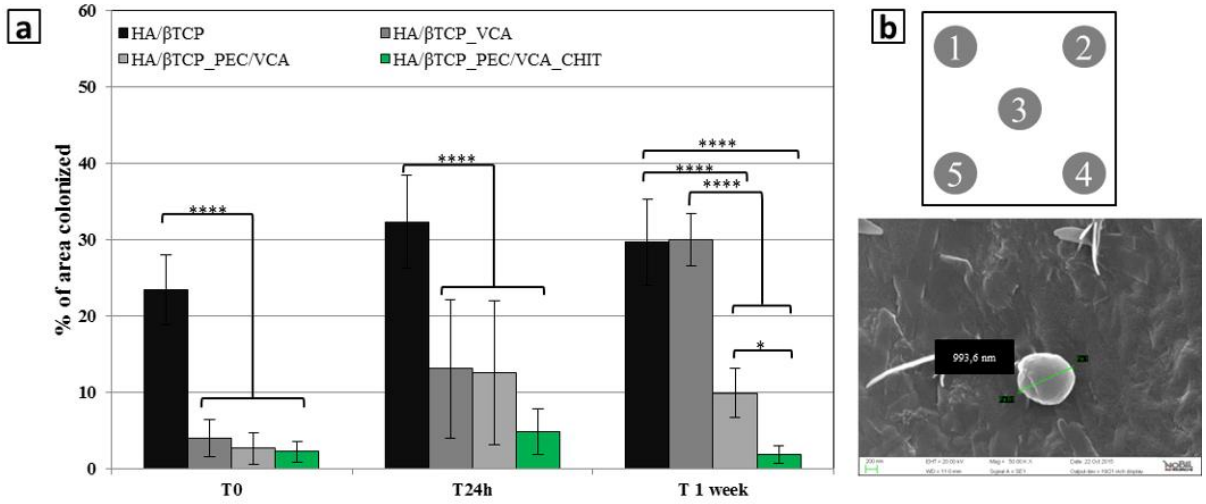

Figure 27. The percentage of surface area covered by bacteria was calculated respect the total area represented on 10000x images (a). The area of *Staphylococcus epidermidis* was calculated as mean value of 180 bacteria, and is equal to 0.71 ± 0.18 µm^2 (diameter of 0.95 ± 0.12 µm (b). The total area analyzed for each images is equal to 660 µm^2. Five points for each scaffold (n=2) were analyzed, as it is represented by the schematic diagram (c). HA/βTCP scaffold was covered by bacteria for 30% of total area, at each time point. The addition of Vancomycin and Vancomycin-Pectin, inhibits bacterial adhesion until 24 h and 1 week, respectively. Only engineered HA/βTCP_PEC/VCA_CHIT scaffold allows antiadhesive properties against *Staphylococcus epidermidis*, up to 1 week. The data are represented as mean ± standard deviation (n=5).

In vitro study

Developing a new composite bone filler using ceramic and polysaccharides material is important to check if some inflammatory responses are generated. Furthermore, prevention strategies of periprosthetic infection should involve bacterial growth inhibition as well as osteoblast proliferation promotion, with the aim to reach new bone formation. Specific ceramic scaffold's microarchitecture could allow cell infiltration and bone growth inside the scaffold, in particular HA and βTCP components have already shown a great potential in bone regeneration, since they are the principle inorganic component of bone. Ideally, PEI coating would guide soft tissue regeneration in the first times after surgery, avoiding connective tissue infiltration inside the bone substitute, since it has a faster growth and through ceramic interaction with osteoblast cells promoting bone formation, meanwhile controlling bacterial infection.

Inflammatory response

We performed an RT-PCR gene expression on HA/βTCP, HA/βTCP_VCA, HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT scaffolds after 4 h of macrophage cells culture. Gene expression analysis on IL-1β, IL-10 and MCP1 were conducted by real time RT-PCR **(Figure 28)**. The target of this test was to understand if the different steps of functionalization could stir up an inflammatory response, compared to HA/βTCP ceramic scaffold, which were set as a control. As it is reported, no material elicits a pro-inflammatory response, IL-1β and MCP1 gene fold expressions are comparable to the HA/βTCP ceramic control, which means that neither HA/βTCP_VCA nor HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT stimulate an inflammatory response. HA/βTCP_PEC/VCA_CHIT shows a 3-fold expression higher compared to other samples tested for IL-10 gene.

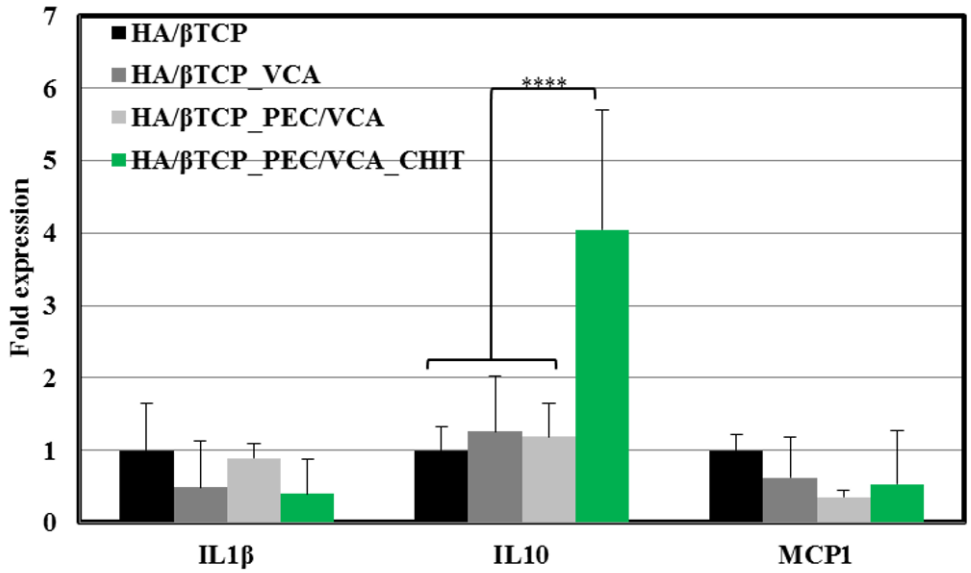

Figure 28. Gene expression of macrophages after 4 h of cell culture. a) Fold expression of cytokines IL-1β, Il-10 and chemokines MCP1 for HA/βTCP_VCA, HA/βTCP_PEC/VCA and HA/βTCP_PEC/VCA_CHIT scaffolds respect the ceramic control HA/βTCP scaffold. Results demonstrated that any material stimulates a pro-inflammatory response, and that PEC_VCA_CHIT coating elicits anti-inflammatory response, with a higher expression of IL-10. The data are represented as mean ± standard deviation (n=3). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Two-way ANOVA with Tukey's multiple comparison test).

If implanted materials rose a local inflammatory response, the immune system would be activated[74]. Propagation of this local inflammation would activate the release of a set of cytokines and, if not controlled, could promote bone resorption and, consequently, implant's failure. Bone resorption occurs when inflammatory mediators reach a critical concentration, which depends on the expression of pro-inflammatory cytokines, such as the Interleukin (IL) family, of which IL-1β is the most studied member due to its role in acute and chronic inflammatory and autoimmune disorders,

and chemokines, a family of chemoattractant cytokines which major role is the selective recruiting of monocytes, neutrophils and lymphocytes. Besides IL-1β primary action, monocyte chemoattractant protein-1 (MCP-1), the first discovered human CC chemokine, regulates migration and infiltration of monocytes/macrophages, thus contributing to raise a response aimed at eliminating invading pathogens through phagocytosis. On the opposite site, the inflammatory level is controlled by the expression of anti-inflammatory cytokines, such as IL-10. In normal physiological conditions, there is a balance between bone formation and bone resorption, and as it happens in certain inflammatory conditions, this balance can be altered. This equilibrium is regulated by the relative expression of proteins such as receptor activator of nuclear factor kappa-B (RANKL) and the soluble decoy receptor osteoprotegerin (OPG). During an inflammation response, RANKL/OPG ratio is altered by the action of pro-inflammatory cytokines, such as IL-1β that induces osteoclastogenesis, increasing the expression of RANKL, while counteraction by anti-inflammatory mediators, such as IL-10, decreases RANKL or increases OPG, in order to inhibit osteoclastogenesis. The results elucidate that PEI coating stimulates the expression of an anti-inflammatory mediator; *in vivo*, this stimulation could help in controlling inflammation and in promoting bone formation.

Osteoblast-like cell culture

Adhesion and proliferation of osteoblastic SAOS-2 cells are demonstrated through SEM investigations **(Figure 29).** An excellent colonization could be observed on HA/βTCP scaffold after 1 week of cell culture, on which substrate cells appeared well adherent with many filipodia protrusions. HA/βTCP_PEC/VCA_CHIT scaffold shows a good osteoblast colonization, in particular where HA/βTCP phase is directly available for cell adhesion, cells appear well spread and strong philipodia protrusions were observed. Polyelectrolyte coating did not interfere with osteoblast proliferation[38], instead as it is possible to observe from SEM images, only pectin coating avoids cell adhesion, on the calcium phosphate surface, too.

HA/βTCP_PEC/VCA_CHIT scaffold provides an excellent substrate for osteoblast adhesion, spreading and proliferation, and it is confirmed that the vancomycin released is not cytotoxic against osteoblast growing cells. HA and βTCP have been widely used as bone filler materials, since they are similar to the inorganic phase of natural bone. Many studies show the biocompatibility and the attitude to promote bone formation *in vivo*, furthermore highly porous calcium phosphate scaffolds have been demonstrated to enhance osteoprogenitor cell proliferation and infiltration[16–18]. However, the major concern against antibiotic release *in situ* is related to the biocompatibility with the surrounding tissues, research work showing that, along all tested

antibiotic, vancomycin shows excellent antibacterial activity against germs and the lowest osteoblasts cytotoxicity[75] .

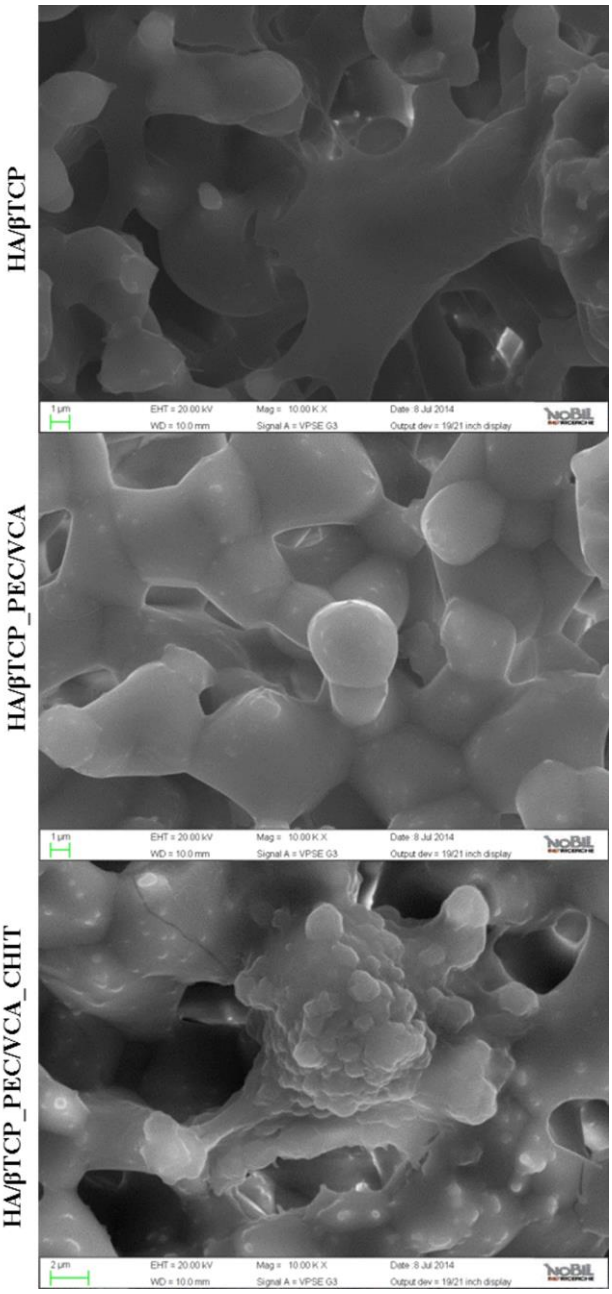

Figure 29. SAOS-2 osteoblast adhesion and proliferation after 1 week were investigated by SEM, after glutaraldehyde fixation. An excellent adhesion and spreading was observed on HA/βTCP scaffold. A very low cell adhesion was present on the surface of HA/βTCP_PEC/VCA scaffold, as well on the ceramic exposed part. A good cell adhesion and philipodia protrusion was observed on HA/βTCP_PEC/VCA_CHIT scaffold, in particular on the ceramic exposed part.

4.4.3 Conclusion

Dental infections caused by infected orthopedic implants may cause devastating consequences, since they involve the removal of foreign body and damage tissue from a wound. A multitude of surgical procedures are necessary, coupled to an intensive systemic antibiotic therapy, furthermore the poor antibiotic penetration inside the infected bone and the specific interaction of the bacteria with the foreign body could result in a re-infection of the site. However, leaving the bone defect untreated could allow connective tissue infiltration and a loss in the mechanical stability. Bone defects have to be filled with a scaffold that could provide a local administration of antibiotic drug, could control the infection and could address bone regeneration before a new implant could be inserted. In this study, we developed a new-engineered biphasic calcium phosphate scaffold functionalized with a pectin and chitosan polyelectrolyte, loaded with vancomycin, HA/βTCP_PEC/VCA_CHIT. Performed tests show good mechanical properties and stability during degradation in different pH test solutions, coupled with an excellent antibacterial property along 1 week. Release studies demonstrated that the developed coating allows a long-term release, confirmed by SEM investigation on biofilm formation on the surface of scaffolds. Anti-inflammatory responses have been stimulated in *in vitro* studies with macrophages and polyelectrolyte functionalization does not interfere with osteoblasts growing. HA/βTCP_PEC/VCA_CHIT scaffold could be used as new and effective tools to prevent or treat periprosthetic infection in bone and dental procedures.

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4.5 NOVEL BIOCERAMIC-REINFORCED HYDROGEL FOR ALVEOLAR BONE REGENERATION

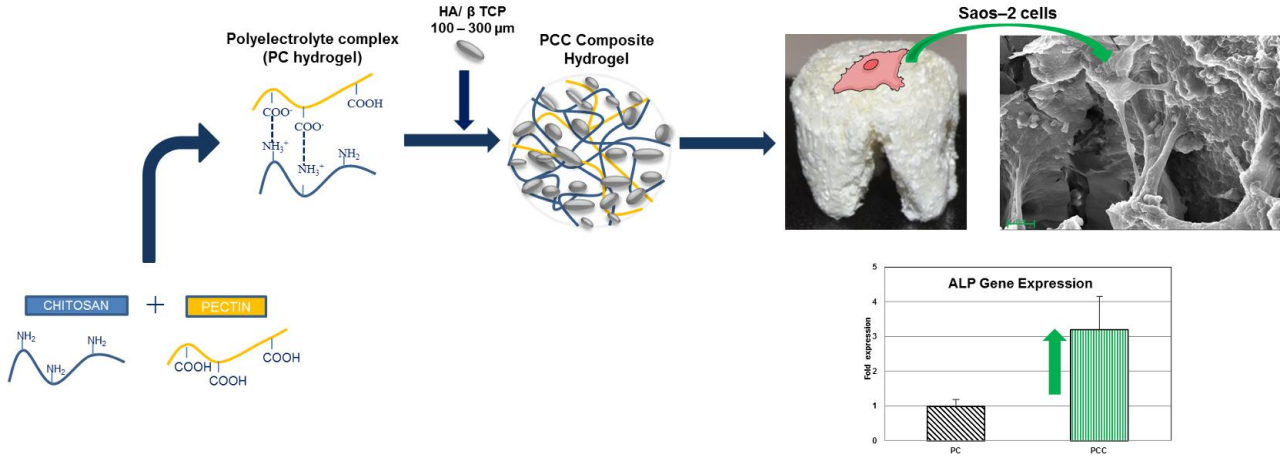


Figure 30. Graphical abstract of the bioceramic-reinforced hydrogel.

4.5.1 Introduction

The restoration of teeth by using titanium dental implants is nowadays a quite common procedure[1]. The American Association of Oral and Maxillofacial Surgeons reported that 69% of adults aged 35 to 44 have lost at least one permanent tooth and, by age 74, almost 26% of adults have lost all of their permanent teeth. More than 300,000 dental implants are placed per year worldwide and, until 2020, this number is expected to increase[1,2]. Tooth loss is a possible consequence of traumas or periodontal diseases, such as gingivitis, periodontitis or tissue decay. The rate of success of dental implants is around 98%, but it should be taken into account that the positive fate of a surgical procedure involving the insertion of a titanium screw still depends on the quality and quantity of alveolar bone which is present in the extraction site. In order to get a successful implant insertion, the alveolar ridge should have a minimum dimension, which is of 5 mm of width in the maxilla and of 10 mm of bone height[3]. If these dimensions are not available, an augmentation strategy will be necessary using grafting procedures. A huge alveolar bone loss of around 2 mm in vertical and almost 50% in horizontal occurs in the first 6 months after surgery; a continuous bone resorption occurs if no treatment is provided during the next years after the extraction procedure, and an average of 60% of bone loss could be reached in 3 years[4–9]. These results show the importance of the implementation of a strategy aimed at augmenting the alveolar bone volume to provide a stable support for the implant and the future crown. The common use of autografts or allografts is affected by the morbidity and the risk of disease transmission associated with the donor site, thus the use of man-made bone graft biomaterials is more and more attractive[10–12]. A bone graft material should be

biocompatible, degradable, osteoconductive, osteoinductive and should mimic the ECM of bone, in order to allow cell infiltration, proliferation and new bone formation. Hydroxyapatite (HA) and β-tricalcium phosphate (βTCP) are well-known ceramic materials, widely used in bone tissue engineering as bone grafts in the form of particles or three-dimensional scaffolds[13–15]. HA ($Ca_{10}(PO_4)_6(OH)_2$) is the most abundant component in native bone, around 65% of inorganic phase, but despite many studies show osteoconduction stimulation and excellent mechanical properties, the slow degradation rate of HA limits its use as a bone filler only[16–18]. Combination of HA with βTCP allows the degradation properties to be managed, since βTCP materials have a degradation rate 3 – 12 times faster than HA, and mechanical properties to be maintained good[19]. The balance between HA and βTCP is a key point to obtain mechanical strength, degradation and, ultimately, to stimulate osteointegration. Animal studies demonstrated that HA/βTCP biphasic materials in the rate of 25/75 allow new bone formation[15]. In the irregular post-extractive site, it is important to use materials which can fill the void, be shaped in an easy manner and be not prone to migration, hence particles and three-dimensional rigid scaffolds are not the best choice. To overcome this issue, we developed a pectin/chitosan-based polyelectrolyte hydrogel reinforced with biphasic calcium phosphate particles. Chitosan is a natural polycationic material derived from chitin extracted from crustaceous exoskeleton, composed of β – (1,4) – glucosamine and N-acetyl-D-glucosamine and, due to its biocompatibility, its intrinsic antibacterial nature, its ability to not stimulate a foreign body reaction, and its promotion of cell adhesion, proliferation and differentiation, it has aroused great interest in finding many applications in bone tissue engineering[20,21]. Furthermore, chitosan has a backbone similar to glycosaminoglycan, the major component of bone ECM[22,23]. In order to stabilize the structure, we coupled chitosan with a natural polyanionic polysaccharide, pectin, which is a major component of cell walls of citrus or apple peel by-products. Pectin consists in a poly(D-galacturonic acid) chain, with a carboxyl group, in part methoxylated[24]. In the last decades, pectin has found promising application in bone tissue engineering as a drug carrier and, in this work, the ionic interactions that occur between chitosan and pectin were used to develop a novel hydrogel which could mimic an ECM-like environment for osteoblast cells[25–27]. Combining ceramics with natural materials provides many advantages, in particular for dental practice: shape control, optimization of adhesion between implant and surrounding bone tissue, easy adaptation of the extraction site, promotion of clot formation and avoiding ceramic particles migration[16,23,28–30].

4.5.2 Results and Discussion

In this work, we designed, developed and characterized a composite hydrogel sponge, which combines the advantages of calcium phosphate materials with those of natural materials, and we compared it with base hydrogel materials.

4.5.2.1 *Chemical characterization*

ATR-IR analysis

ATR-IR spectra reported in **Figure 31 a** show the spectra of pectin, chitosan, pectin/chitosan polyelectrolyte complex (PC), HA/βTCP particles and pectin/chitosan_HA/βTCP composite hydrogel (PCC). The analysis confirms that all peaks belong to inorganic material; in particular, triply degenerated asymmetric stretching mode (ν3) of the P-O bond of the phosphate group is associated to the peaks at 1125 cm^{-1} for tricalcium phosphate and at 1025 cm^{-1}-1010 cm^{-1} for hydroxyapatite[15,31]. Typical spectra of polysaccharides were shown by pectin and chitosan powder. The region between 3700 cm^{-1} and 3000 cm^{-1} for pectin and chitosan is assigned to the O–H stretching vibration (νOH), while the region between 3000–2800 cm^{-1} belongs to C-H stretching vibration (νCH) **(Figure 31 a)**. Deeper analysis on pectin spectra shows two bands associated with the stretching vibration at 1740 cm^{-1} of carbonyl group, corresponding to the methyl ester group ($COOCH_3$) and carboxyl acid (COOH), while the band at 1606 cm^{-1} belongs to the stretching vibration of the carbonyl group of the carboxylate ion (COO^-) **(Figure 31 b).** Concerning chitosan spectra, the band at 1647 cm^{-1} is due to the C=O stretching vibration of amide I, whilst the band at 1580 cm^{-1} is due to the NH bending amide II, maybe overlapped to the N-H vibration of the amine groups **(Figure 31 b)**. Band assignment is consistent with available literature[32,33]. PC spectra show the formation of PEI complex; a shift of amine band to 1557 cm^{-1} due to the interaction between the positive charge of chitosan, NH_3^+, and the negative charge of pectin, COO^- , was detected **(Figure 31 b)**. As expected, the spectra of PCC sample show bands associated with both the PEI complex and the inorganic phase; in particular, the band at 1557 cm^{-1} and those between 950 cm^{-1} and 1140 cm^{-1}, respectively **(Figure 31 b)** [34].

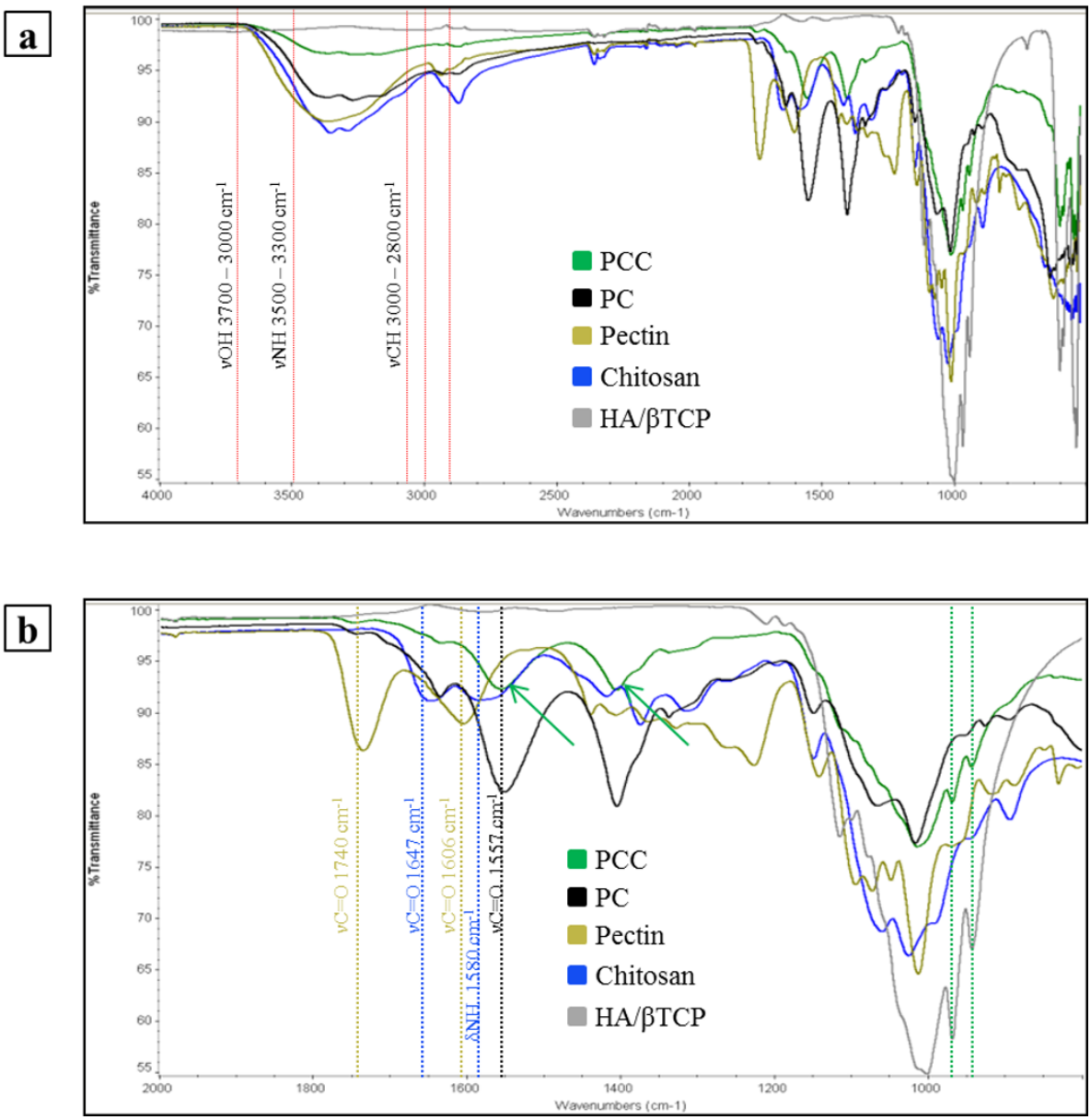


Figure 31. (a) ATR-IR spectra of pectin and chitosan powder, HA/βTCP ceramic particles, PC hydrogels, PCC composite scaffolds. (b) ATR-IR spectra focus between 2000 cm^{-1} and 500 cm^{-1} of materials, show the shift of the amine band to 1557 cm^{-1}, and the bands belonging inorganic phase, at 950 cm^{-1} and 1140 cm^{-1}.

4.5.2.2 Structural characterization

µCT and SEM analysis

Three-dimensional scaffolds should allow cell-infiltration and facilitate vascular invasion. Furthermore, it is necessary that the ceramic particles are homogenous dispersed in the natural matrix, in order to promote uniform osteogenesis. Both, micro-porosity and macro-porosity, are important morphological properties[35,36]. Micro-porosity promotes blood vessel infiltration, nutrients transportation and allows the clot formation that is important to promote the healing process. On the

other side, macro-porosity allows cell infiltration and new bone growth[37,38]. Capillary test using red ink, qualitatively shows the porosity interconnection of the PCC material, which could adsorb the solution in around 5 seconds (**Figure 32**). Furthermore, after hydration, if a compressive stress is applied on the samples, the solution is expulsed from the matrix, but after removal of the stress the PCC material is able to re-adsorb all the solution, without any permanent deformation. The material works as a bearing, which is an important characteristic in order to avoid inflammatory response on the surrounding tissue due to the stiffness of the material, in particular in application at the interface between hard and soft tissues. Furthermore, the ability to adsorb water and retain it into the matrix allows formation of a clot in the wound site, which is the first step to stimulate the migration of bone cells into the scaffold, and to promote regeneration of alveolar bone. In alveolar bone regeneration, the use of sponges is desirable, since they could easily fit into the irregular alveolar bone defect, be simply cut with scissors or a lancet, and easily molded in the periodontal cavity (**Figure 32**)[16,38,39].

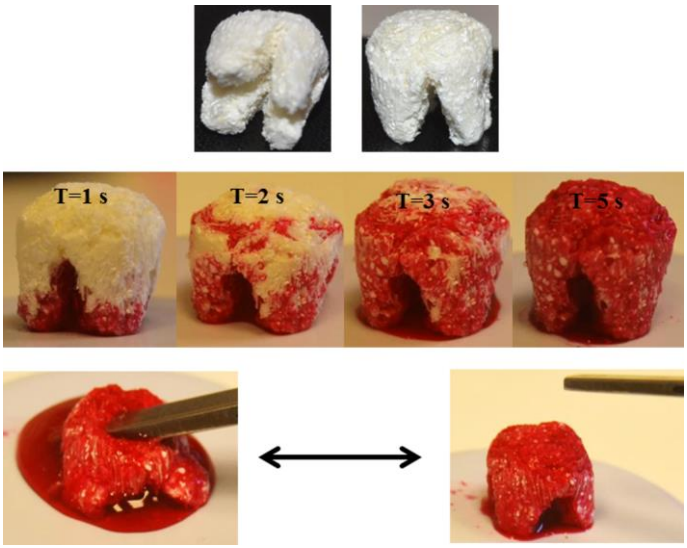


Figure 32. Easy mouldability of composite scaffold, highly hydration properties (water colored with few drops of red ink was used for this qualitative hydration test) and capacity to recover the initial shape after a compressive stress.

A µCT study (**Figure 33 a**) confirmed the results of SEM analysis. Pore size distribution for PCC sample is in the range around 100 - 250 µm, while the PC scaffold has a pore size distribution which goes until 600 µm, with most pores located between 250 and 300 µm (**Figure 33 b**). Direct comparison of mean pore sizes (St.Sp) further confirms the decrease of pore dimension due to the introduction of ceramic particles (250 vs. 120 µm). The total porosity decreases by adding ceramic particles, too, from around 74% for PC sample to 55 % in the case of PCC scaffold.

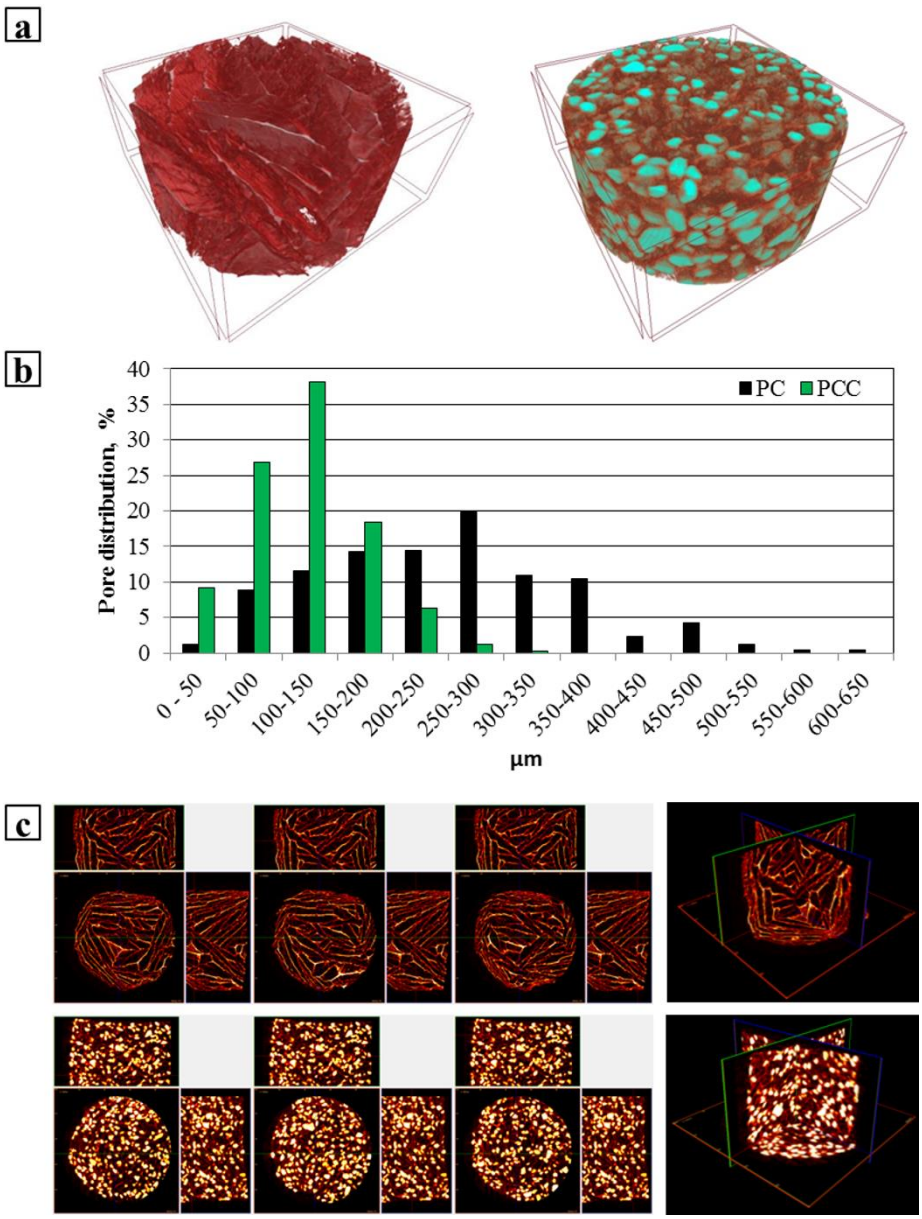


Figure 33. (a) µCT images confirm SEM investigation and reveal uniform distribution of ceramic particles in the pectin_chitosan PEI matrix. (b) Pore size distribution of PC and PCC scaffold, the addition of ceramic particles reduces the pore size. (c) 2-D development of PC and PCC scaffold, obtained by placing the analysis planes [xy] and [yz] in the mid-length, and moving the [xz] plane from the bottom to the middle and until the top of the length of the scaffold; and 3-D reconstruction of the central (approximately mid-length) cross-section along the [xy], [xz] and [yz] orthogonal planes.

In **Figure 34,** SEM analysis at different magnifications for PC and PCC samples are reported. The addition of ceramic particles reduces the pore size and the total porosity, but reinforces the trabeculae and the structure of the materials. Furthermore, the dispersed particles are well distributed in the PEI matrix. *In vitro* and *in vivo* studies show that a porosity up to 50 %, and pore size in the range between 100 to 400 µm are the optimum for bone healing and show higher alkaline

phosphatase activity and new bone formation compared with materials with a porosity lower than 100 μm and higher than 400 μm[40].

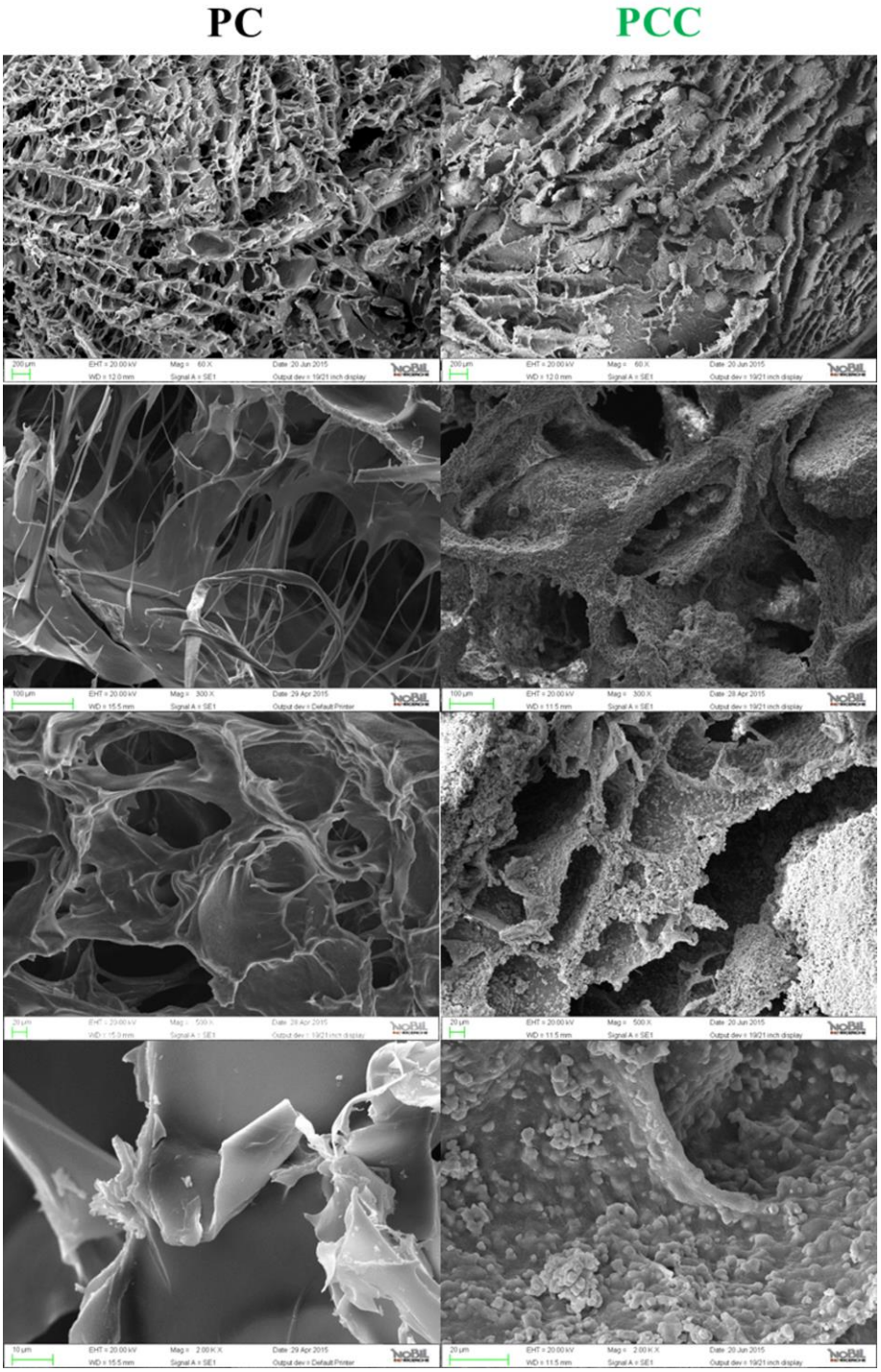


Figure 34. (a) SEM investigation of PC and PCC scaffolds, highly interconnected pores are shown for PC scaffold; the incorporation of ceramic particles reduces the porosity and pore size of the material.

4.5.2.3 *Mechanical characterization*

Compression test

The incorporation of ceramic particles into soft hydrogel scaffolds is a valuable strategy to improve the mechanical properties of the base material[41,42]. A compressive test was performed on PC and PCC scaffolds, and the results are show in **Table 3** and in **Figure 35**. The addition of HA/βTCP particles increases the compressive strength, the stress strain and the toughness of the material, both in as prepared and hydrated condition. Compressive elastic modulus for PC scaffolds was 1005.3 ± 250.0 kPa in as prepared condition, instead was two-fold higher for PCC scaffold, 2559.33 ± 595.6 kPa. Hydration kinetics data show that the water uptake at pH 7.4 was higher in both case and, therefore, the compressive elastic modulus decreases to 33.3 ± 7.6 kPa for PC material and until 65.4 ± 8.97 kPa for PCC scaffolds. Alveolar bone regeneration is a low-bearing application, where providing stability and three-dimensional shape for functional and aesthetic reasons is more important than mechanical strength[16,43]. PC materials show a soft structure, and the incorporation of ceramic particles inside the PEI matrix makes the material tougher (**Table 3)**. For example, the toughness was 34.28 ± 4.02 kJ/m^3 and 222.86 ± 1.94 kJ/m^3 for PC and PCC scaffold respectively, in as prepared conditions. There is a relative paucity of literature dealing with the toughness of bone tissue engineering scaffolds and, thus, comparison with previous works is difficult; however, it is interesting to point out that the toughness of PCC scaffold is ten times higher than that assessed for single-phase glass-ceramic porous scaffolds[44]. After 24 h at 37 °C in a solution with pH 7.4, both hydrated samples show a decrease of toughness (2.38 ± 0.46 kJ/m^3 for PC scaffold and 12.04 ± 2.05 kJ/m^3 for PCC scaffold) compared to as prepared materials. A similar behavior was also found by Liu et al. on bioglass scaffold[45]. The toughness of PCC material in hydrate conditions is comparable, as order of magnitude, to the value found by other authors for hydroxyapatite scaffolds[46]. Ceramic particles, as expected, reduce the porosity but mechanically reinforce the scaffolds, maintaining a range and a degree of porosity that should still allow cell infiltration and proliferation[35].

SAMPLE	AS PREPARED			HYDRATED		
	Compressive Modulus [kPa]	$Stress_{40\%}$ [kPa]	Toughness [kJ/m³]	Compressive Modulus [kPa]	$Stress_{40\%}$ [kPa]	Toughness [kJ/m³]
PC	1005,33 ± 250,00	127,00 ± 5,57	34,28 ± 4,02	33,33 ± 7,56	13,27 ± 3,00	2,38 ± 0,46
PCC	2559,33 ± 595,61	1525,33 ± 60,93	222,86 ± 1,94	65,44 ± 8,97	120,67 ± 27,01,	12,04 ± 2,05

Table 3. Table reporting compressive modulus, stress and toughness parameters.

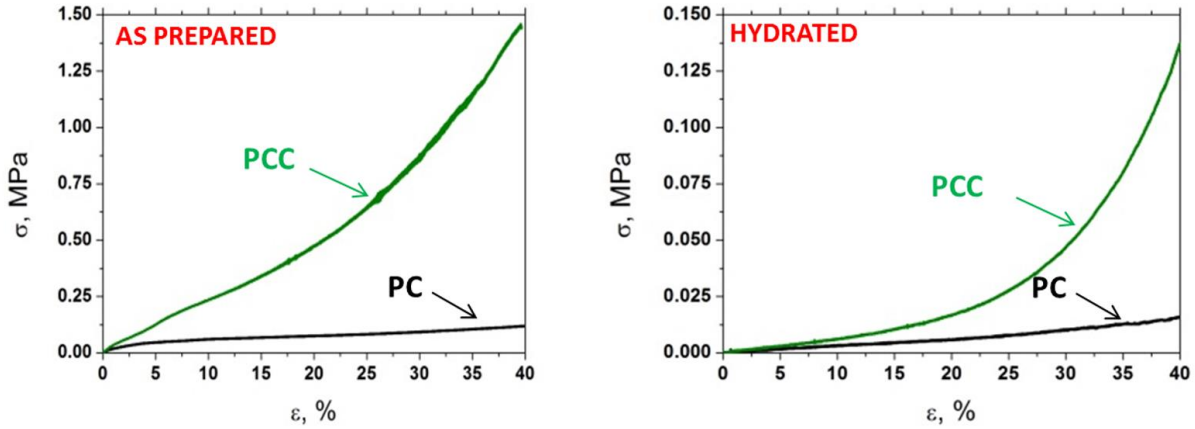


Figure 35. Stress – strain curves for PC and PCC scaffolds in as prepared and hydrated conditions.

Cyclic stress is a common situation under which the scaffolds used for alveolar bone regeneration are subjected. The stability and integrity of the scaffolds are the most important properties that they should have, since material fragmentation could provoke an inflammatory response and a huge deformation could cause the collapse of the defect. Hydrogel PC scaffolds show a stable energy adsorbed (and recovery network) from the 5th to the 100th cycle, the addition of 90% of ceramic particles does not affect too much the trend, the energy adsorbed was slightly higher for the PCC composite due to the reduced mobility of the PEI network. The results are affected by the deformation of swollen surface of the samples, that does not return instantaneously to the original shape after cessation of compression stress. After 100 cycles, no fragmentation was detected for both samples, furthermore the sample left without load to recover the shape for 5 minutes, showed a variation of the final diameter with respect to the initial diameter of 1.4 ± 0.59 % and 7.52 ± 1.32 % respectively for PC and PCC materials (more than 90 % of recovery for both samples). This means that, *in vivo*, the PCC scaffold is able to maintain the shape and to avoid the collapsing of the site (**Figure 36**). Furthermore, tooth extraction site has a non-regular shape, hence ensuring the possibility to customize the dimension of the material in order to fit the socket is an important characteristic for dental practitioner. In alveolar bone regeneration, the use of sponges is desirable, since they could easily fit in to the alveolar bone defect, and could be simply cut with scissors or a bistoury, and easily molded in the periodontal cavity[16,38,39]. The addition of calcium phosphate particles, doesn't influence the mouldability of the PC hydrogel base material.

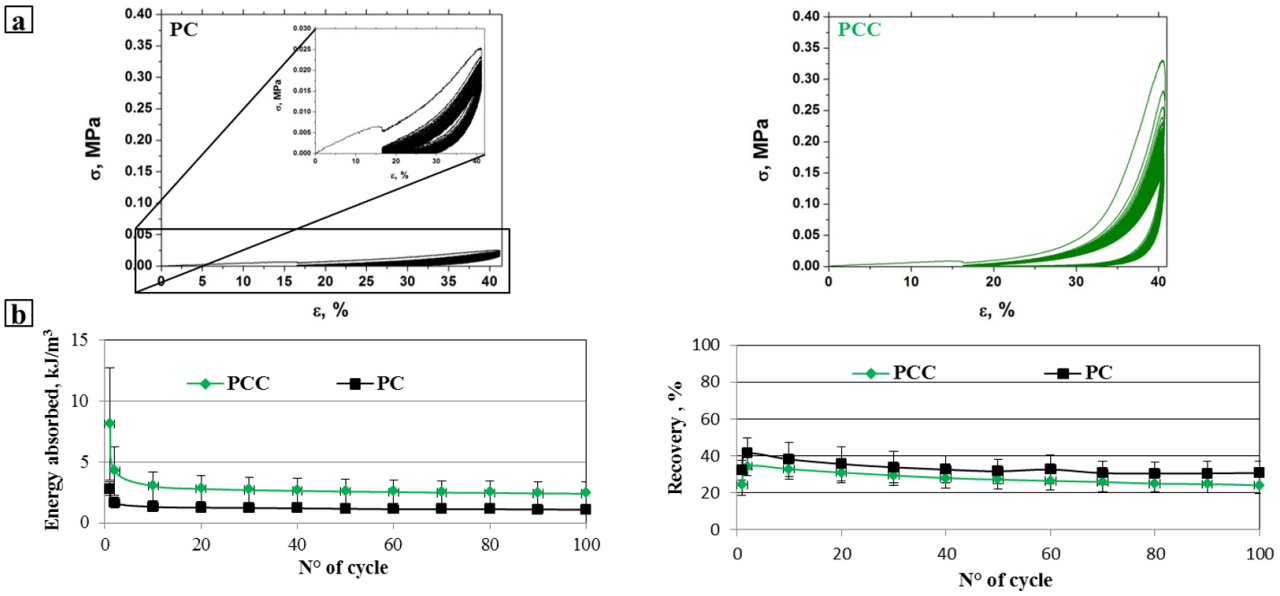


Figure 36. PC and PCC samples were subjected to cyclic strain, in fully hydrated condition and the loading and unloading curves were monitored for hundred cycles. Energy adsorbed and recovery percentage were monitored for PC and PCC samples every 10 cycle. The incorporation of calcium phosphate particles increases the strength of the scaffold, but does not reduce the recovery ability of the PCC scaffold, which is similar to the PC material. The data are represented as mean ± standard deviation (n=3).

4.5.2.4 *Physical characterization*

Hydration studies at different pH

A high degree of swelling allows cell infiltration into the scaffolds and maximizes the probability of cells growth in a three-dimensional structure[29]. Furthermore, high swelling behavior improves the ability of the scaffold to adsorb nutrients from the culture media; chitosan/pectin complexes are formed by a ionic interaction between positive charges of chitosan and negative charges of pectin, hence, the PEI network exhibits a pH-sensitive swelling[47,48]. We tested the swelling behavior in three different solutions with three different pH 2.5, 5.5 and 7.4, and the results are shown in **Figure 37.** PC based hydrogels showed a significant different behavior depending on the pH of the solution. Changing the pH of the solution, the degree of interaction between pectin and chitosan changes and the swelling increases or decreases, depending on the degree of dissociation of the complex (**Figure 37 a**). At low pH values (2.5), pectin is neutralized and free positive charges (NH^{3+}) appear in the network; COOH from pectin chain allows the swelling of the material until the value of 1460.42 ± 81.33 % of the initial mass, after 6 h. After 24 h PC, hydrogels soaked in a solution of pH 2.5 showed a huge increase in the water uptake; this behavior could be associated to the total degradation of pectin and to the loss of PEI network that allows the highest water

adsorption, 2495.34 ± 33.87%. At alkaline pH (7.4), free negative charges appear inside the network, since at higher pH chitosan is partially neutralized, and the PEI network will be loosen, allowing a percentage of solution uptake equal to 1880.16 ± 218.8 %, after 6 h. At pH 7.4, the partial neutralization of NH^{3+} has not caused a total loss of the PEI network, since chitosan has still some ionized group bonded with COO^- of the pectin chain. However, after 24 h, the swelling ratio started to decrease, due to an initial degradation of the network. Since the pK_a of pectin is 4.0 and that of chitosan is 6.0, at the pH of 5.5 over 99% of pectin is still in its ionized form and chitosan exists as both ionized form NH^{3+} and unionized form NH_2. The swelling percentage of PC network in the solution with a pH of 5.5, was 1443.77 ± 14.4 % after 6 h; then, the ability of the network to adsorb the solution reduces. However, due to the presence in the network at pH 5.5 of intramolecular H-bonding between $COOH_3$ and OH, which makes more stable the PEI matrix, the PC hydrogels after 24 h at pH 5.5 show a lower swelling degree (687.69 %) compared with the PC hydrogel at the pH of 7.4 and 2.5. Addition of HA/βTCP particles stabilizes the swelling properties of the PC based hydrogel (**Figure 37 b**). Indeed, no significant differences were measured (two-way ANOVA test reveals no significant difference between PCC scaffold at different pH) during the hydration kinetics in the three different solutions (pH of 2.5, 5.5, 7.4) (**Figure 37 b)**. Furthermore, as expected, the maximum water uptake was lower for PCC scaffold compared with the PC hydrogel: for example, the swelling degree for PC scaffold at pH 7.4 was after 6 h about 275.05 ± 35.45 %, almost 10 folds lower than the value reported for PC hydrogel at the same pH. The maximum percentage of solution uptake for PCC material was reached after 2 h and was stable until 24 h. As already reported for PC hydrogels, also in the case of PCC samples the lowest value of water uptake was shown for the sample soaked in the solution at pH 5.5. Providing a stable material could allow the dental practitioners to use the scaffold independently of the environment, since it will work always at the same manner **(Figure 38)**. Furthermore, the swelling of the PCC scaffold is high enough to ensure cell infiltration and nutrient transportation, and a mechanical strength is still maintained. The incorporation of ceramic particles into the PC matrix reduces the swelling capability of the materials, which is maybe due to the interaction between the particles and the network. In particular, pectin carboxylic groups could be ionically crosslinked by calcium ions (Ca^{2+}), thereby forming the so-called “egg box” structure, where a divalent cation is bonded with different carboxylic anions[24]. Furthermore, the high percentage of ceramic particles used in this work (90 wt.%) reduces the influence of the PC matrix in the swelling properties **(Figure 38)**.

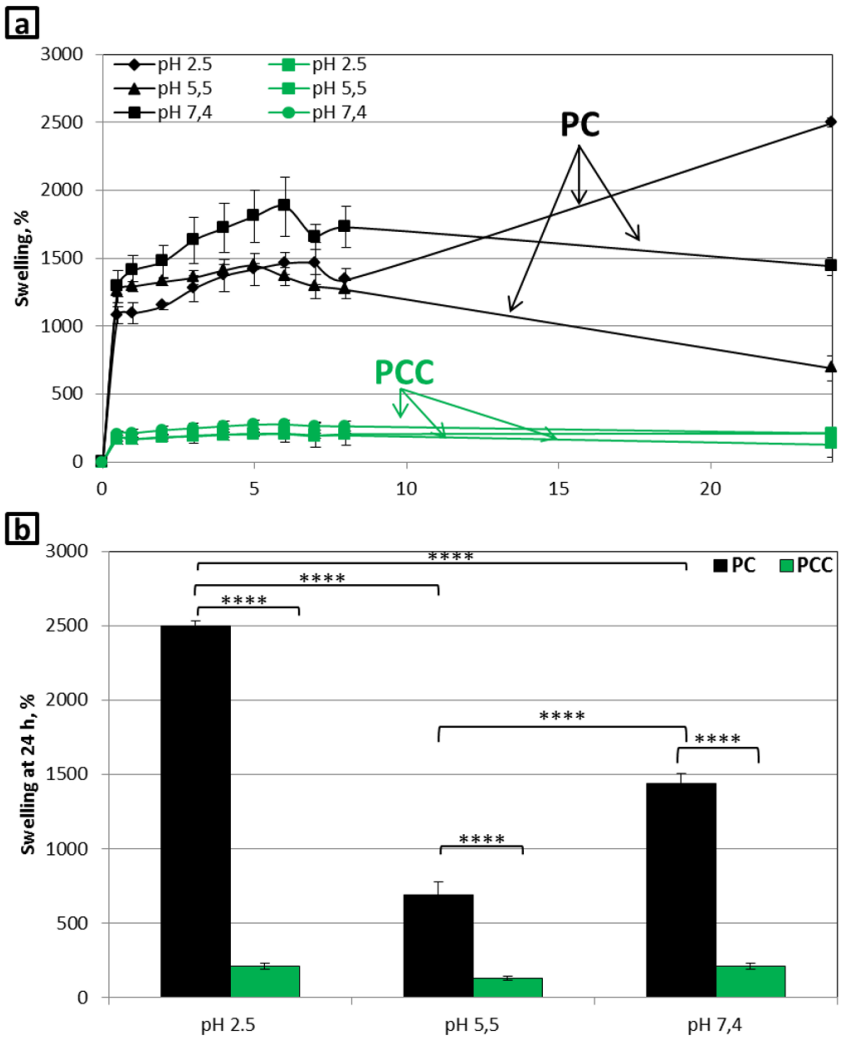


Figure 37. Hydration kinetics, of PC and PCC scaffolds, was tested in different pH conditions, 2.5, 5.5 and 7.4 within 24 h (a). PCC material results more stable and less sensible at pH variation than PC scaffold that show high water uptake at pH 2.5 and 7.4 due to the neutralization of NH^{3+} at high pH and COO^{-} at low pH, which causes the loss of the PEI network (b). The results are reported as mean ± standard deviation (n=3), (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-way ANOVA with Tukey's multiple comparison test).

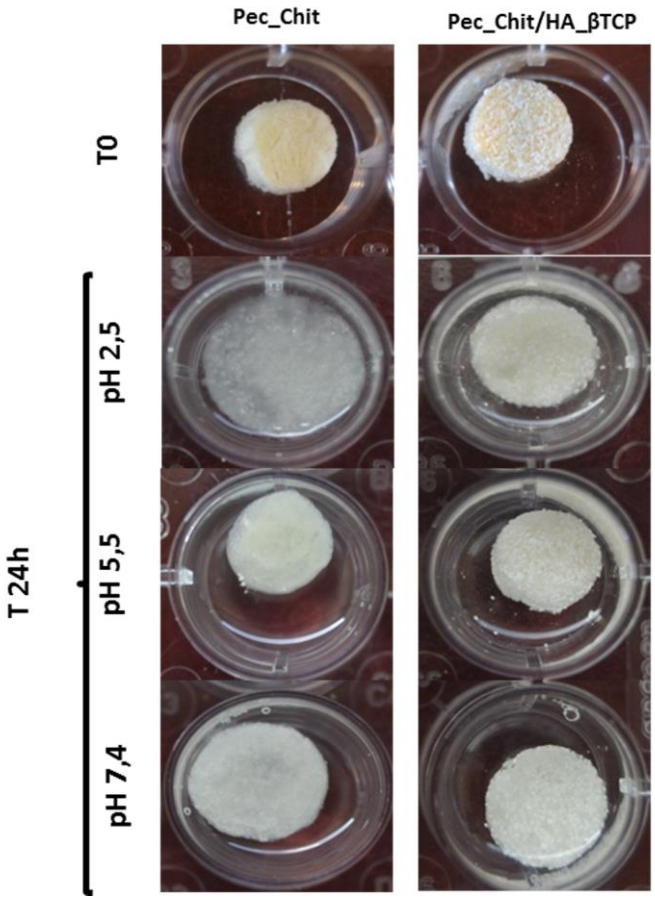


Figure 38. Optical images show the samples in as-prepared condition (T0) and the swollen sample at different pH, after 24 h.

Degradation properties at different pH

After implantation, the composite hydrogel should degrade, and new bone should form and replace it. Furthermore, during degradation, the osteconductive HA/βTCP ceramic particles come into direct contact with the newly formed bone and should further promote osteointegration. The degradation behaviors of PC and PCC samples have been tested in three different conditions of pH (2.5, 5.5, 7.4) for 1 week and the results are shown in **Figure 39**. During the degradation process, the mechanisms are similar to those obtained during the hydration kinetics, since the solutions used were the same[49]. PC hydrogel shows the highest degradation rate at pH 2.5 with almost 100 % mass loss after 1 week; this mass loss was twice compared to that of PC at pH 7.4 and almost three times with respect to the base hydrogel at pH 5.5, which lost around 40% of the initial mass (**Figure 39 a, b**). At the pH of 2.5, the PEI network will be loosen due to the neutralization of the pectin. The same behavior is reported for the PCC sample, that lost around 40 % of the initial mass after 1 week in the solution at the pH of 2.5. These results are partly due to the neutralization of COO^- ions, and in part to the dissolution of ceramic particles in a highly acidic environment. Furthermore, the ionic interaction between the two polymer chains is reversible and non-permanent, so in aqueous solution

this association is gradually reduced and then lost with time. PCC scaffolds tested in the solution at pH of 5.5 and 7.4 showed a similar percentage of initial mass loss, around 10 %. The addition of ceramic particles makes the materials more resistant and less susceptible to pH variation (**Figure 38 c**).

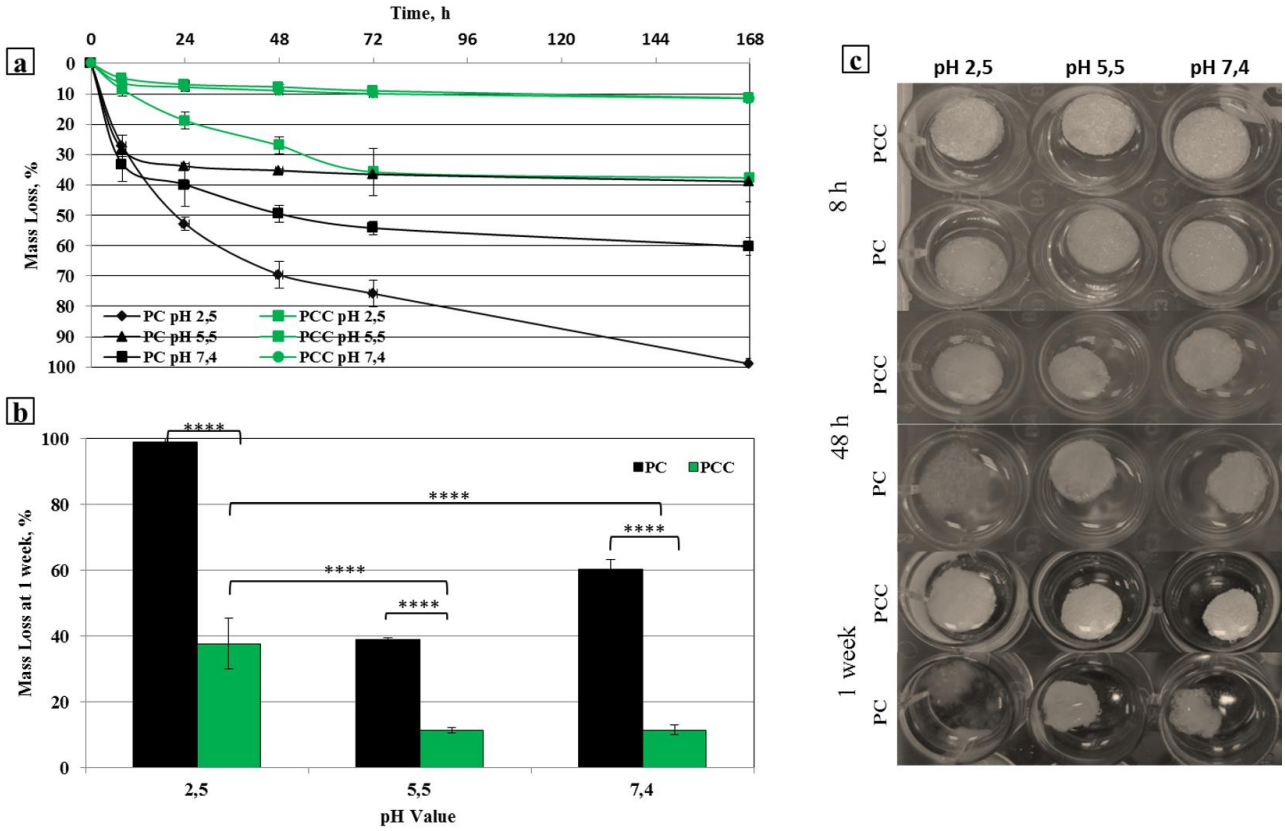


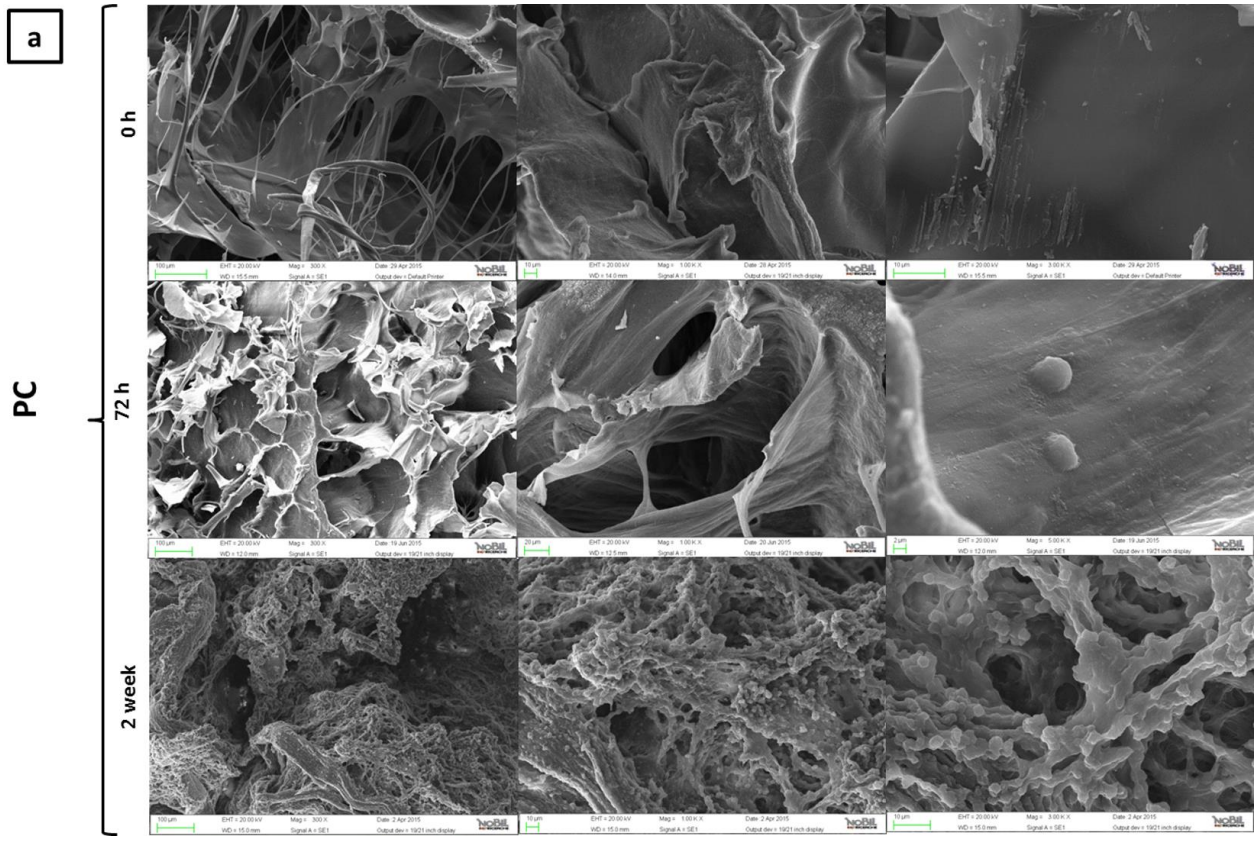
Figure 39. Degradation behavior of the PC and PCC materials was studied during 1 week in different pH condition. Slow degradation rate was recorded for PCC scaffold at pH 5.5 and 7.4 (around 10 %), while a higher degradation percentage with respect to the initial mass was observed at pH 2.5, maybe due to the high solubility of calcium phosphate at low pH (a). PCC material showed high degradation rate in particular at low pH 2.5, and a pH 7.4; slower degradation rate was calculated at the pH of 5.5 (b). Optical images of PC and PCC scaffold during degradation test (c). The results are reported as mean ± standard deviation (n=3), (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-way ANOVA with Tukey's multiple comparison test).

4.5.2.5 Biological characterization

Osteoblast-like cell culture

Adhesion, spreading and morphology of osteoblast-like cells cultured for 14 days are shown in **Figure 40**. SEM images show that, after 72 h, SAOS-2 cells seeded on the PC scaffold surface exhibit a round shape and low density, followed by high proliferation and formation of a network of cells. In particular, SEM images after 2 weeks show the formation of large cords of cells, which prefer to stay together rather than adhering and spreading on the surface of the materials (**Figure 40**

a). The addition of ceramic particles increases the adhesion and proliferation of osteoblast cells at 72 h, and the SEM images reveal that, after 2 weeks, SAOS-2 cells are completely adhered on the surface and infiltrated inside the porosity, forming bridges and creating a strong and consistent layer of cells (**Figure 40 b**). The higher attachment and proliferation of cells on the PCC material are due to the presence of the calcium phosphate phase that conducts osteoblast proliferation[50]. Furthermore, the addition of micro-particles increases the roughness and the area of the surface, which promote the osteoblast proliferation and adhesion[30]. Since PC and PCC materials show different cell morphology at two weeks but both demonstrated cytocompatibility and high proliferation, we thus performed ALP gene-expression after 7 days of SAOS-2 cell culture. The results are reported in the bar graph in **Figure 41,** demonstrating that the presence of HA/βTCP particles in the PCC composite scaffold promotes ALP activity, which is 3-fold higher than that of PC-based hydrogel. *In vitro* studies show a good biocompatibility of PCC scaffold, furthermore the addition of ceramic particles could promote alveolar bone formation and infiltration inside the PCC composite hydrogel.



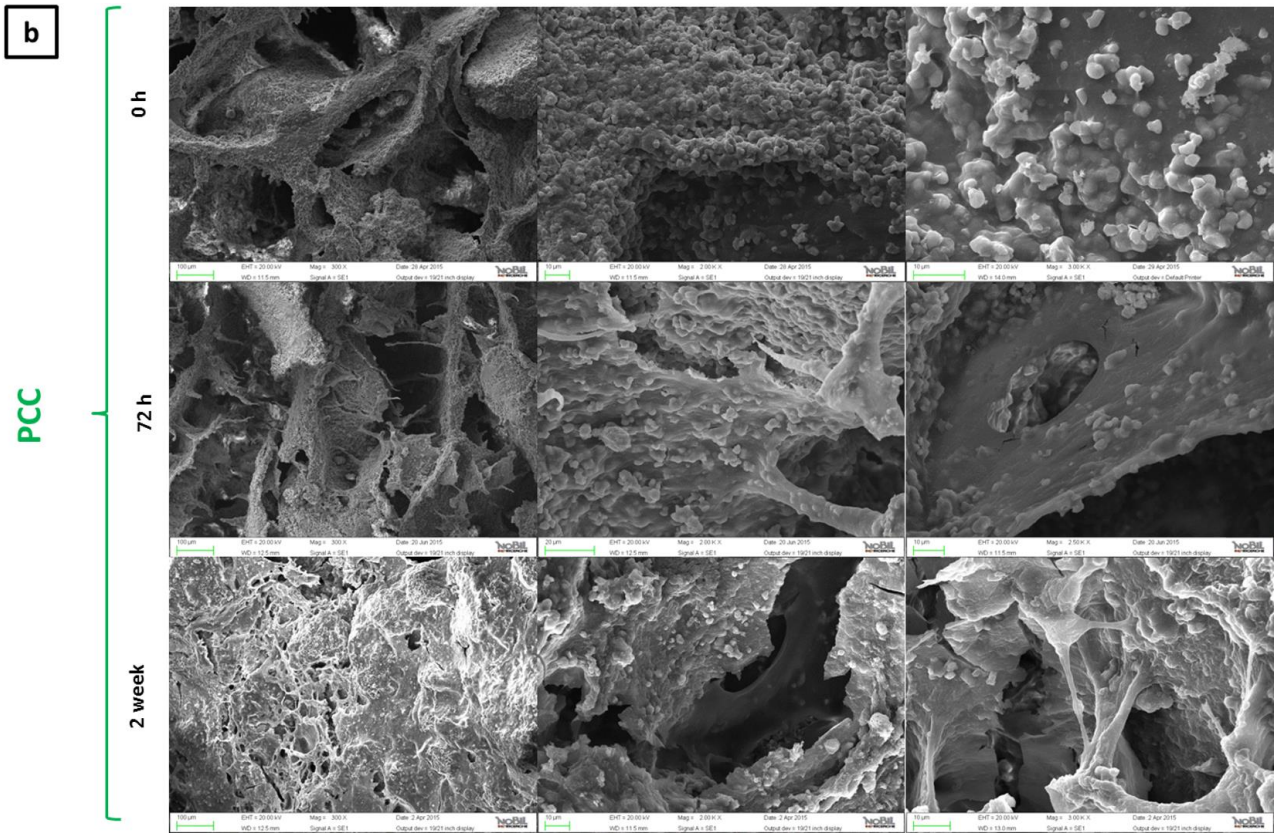


Figure 40 (a,b). Adhesion and proliferation of osteoblast-like cells were investigated by SEM analysis on PC (a) and PCC (b) scaffolds at 72 h and after 2 weeks. HA/βTCP particles promote adhesion and proliferation of SAOS-2 cells after 72 h and a confluent layer of cells after 14 days was detected.

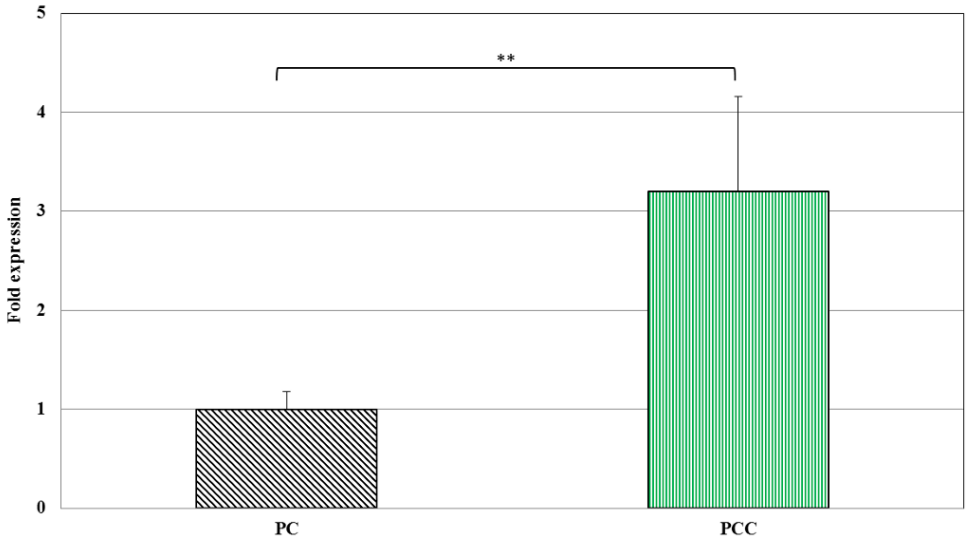


Figure 41. ALP gene expression after 1 week of osteoblast-like SaOS-2 cells culture. Gene expression analysis reveals that PCC scaffold promotes Alkaline Phosphatase expression, 2 folds more than PC based hydrogel. Both materials are cytocompatible and promote cell proliferation, but the presence of ceramic particles enhances the formation of new mineral matrix, which means that it could be an excellent tool for alveolar bone regeneration. The results are reported as mean ± standard deviation (n=4), (*p<0.05, **p<0.01, Student's t-test).

In order to better analyze the osteogenic response of the cells in contact with the material developed, Saos-2 cells were grown in medium culture with insertion of the two different scaffolds and, at different time points (24 h and 72 h), were collected in order to perform RT-qPCR **(Figure 42)**. The RT-qPCR results show, for PCC scaffold, a progressive increment in the expression fold values of Collagen I (2.0 at 24h, 3.0 at 72h), Osteopontin (3.0 at 24h and 13.0 at 72h), Runx2 (2.3 at 24h and 3.0 at 72h), ALP (2.0 at 24 and 4.0 at 72h) and SPARC (2.3 at 24h and 7.0 at 72h) compared to controls (polystyrene), and PC scaffold providing the hypothesis that cells in culture medium with the presence of ceramic particles produce more elevated levels of early osteogenic markers implicated in matrix deposition and differentiation phases, compared to controls. A different trend is seen in the expression of osteocalcin, which is downregulated at time point 24h, while at 72h a three-fold expression compared to controls can be appreciated. Given that OCN is a late osteogenic marker, whose expression is regulated by the transcription factor Runx2, it can be hypothesized that the increment in fold expression of Runx2 seen at time point 72h, positively regulates OCN after time point 24h.

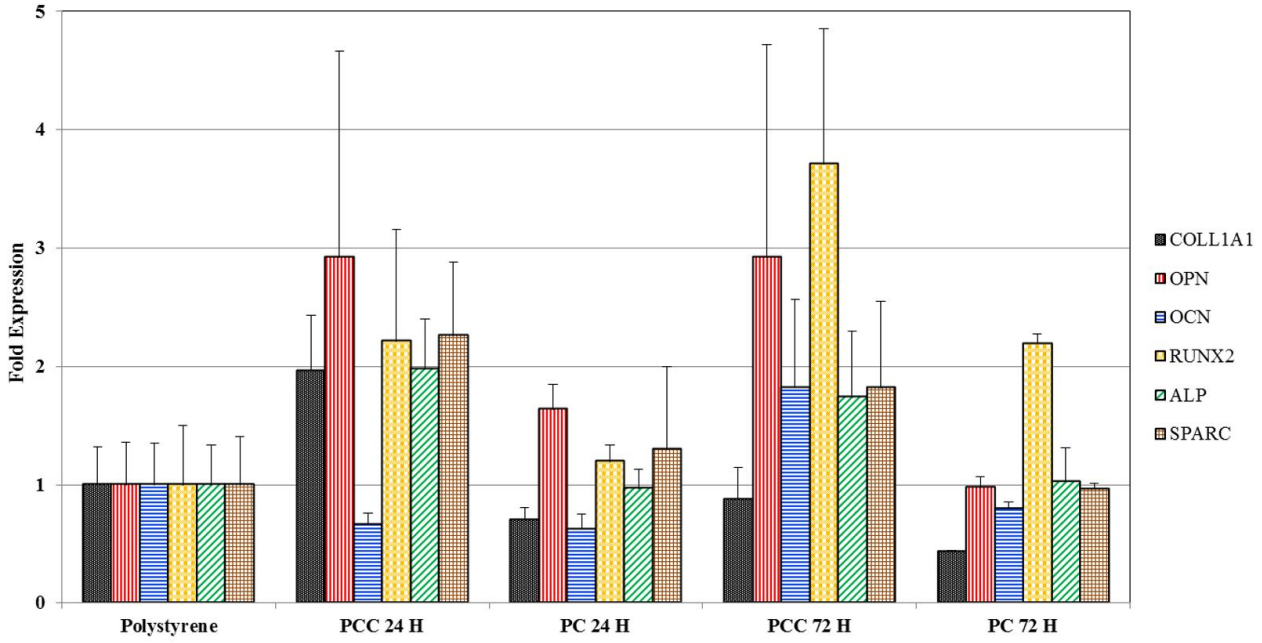


Figure 42. Gene expression after 24 h and 72 h of osteoblast-like SAOS-2 cells culture on PC and PCC scaffolds. The principal osteogenic genes have been analyzed (COLL1A1=Collagen, OPN= Osteopontin; OCN=Osteocalcin; RUNX2=Runt-related transcription factor 2; ALP= Alkaline Phosphatase; SPARC= Osteonectin), and the results confirm that the presence of ceramic particles in the polysaccharides matrix allow the production of more elevated levels of early osteogenic markers, compared to the control. The results are reported as mean ± standard deviation (n=4).

Inflammatory response of the surrounding cells

Gene expression analysis on IL-1β, IL-6 and IL-10 is reported in **Figure 43**. Inflammatory response was evaluated on cells grown on the polystyrene in presence of PC, PCC and without the scaffold. The test was assessed in order to evaluate the possible inflammation that could be provoked by the implanted material on the surrounding tissue. Degradation products, chemical composition and swelling behavior could influence the response of the surrounding tissue; if the material does not stimulate any cytokines expression, a foreign body reaction is avoided. As shown in **Figure 43**, no material elicits a pro-inflammatory response, the expression of IL-1β and IL-6 are comparable between the cells grown in presence of PC and PCC and with the control polystyrene alone. A slight increase in the expression of IL-10 was reported for PCC material compared with the polystyrene control, conversely no significance was assessed to the increase of IL-10 for PC material. Bone resorption occurs when inflammatory mediators reach a critical concentration, which depends on the expression of pro-inflammatory cytokines, such as the interleukin (IL) family, of which IL-1β is the most studied member due to its role in acute and chronic inflammatory and autoimmune disorders. On the opposite site, the inflammatory level is controlled by the expression of anti-inflammatory cytokines, such as IL-10. In normal physiological conditions, there is a balance between bone formation and bone resorption, and as it happens in certain inflammatory conditions, this balance can be altered. This equilibrium is altered by the action of pro-inflammatory cytokines, such as IL-1β that induces osteoclastogenesis, while counteraction by anti-inflammatory mediators, such as IL-10, inhibits osteoclastogenesis. An anti-inflammatory response seems to occur in both PC and PCC scaffold, although a slight increase of IL-10 gene expression was detected in the latter compared to the former (**Figure 43**). The hypothesis is that this anti-inflammatory could be due to the pectin properties, which has shown an anti-inflammatory behavior in many studies[51–53]. Further and in-depth studies have to be done in order to fully elucidate this behavior, but this study confirms that PCC could be used as a safe bone graft for alveolar bone regeneration, without the risk of inducing inflammation in the surrounding tissue.

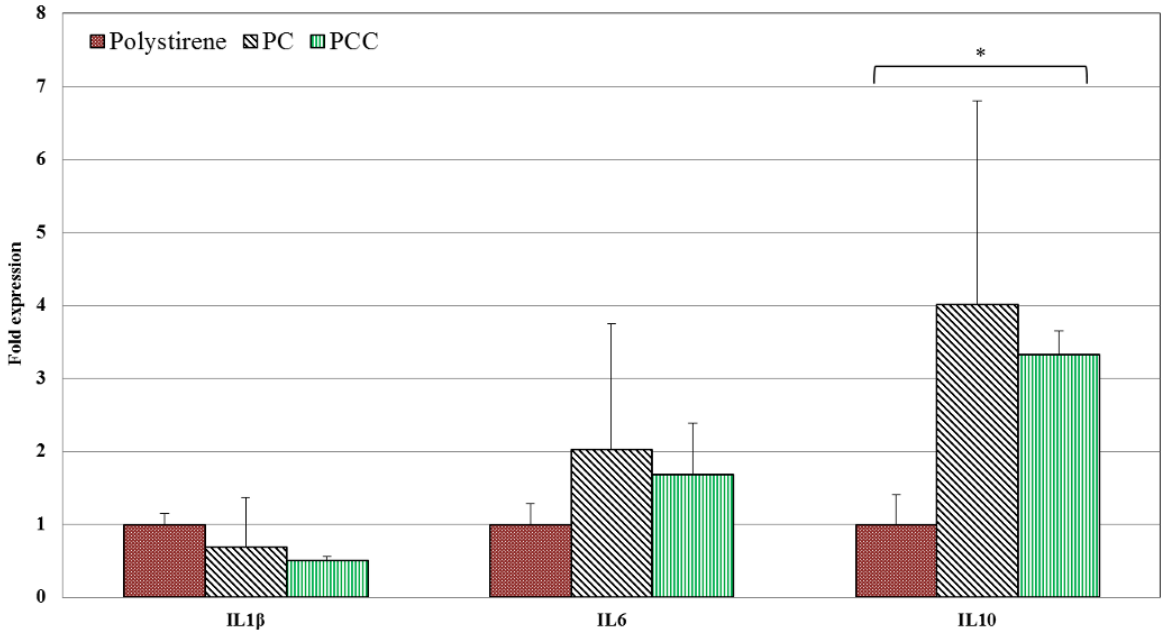


Figure 43. Inflammatory response of the macrophages cells surrounding the material. Gene expression of IL-1β, IL-6 and IL-10 of cells grown in contact with PC and PCC scaffold. Both material do not show any pro-inflammatory response. The results are reported as mean ± standard deviation (n=4), (*p<0.05, two-way ANOVA with Tukey's multiple comparison test).

4.5.3 Conclusion

Alveolar bone regeneration is a procedure that requires a biomaterial with some peculiar characteristics such as mouldability in order to fill the irregular void, biodegradability, swelling capacity to promote infiltration of nutrients and avoiding inflammatory response on the surrounding tissue, osteoconduction and promoting new bone formation. Composite scaffold comprising a pectin/chitosan base hydrogel filled with HA/βTCP particles (PCC) were successfully prepared and characterized. The results show that PCC material has a good stability in different pH condition, with a high swelling degree (up to 200 % of the initial weight). Mechanical characterization demonstrated that the addition of ceramic particles increases the mechanical strength compared to the base hydrogel (toughness of PCC scaffold was around 220 kJ/m^3, and compressive elastic modulus was 2.5 MPa in dry condition). The scaffold morphology and porosity as well as the presence of osteoconductive HA/βTCP micro-particles promote highly osteoblast adhesion and proliferation with a 2-fold higher ALP gene expression at 1 week compared to PC scaffold. Gene expression results demonstrated that PCC scaffold elicits anti-inflammatory and pro-osteogenic responses; this results confirm that PCC biomaterial could be an excellent tool for application in alveolar bone regeneration.

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4.6 NOVEL PECTIN/CHITOSAN GUIDED TISSUE REGENERATION MEMBRANE COATED WITH HYALURONIC ACID

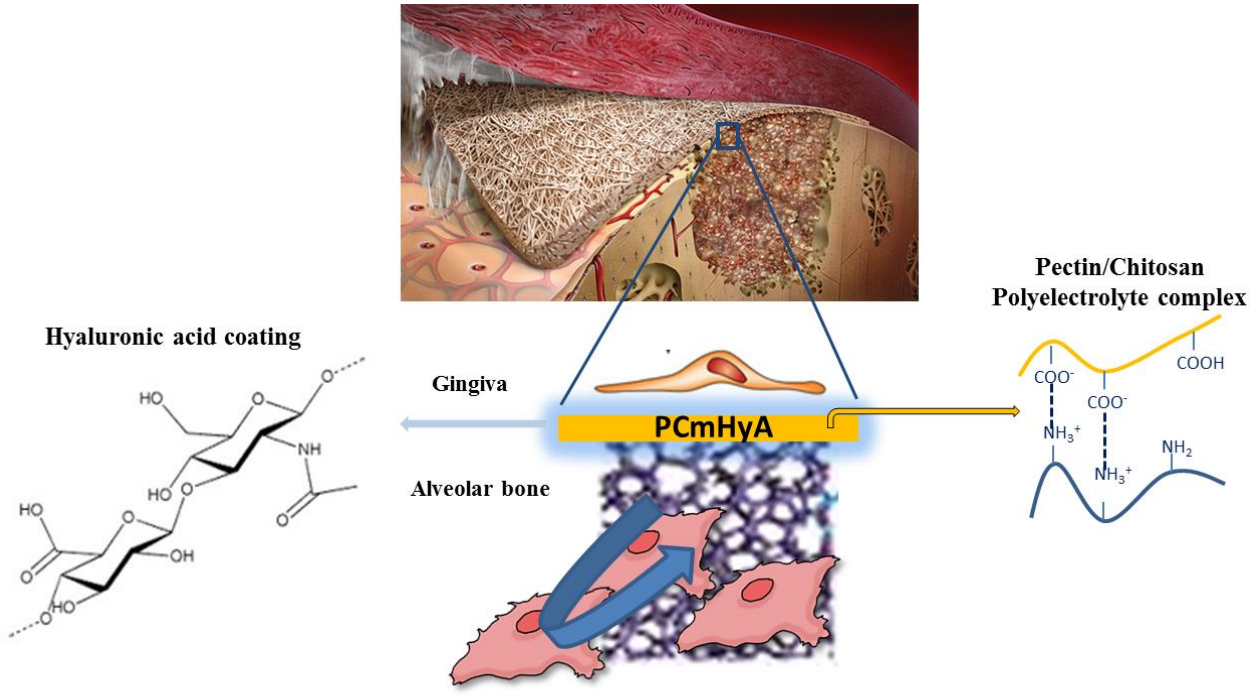


Figure 44. Schematic diagram of the function of guided tissue regeneration membrane.

4.6.1 Introduction

Traditional periodontal surgery techniques include gingivectomy, open flap debridement, and osseous surgery[1–4]. These techniques allow gaining access to diseased tissues and removing local factors, such as plaque, calculus, and endotoxins[5–7]. Chronic periodontitis is a disease caused by an untreated bacterial infection, which causes the loss of soft and hard tissue of the periodontal site and, in many cases results in the tooth loss[6,8–10]. Whenever the tooth is lost or extracted, surgical procedures involve the replacement of it, using titanium dental implants[11,12]. In many cases, bone resorption due to the activation of specific cytokines by an inflammation process, does not allow the insertion of dental implants. In these cases, it is necessary to promote the regeneration of new alveolar bone using the so called bone grafting material, which is usually made of ceramic materials in form of particles, porous scaffolds or paste[13,14]. However, the regeneration following these techniques usually results in a formation of long junctional epithelium, and the formation of new connective tissue in the periodontal wound[15]. The predictability of dental implants to be integrated with the surrounding bone is essential to restore functions in patients. According to the hypothesis formulated by *Melcher*, in order to achieve an excellent bone regeneration which could support the implant insertion, it is necessary the use of a guided tissue membrane[16]. Epithelial tissue and

fibroblasts from the gingiva could grow faster than osteoblast cells and, if not excluded from the root socket, they could inhibit formation of new bone tissue[15,17]. Membranes are used to create a space and to protect the blood clot in order to preferentially allow bone growth into the space. Karring et al. was the first who experimentally and histologically demonstrated the Melcher's hypothesis[16,18]. They showed that periodontal regeneration is achieved when epithelial cells and fibroblasts are excluded from the wound space and the pre-osteoblast cells are allowed to migrate in the bone graft and to produce mineralized matrix, in order to regenerate bone tissue. The first material used for GTR was a cellulose acetate laboratory filter, and it was the first time when periodontal regeneration was achieved using GTR technique[19]. From that moment, many studies have been done in order to develop membranes with several important properties, such as biocompatible, cell exclusion properties, space maintenance, tissue integration, and easy to use[20–22]. Many types of materials have been used for GTR membranes, both non-resorbable and resorbable. Non-resorbable membranes have the disadvantage that they need a second surgery procedure to be removed, the most used non-resorbable membrane is made of ePTFE material, which has the advantage that does not generate antigenicity[23]. On the other side, resorbable materials, in particular from natural sources, have the advantages that do not require an additional surgery and are biocompatible and degradable during tissue formation. The most used material for resorbable membranes is collagen from bovine or porcine sources[24,25]. The main disadvantage of xenografts is the antigenicity and, for this reason, tissues are previously processed to remove all organic constituents, but this process generates high costs that reflect on the final product[26]. Furthermore, collagen degradation involves an enzymatic process, due to the collagenase enzymes, and during periodontitis bacteria stimulate the production of collagenases which could increase the degradation of the membranes, thus making the regeneration unpredictable[27–29]. In order to avoid an un-controlled degradation rate, the collagen fibers are crosslinked using chemical compounds such as glutaraldehyde, which could cause cytotoxicity and inflammation response in the surrounding tissue[30,31]. In this work, we developed and functionalized, on its surface, a novel GTR membrane made of pectin and chitosan polysaccharides, with hyaluronic acid which gives antiadhesive properties to the materials without compromising the membrane's cytocompatibility.

Plant-derived biomaterials have aroused great interest in the last years, since they could mimic the extracellular environment and avoid a foreign body reaction, promoting cell proliferation and tissue formation[32]. Thus, pectin are nowadays under enthusiastic investigation in the biomaterial field as novel candidates for soft and hard tissue engineering. Pectin, in plants, creates a wall matrix which plays several roles, such as mechanical support, physical barrier against pathogens, and acts as bearing for vegetable cells[33]. Furthermore, pectin showed anti-inflammatory properties *in vitro*, and

in some cases, has been shown to be immunologically inert[34,35]. Another great advantage of pectin chain is its polyanionic nature, which allows such a polymer to be ionically crosslinked with polycationic polysaccharides such as chitosan[36]. Chitosan is a widely used material in tissue engineering, due to its biocompatibility, its intrinsic antibacterial nature, its ability to not induce a foreign body reaction and to promote cell adhesion, proliferation and differentiation[37–39]. Polyelectrolyte complex (PEI) could be generated coupling pectin and chitosan materials, in order to create a stable and non-toxic crosslinked membrane with a predictable degradation rate[36,40–42]. Furthermore, the non-immunologically and antimicrobial properties of the complex allow reducing the inflammation response. PEI membranes could mimic the natural extracellular environment promoting cell proliferation. In order to avoid infiltration, we coated the membrane with hyaluronic acid, a well-known molecule, which has antiadhesive properties[43,44]. Hyaluronic acid is a polyanionic polysaccharide with excellent lubricity, non-adhesive and anti-bacterial nature[45–47]. Among all different surface modification techniques, the layer-by-layer deposition has attracted much attention since it is versatile and any type of chemical crosslinker is needed[48,49]. Since chitosan is a polycationic material, it is able to form a polyelectrolyte with a polyanionic hyaluronic acid, and to form a stable surface layer on the membrane surface. In this work, we developed a membrane following the concept of layer-by-layer technique, in order to modify the surface of the bulk; a single layer of hyaluronic acid was deposed on the surface using ionic interaction with chitosan polysaccharides.

In this way, it is possible to promote proliferation of the fibroblasts avoiding the infiltration and maintaining the root space available for osteoblast proliferation and new bone formation. We designed and characterized a GTR membrane, made with pectin and chitosan polysaccharides, which are bonded together with a ionic crosslinking (PCm). We coated the PCm membrane with hyaluronic acid, in order to obtain an antiadhesive surface, to guide fibroblast regeneration without infiltration in the membrane matrix (PCmHyA). Chemical, mechanical and biological characterizations have been analyzed, and we demonstrated that the novel PCmHyA membrane could be used in GTR procedures.

4.6.2 Results and Discussion

4.6.2.1 *Chemical characterization*

ATR-IR and XPS analysis

ATR-IR spectra in **Figure 45 a** show spectra of pectin powder, chitosan powder, hyaluronic acid powder, pectin/chitosan polyelectrolyte complex membrane (PCm) and pectin/chitosan membrane functionalized with hyaluronic acid (PCmHyA). The analysis confirms that all peaks belong to polysaccharide, the region between 3700 cm^{-1} and 3000 cm^{-1} for pectin and chitosan is assigned to the O–H stretching vibration (νOH), while the region between 3000–2800 cm^{-1} belongs to C-H stretching vibration (νCH) (**Figure 45 b**). Deeper analysis on pectin spectra show two bands associated with the stretching vibration at 1740 cm^{-1} of carbonyl group, corresponding to the methyl ester group ($COOCH_3$) and carboxyl acid (COOH), while the band at 1606 cm^{-1} belongs to the stretching vibration of the carbonyl group of the carboxylate ion (COO^-) (**Figure 45 c**). Concerning chitosan spectra, the band at 1647 cm^{-1} is due to the C=O stretching vibration of amide I, whilst the band at 1580 cm^{-1} is due to the NH bending amide II, maybe overlapped to the N-H vibration of the amine groups. Band assignment is consistent with available literature (**Figure 45 c**)[36,40,50]. Hyaluronic acid showed a peak around 1040 cm^{-1} and one around 1200 cm^{-1}, which are probably due to the stretching of C-O-C, C-O and C-O-H, furthermore the weak band around 1650 cm^{-1} could be associated at C=O of the amide I (**Figure 45 c**)[51].

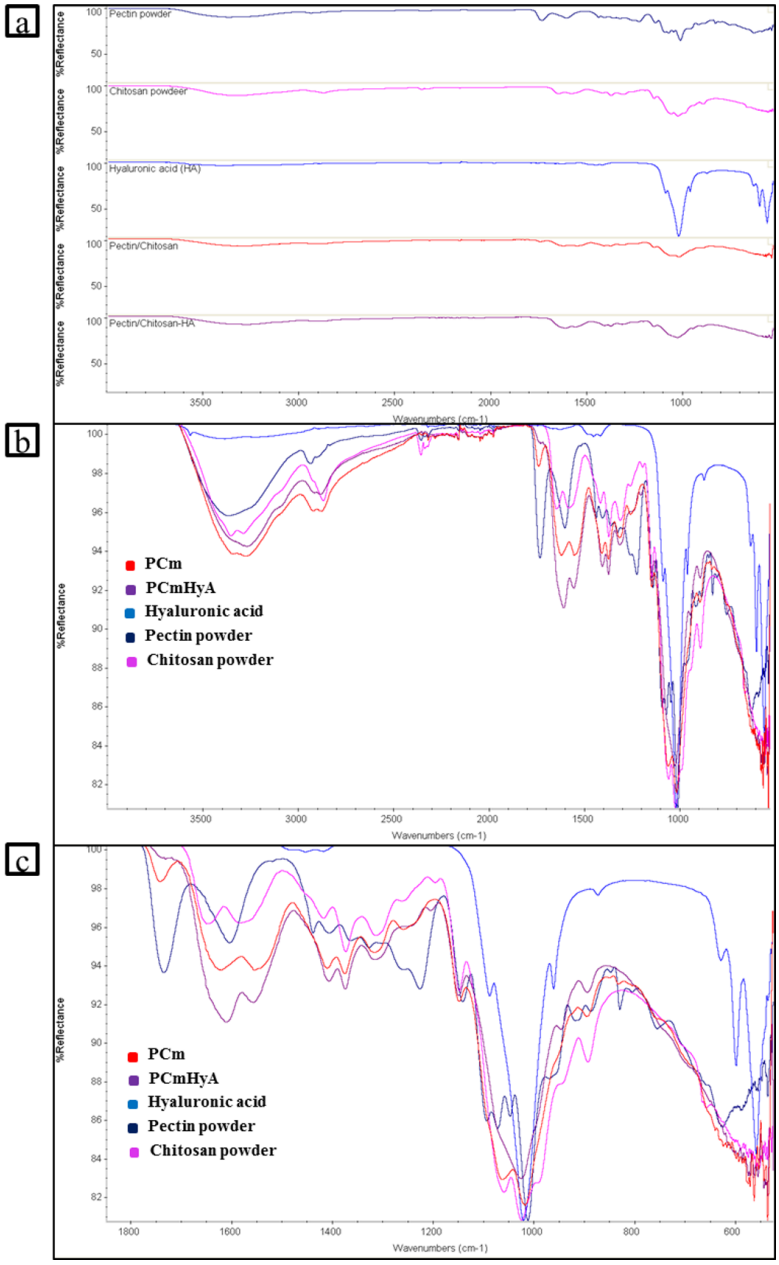
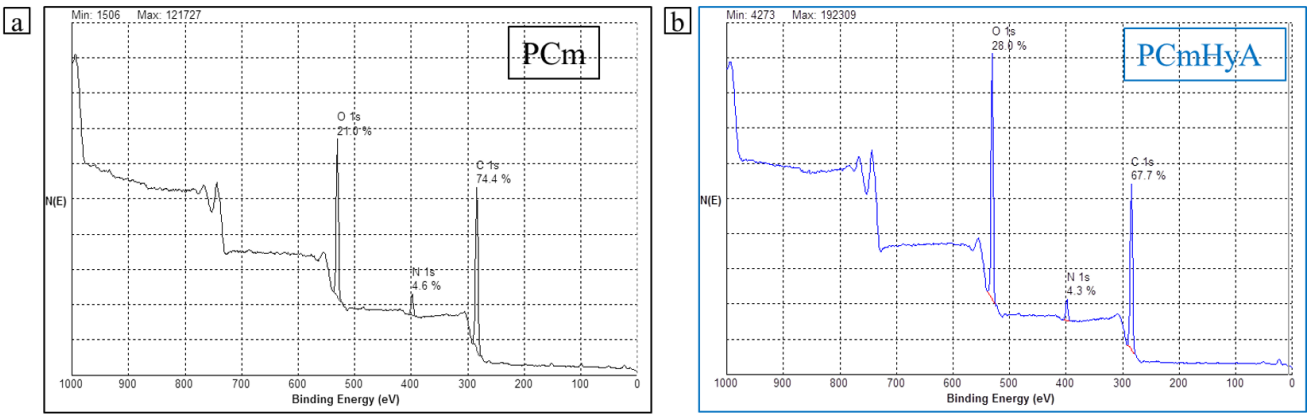


Figure 45. (a) ATR-IR spectra of pectin and chitosan powder, PCm and PCmHyA between 4000 – 525 cm^{-1}. (b) ATR-IR of all spectra overlapped between 4000 cm^{-1} and 500 cm^{-1} show the shift of the amine band to 1557 cm^{-1}, and the bands belonging inorganic phase, at 950 cm^{-1} and 1140 cm^{-1}. (c) ATR-IR spectra focus between 2000 cm^{-1} and 500 cm^{-1} of materials. The presence of HyA on the surface could be associated to the presence of a specific peak at 1200 cm^{-1}, usually attributed to the C-O stretch.

PCm spectra show the formation of PEI complex; a shift of amine band to 1557 cm^{-1} due to the interaction between the positive charge of chitosan, NH_3^+, and the negative charge of pectin, COO^-, was detected. As expected, the spectra of PCmHyA membrane sample show bands associated with both the PEI complex and the hyaluronic acid polymer. In particular, the peak at 1200 cm^{-1} which is present in both HyA and PCmHyA spectra but does not appear in the spectra of PCm material; this could be due to the carboxylic acid of hyaluronic acid. Furthermore, between 900 cm^{-1} and 1100

cm^{-1} the PCmHyA spectra show a different shape, probably due to the presence of hyaluronic acid, a slight difference was also observed in the shape of the curve around 1500 cm^{-1} . In order to confirm the success of the coating process, we also performed XPS analysis on the PCm and PCmHyA membrane (**Figure 46).**



c

Sample	O	N	C	O/C
PCm	21	4,6	74,4	0,28
PCmHyA	28	4,3	67,7	0,41

Figure 46. Wide scans of XPS for PCm and PCmHyA substrates. The chemical composition (at.%) indicates that the substrate surface is nearly fully covered with the hyaluronic acid layer.

As expected for both samples, the main peaks are associated to oxygen, nitrogen and carbon. The presence of hyaluronic acid on the surface, changes the ratio between O and C, in particular the atomic concentration associated to the peak of O_{1s} increases and that associated to the peak of C_{1s} decreases, and the ratio O/C increases of two fold, as compared with the PCm material[52]. These results confirm the presence of hyaluronic acid on the PCm surface, and assess the success of the coating process.

4.6.2.2 Structural characterization

SEM Analysis

The structure of the developed membranes has been evaluated through SEM analysis. PCm and PCmHyA were analyzed in section and in flat direction, in order to investigate if the surface coating by hyaluronic acid influences the structure of the PCm material. In **Figure 47,** three different magnification (100x, 200x and 500x) of the section for both PCm and PCmHyA are reported. Membranes have a highly porous inner structure, and the hyaluronic acid coating does not affect the

pore size and shape. It seems that the coating process does not infiltrate inside the membrane, and does not fill the pore but remains, as expected, on the surface of the membrane.

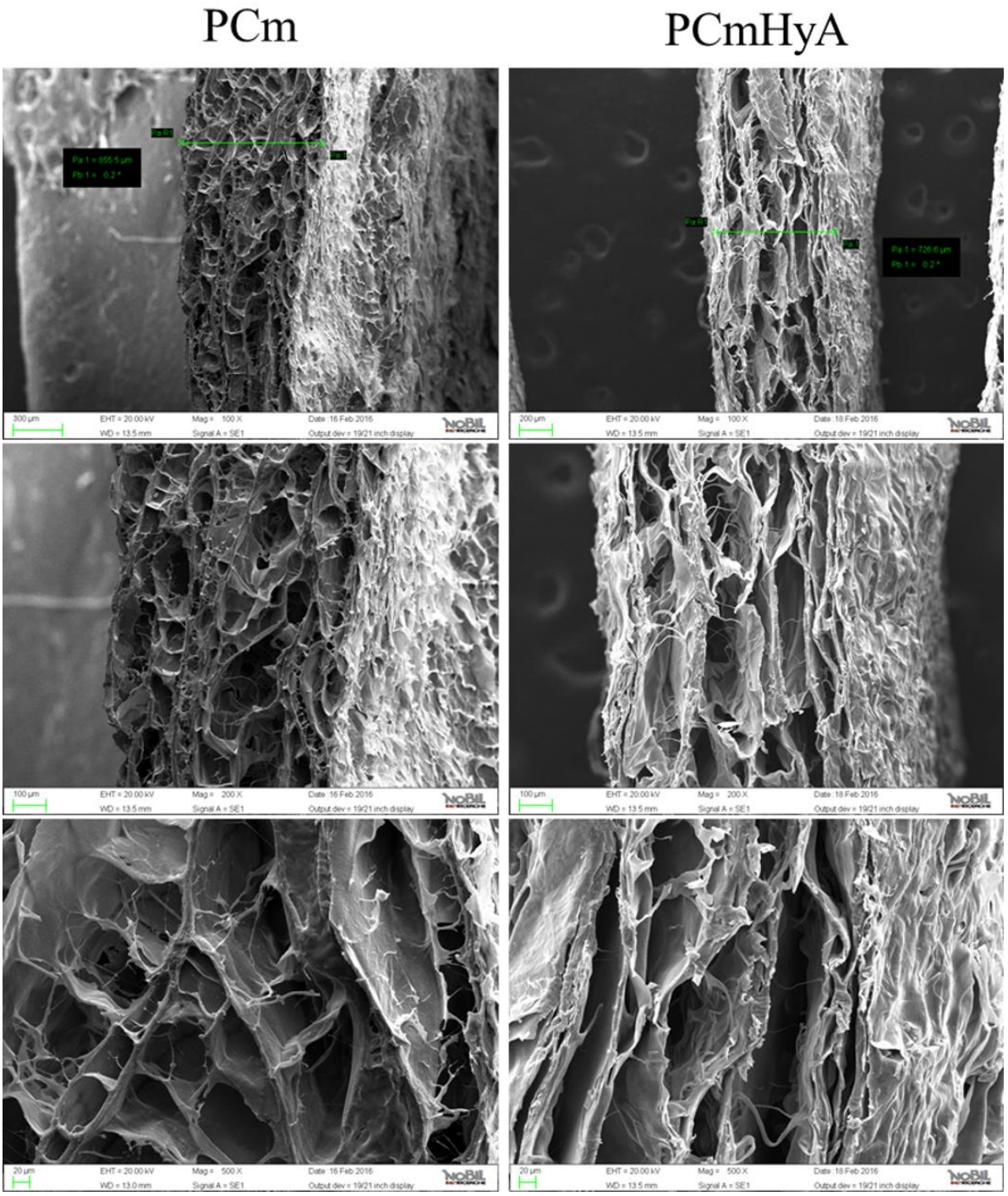


Figure 47. SEM images of porous section for PCm (thickness of 855.5 µm) and PCmHyA (thickness of 726.6 µm) materials. The coating with hyaluronic acid, does not affect the inner structure of the membrane.

Porosity is necessary, because it allows nutrients transportation and blood vessels infiltration, which is mandatory to achieve a correct periodontal regeneration[53]. The porosity is due to the production process, which involves a freeze drying step where the water embedded in the PEI matrix leaves and generates the interconnected pores[53–55]. PCmHyA involves two steps of freeze dry, one to generate PCm and the second after the coating with HyA. The presence of HyA coating is clear in the SEM images reported in **Figure 48.** HyA layer is present on the membrane surface, forming a dense network. HyA is a widely studied polymer for applications requiring minimal cellular adhesion[46,48,56]. Barriers made from cross-linked HyA have been effectively used to prevent adhesion between adjacent tissue layers in postoperative surgery[57,58]. Furthermore, since HyA is naturally present in high concentrations in the soft connective tissues, it is an appropriate choice for supporting fibroblast and epithelial regeneration and augmentation. It was demonstrated that cross-linked HyA films accelerate tissue healing in full-thickness wounds[59]. Highly hydration and non-immunogenicity of HyA, provide a conductive environment for tissue repair. Keratinocytes and fibroblast *in vitro*, generate soft tissue layers, on the surface of HyA coated materials[59]. The presence of ionic cross-linked HyA layers on the surface of the PCmHyA material, could allow fibroblast and epithelial proliferation avoiding the infiltration into the defect socket. Furthermore, there are many studies which demonstrated the effectiveness of HyA layers to prevent bacterial adhesion to dental implants[44,47,49]. These properties might be a further useful characteristic in such application as periodontal tissue engineering, where the presence of bacteria in the oral cavity could inhibit the correct regeneration. The porous structure of the membrane could be used as a carrier to delivery drugs or growth factors *in situ* in a controlled manner[53].

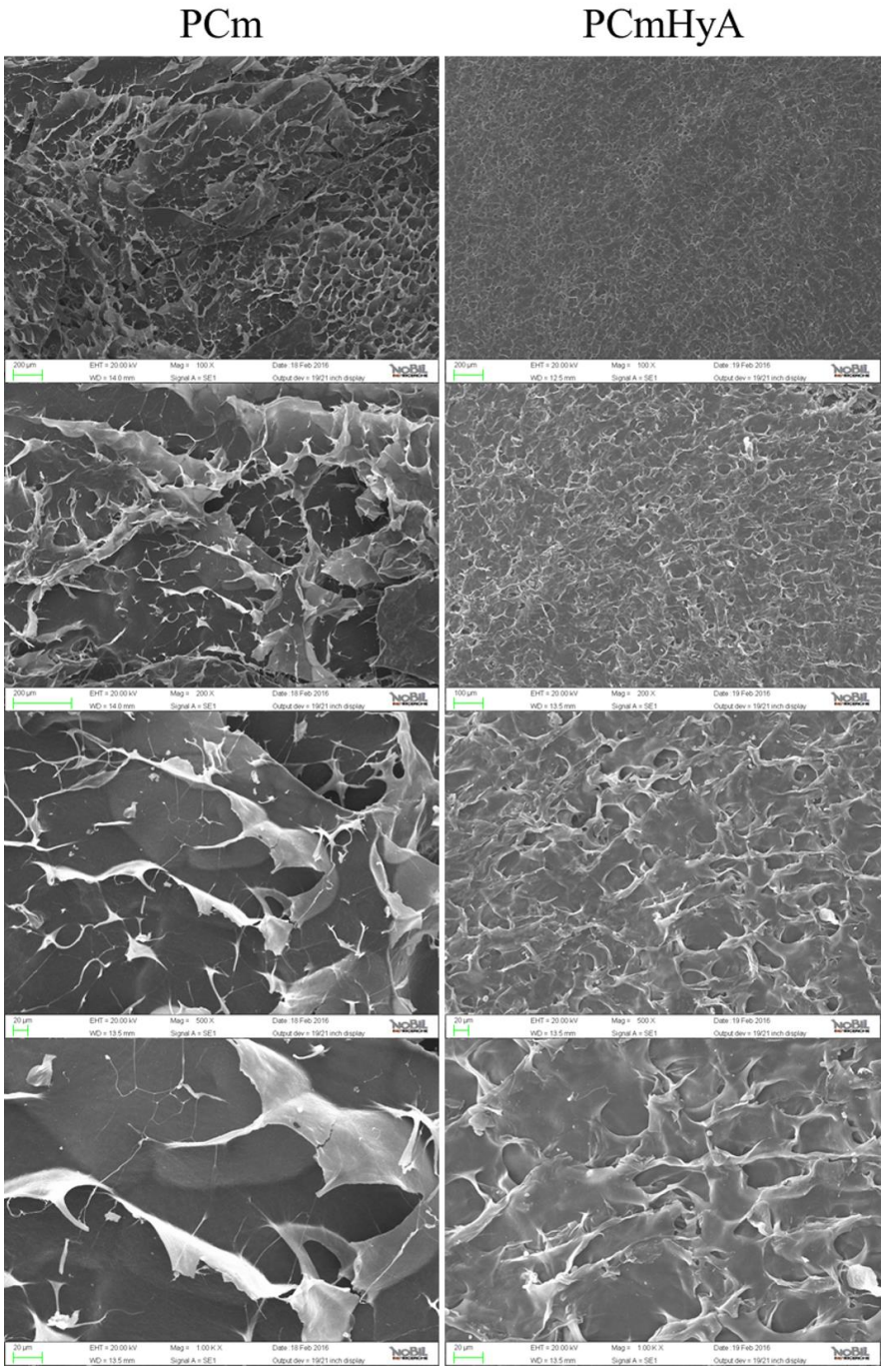


Figure 48. SEM images on the flat direction for PCm and PCmHyA materials. The presence of hyaluronic acid is clear visible on the surface, as a dense network.

4.6.2.3 *Mechanical characterization*

Tensile test

Tensile test was conducted on PCm and PCmHyA samples. Five samples for each type were tested, using a tensile machine Bose Electroforce 5500 equipped with a load cell of 100 N. The testing conditions are reported in the paragraph 4.2.4 of the section 4.2 Methods. Both conditions, dry and

hydrate, have been tested, and as expected the behavior of the membranes changes from stiff to soft and elastic.

In **Figure 49** the Ultimate Tensile strength (UTS) and Elongation (ε_r) of the membrane in dry and hydrated conditions are reported. PCm is made of two natural polysaccharides, which are crosslinked with a low ionic interaction, hence the resulting material has a softener characteristics, in particular during hydration. In as prepared conditions, the material results indeed rigid and strong enough to reach 2 MPa of Ultimate Tensile Stress, which is a comparable result of gold standard GTR membranes produced by Geistlich, Biogide® made of non-crosslinked porcine collagen (data not shown). A slight increase was detected for the PCmHyA membrane. However, the differences has no significant relevance, and this behavior is not confirmed in hydrate conditions. As well as elongation follows the trend of UTS, indeed the elasticity of the dry material is slight lower for the PCmHyA material compared with PCm material. Dry conditions are not the working conditions of the material, which in clinical applications is usually hydrated, hence we tested the mechanical tensile properties in this condition. If in dry condition the crosslinking factor allows a higher resistance, in hydrated condition the presence of hydrophilic hyaluronic acid on the surface allows a higher amount of water uptake, and this results in a decrease in the mechanical properties compared with PCm material. For both materials, there is a shift from stiffness to elasticity, indeed the maximum elongation is similar for both materials, and reaches value around 50 % of the initial length, 5 times more than in as prepared condition (dry) and a value comparable with the commercially available membrane. As expected, for a highly natural porous material, the water uptake drastically decreases the ultimate tensile stress, that was 0.05 MPa for PCm and 0.03 MPa for PCmHyA, two order of magnitude lower than in dry condition. GTR application is usually a low load application, furthermore the stress under which the material undergoes is different from a classical tensile stress. The main important property that the membrane should have is the capacity to sustain the soft tissue avoiding the collapsing of the soft tissue, and from our test analysis the PCmHyA material has this properties[60].

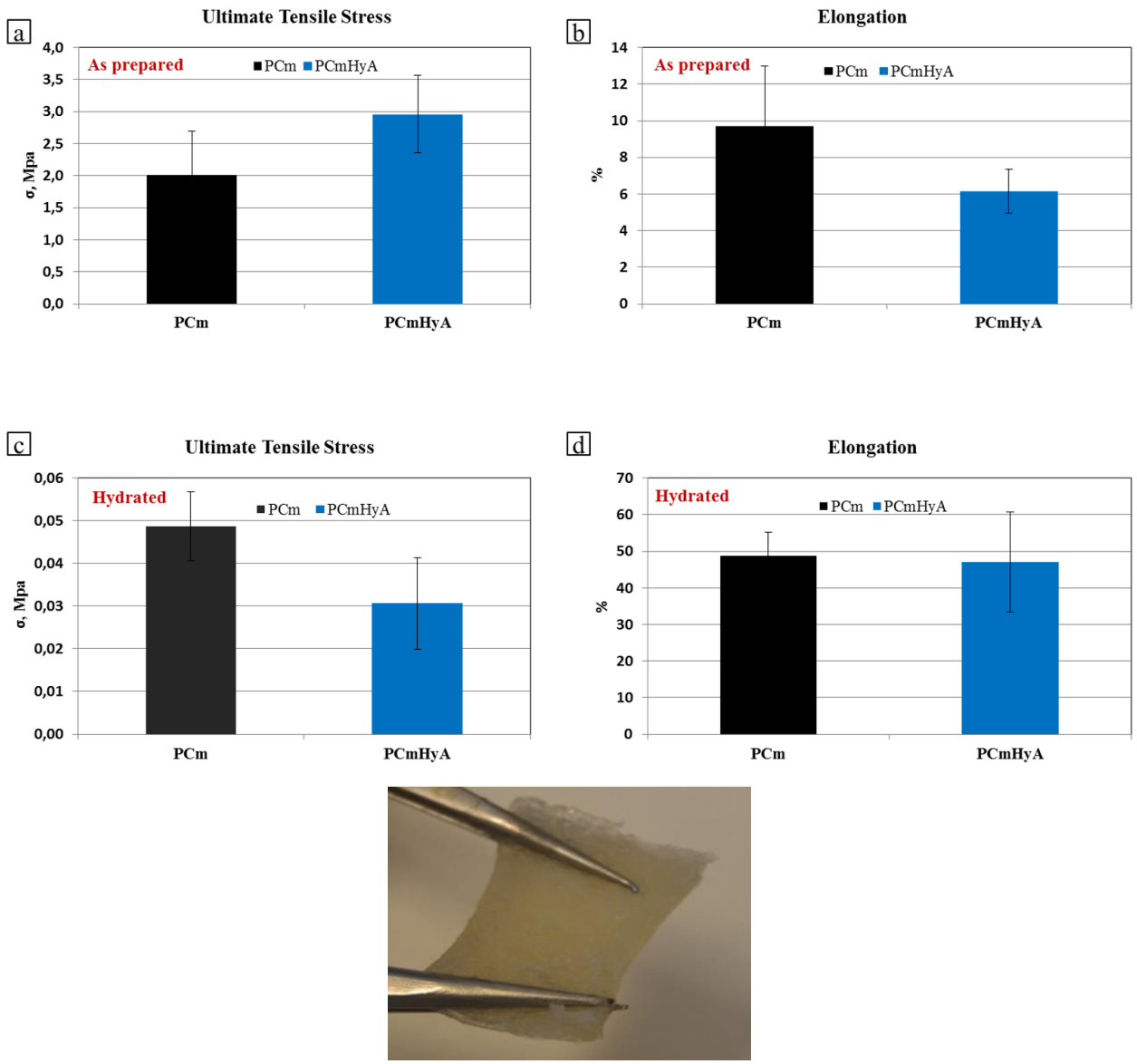


Figure 49. Ultimate tensile stress (a and c) and elongation (b and d) for PCm and PCmHyA membranes in as prepared and hydrated conditions. The presence of hyaluronic acid does not significantly affect the mechanical properties of the membrane. The higher hydrophilicity of the hyaluronic acid layer compared with the PCm material slightly decreases the ultimate tensile stress in hydrated condition. The data are represented as mean ± standard deviation (n=5).

Suture Retention Strength

GTR application is usually a low bearing application, for which is rarely necessary a suture in order to fix the material, but in some cases it could be necessary. Suture retention strength was conducted following the ANSI/AAMI/ISO 7198:1998/2001/(R) 2004 "Cardiovascular implants-tubular vascular prostheses"[61] procedure, and the setup is shown in **Figure 50**. A classical PLA suture thread was used.

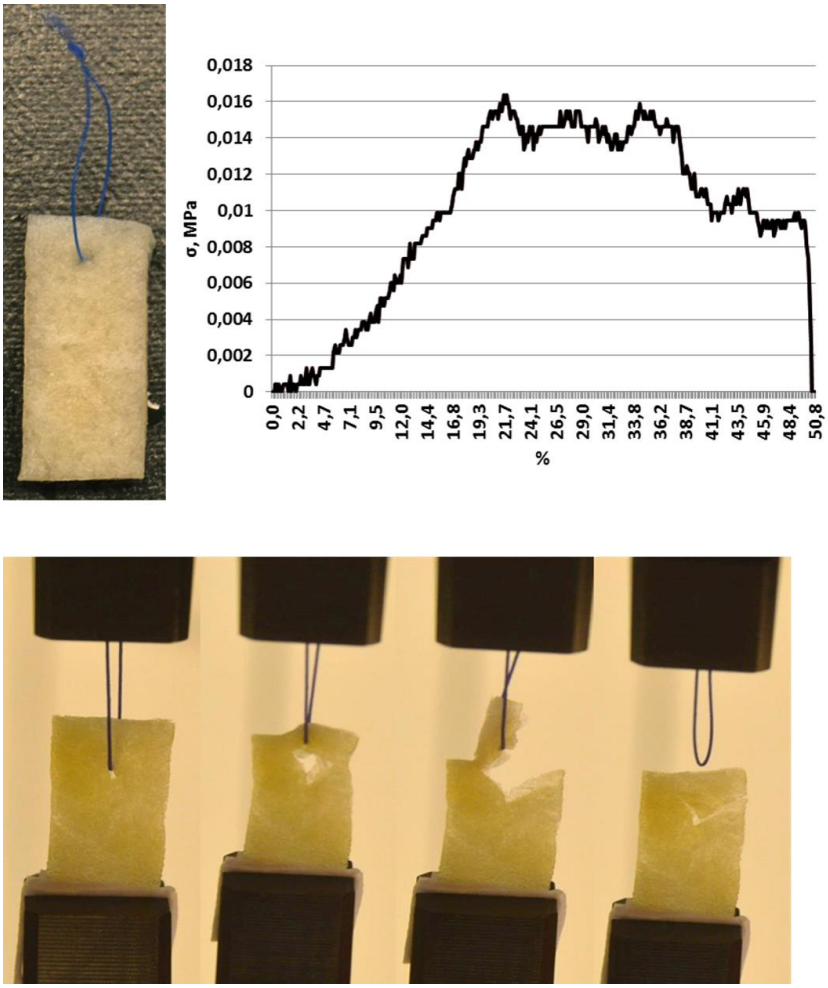


Figure 50. Stress-strain curve of the suture retention strength test for PCmHyA material, and optical images of the sequence test. The PLA suture thread, acts as knife on the soft structure of the membrane.

The test was performed in hydrated conditions on the PCmHyA membrane. The stress-strain curve shows a retention strength of 16 kPa, and a maximum elongation of 20 %. Considering the natural source of the material and the absence of any kind of chemical crosslinking, but just ionic interactions, this is a promising results, in particular about the elongation which confirms the elasticity of the material. The suture thread acts as a knife and cuts the soft structure of the material. In the optical images in **Figure 50** it is possible to see the sequence of the test.

4.6.2.4 *Physical characterization*

Degradation and swelling properties

A degradation test was performed in a phosphate buffered saline solution (PBS at pH 7.4), for 1 month under agitation at 37 °C. PCm and PCmHyA have been tested, the initial weight was registered and at each time point the membranes were taken out, washed in ultrapure water and lyophilized, then the final weight was registered and the percentage of mass loss was calculated.

The results are reported in the bar graph in **Figure 51**. PCm lost 41.28 % of initial mass, a slight increase was calculated for PCmHyA sample which lost after 1 month 46.55 % of the initial mass. As it is possible to note, no differences are detected among the two different samples at each time point. We expected to calculate a lower degradation rate for PCmHyA material, since it was more stable, indeed an opposite behavior was reported. We hypothesize that the highest hydrophilicity of the hyaluronic acid and the second lyophilization step, which allows the formation of bigger pores, allow the incorporation of more water inside the matrix, and its presence affects the ionic interactions and degrades the molecules faster than in the PCm membrane which takes more time to degrade. However, the percentage reached for both samples PCm and PCmHyA, was around 40 % of the initial mass (no significant difference), and it is a value comparable with the commercially available membranes, that means a total resorption in 2 – 3 months that is the time necessary to achieve a good bone regeneration excluding the fibroblast cells from the defect site.

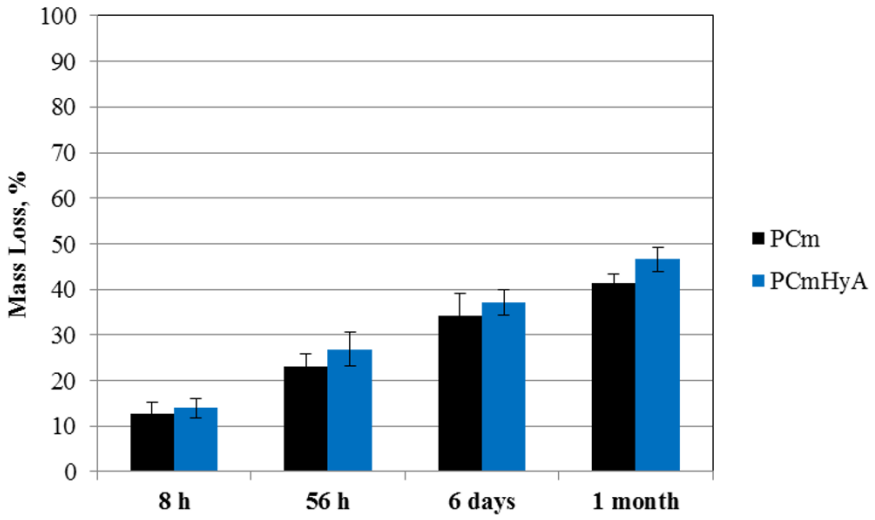


Figure 51. The physiological stability of copolymer network determined by in vitro degradation of PCm and PCmHyA polymers under physiological conditions (PBS, 37 C°). The weight loss of membranes was monitored over the period of 1 month. The increase in the hydrophilicity due to the addition of hyaluronic acid, results in slight increase of water uptake that accelerates in a non-significant manner the hydrolysis of the polyelectrolyte. The data are represented as mean ± standard deviation (n=3).

At pH 7.4, the partial neutralization of NH^{3+} has not caused a total loss of the PEI network, since chitosan has still some ionized group bonded with COO^- of the pectin chain. Furthermore, in the network, there exists the possibility of intramolecular H-bonding between $COOH_3$ and OH, which makes more stable the PEI matrix. Other studies reported a higher degradation percentage of pectin/chitosan complex at alkaline pH, compared with what we found[41,62]. This is probably due to two important factors. Firstly, the porosity of the material influences the water uptake capacity, and

more water could infiltrate in the structure, higher is the degradation of the structure. And second, the ratio between pectin and chitosan inside the PEI complex. Pectin is a water soluble polysaccharide, which has the ability to uptake enormous quantity of water; the addition of chitosan reduces this ability and in our material we have a higher amount of chitosan than pectin, 80 % and 20 % respectively (w/w), so the material properties are closer to the chitosan properties, which is more resistant in physiological condition (pH 7.4). The pH of the solution where samples were soaked, was monitored after 1 month, and any significant difference was detected (data not show), that means that the degradation products of the membranes do not cause a changing in the pH. In particular, many materials used for GTR, for example synthetic materials based on lactic acid or glycolic acid, tend to decrease the pH creating an acid environment, which could activate the immune system response and cause an inflammation, and a foreign body reaction[63].

The hydration properties of the biomaterials are important factors in determining their targeted application. The hydration properties of PCm and PCmHyA were investigated by evaluation bulk hydration characteristics (**Figure 52**). The samples (*n=3)* were subjected to physiological conditions (37 °C and PBS) and uptake of water was calculated after 24 h. The swelling study reveals the maximum equilibrium of water content within 24 h. The percentages of water uptake for PCm and PcmHyA biomaterials, were of 86% of the initial mass and 90% respectively. The addition of hyaluronic acid as coating material slight increases the water uptake. As it is possible to see in the optical images, in **Figure 52** before and after hydration, a change in the dimension of the thickness of the materials is visible. Moreover, the network becomes translucent in the swollen condition, which is the characteristic of an hydrophilic copolymer network. An increase of 50% of the initial thickness, was calculated for both biomaterials.

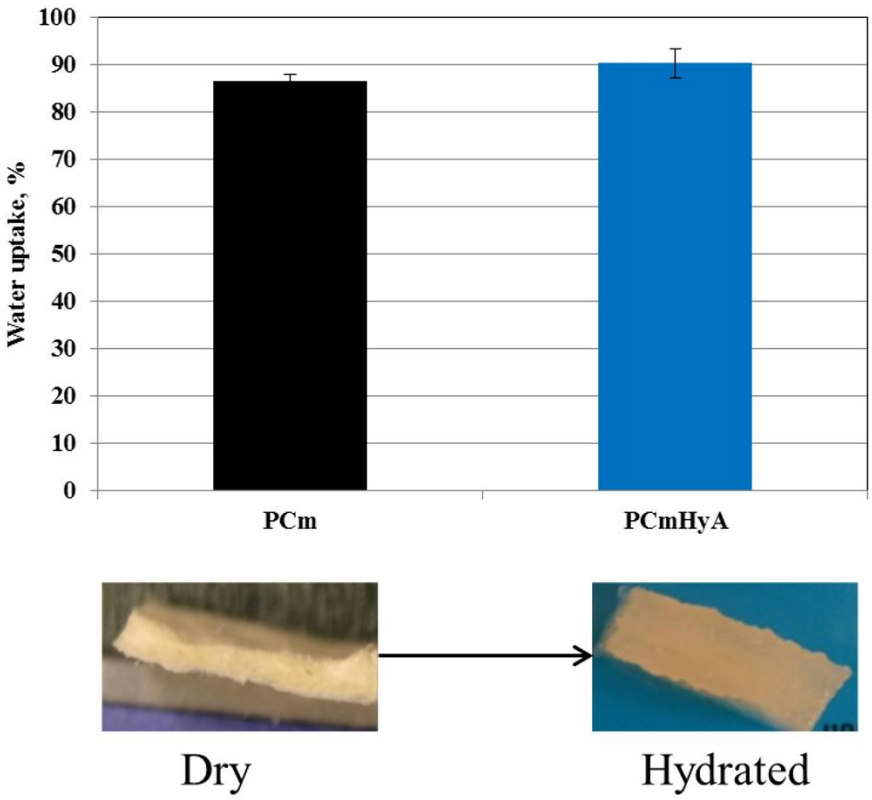


Figure 52. Effect of hyaluronic acid on hydrophilicity of PCm network. The presence of HyA layer slightly increases the water uptake, which is for both materials up to 80 % of the initial weight. Optical images, show the swollen PCmHyA, which acts as bearing after hydration. The data are represented as mean ± standard deviation (n=3).

The membrane works as bearing, since through the application of a compressive stress on the hydrate membranes, the water was expulsed from the PEI matrix, but after the release of the stress, the material adsorbed again the water expulsed. These properties are useful for applications in which the material is in contact with soft tissues, or if the application of the materials is between different tissues or organs, since their ability to adapt to the movement of the surrounding tissue avoids inflammation and foreign body reaction. In GTR procedure, it is important to hydrate the PCmHyA membrane before the implantation in the gingiva, in order to reach a maximum water uptake and to avoid a possible stretching of the gingiva layer. The ability to adsorb water allows formation of a clot which is the first step to achieve tissue regeneration in periodontal site.

4.6.2.5 *Biological characterization*

Fibroblast cell culture and adhesion test

In GTR procedures, the most important property that the membrane should have, is to guide the fibroblast cells on its surface, avoiding the infiltration inside the wound site[20,64,65]. It is further important that the membrane does not cause cytotoxicity, but does promote the proliferation of the cells from the gingiva on its surface. We assume that a possible solution is to develop an antiadhesive surface which promotes cells proliferation. Hyaluronic acid is a well-known material, widely used in tissue engineering, in particular for its biocompatibility, and to its ability to promote cell proliferation[43]. Many studies on the biological properties of hyaluronic acid have been done, furthermore it is also known for its antiadhesive properties. PCm membrane was functionalized with hyaluronic acid, and then the antiadhesive properties of the PCmHyA material were tested using L929 fibroblast cells in comparison with PCm. The results are reported in **Figure 53**, and show cells stained with neutral red on the surface of PCm and PCmHyA. As expected, on both materials the cells have a round shape with a low adhesion properties. However, in the case of PCmHyA, more cell aggregates are present, which are due to the presence of HyA on the surface. Cells prefer to stay together than to adhere on the material surface, but in both cases cells are still alive, that means no cytotoxicity is highlighted. In order to confirm the low adhesion of fibroblasts on the PCmHyA surface, we spectrophotometrically analyzed the DNA quantity of cells on the surface. After a gently wash of the material, the nucleic acid has been extracted from remaining cells on the surface, and the absorbance at 260 nm proper of DNA material was detected. As it is possible to see in **Figure 53**, the value in mg/ml, associated to the PCm material are higher than the value associated to the PCmHyA material, which means a higher adhesion was present on PCm than PCmHyA. This test confirms the initial hypothesis, that considers HyA an antiadhesive material, and demonstrated that the functionalization could exclude the fibroblast cells from the wound site, allowing an excellent bone regeneration on one side and an excellent soft tissue regeneration on the other side.

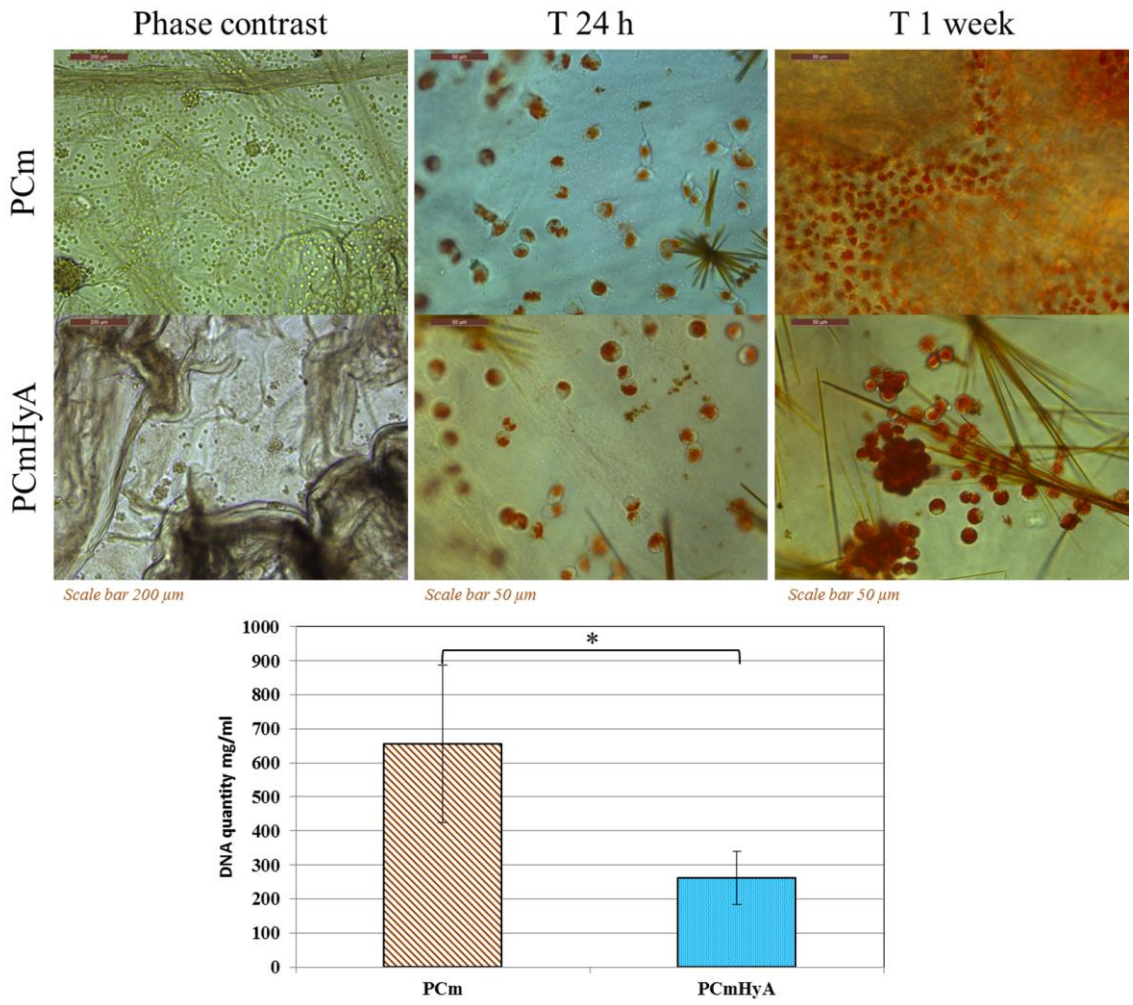


Figure 53. Neutral red staining of fibroblast cells, on the PCm and PCmHyA membranes. DNA quantity, is related to the amount of cells that strongly adhered on the surface. HyA layer reduces the amount of cells adhered on its surface and avoids the infiltration inside the defect site. No cytotoxicity was detected. The data are represented as mean ± standard deviation (n=3), (*p<0.05, **p<0.01, Student's t-test).

4.6.3 Conclusion

In order to achieve an excellent periodontal regeneration, the combination of bone graft materials and guided tissue membranes is mandatory[13,16]. In this work, we designed, developed and characterized a pectin/chitosan membrane functionalized with hyaluronic acid. The membrane is based on the formation of polyelectrolyte complex between pectin and chitosan, which mimics the extracellular matrix, modified on the surface with a second polyelectrolyte complex with hyaluronic acid. The antiadhesive properties of the HyA molecules were demonstrated through *in vitro* studies using fibroblast cells. The membrane shows a degradation rate around 3 months, that is the time recommended to exclude the cells of soft tissue in order to allow bone cells to populate the bone graft and to generate new bone tissue. Considering the nature of the materials and the absence of any chemical crosslinker, the mechanical properties of the material could ensure the stability of the periodontal site avoiding the collapse of the gingiva. The ability to uptake water, reduces the possible inflammatory response due to the contact between the surrounding soft tissue and the membrane, because the PCmHyA works like a bearing and could be fitted in irregular shape defect. Furthermore, it enhances the formation of a clot, which could promote the tissue regeneration.
Further *in vitro* and *in vivo* studies will be necessary, however these results confirm a possible use of this novel membrane in periodontal tissue engineering.

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CHAPTER V

FINAL DISCUSSION AND CONCLUSIONS

Periodontium refers to the specialized tissues that both surround and support the teeth, maintaining them in the maxillary and mandibular bones[1,2]. Tooth loss is a possible consequence of trauma or periodontal disease, such as gingivitis, periodontitis or tissue decay[3]. Periodontal tissue engineering scope is to regenerate the tooth's supporting tissue through a combination of materials, which stimulate cells and signaling molecules to produce new healthy tissue[4]. Many advances have been made in the last decade in the regeneration of complex periodontal and alveolar bone defects[5]. Research efforts in polymeric and ceramic scaffolding systems for cell, protein, drug and gene delivery have led to develop complex systems with predictable response. In the research world there is still some debate as to the best treatment modality for obtaining periodontal regeneration[6]. Some groups advocate the use of bone replacement grafts alone, others suggest that a guided tissue membrane (GTR) alone might be sufficient, and still others recommend a combination of both. *Tobon et al.*[7] conducted a study comparing three different treatment modalities for achieving periodontal regeneration and GTR after endodontic surgery: one control group without bone graft and membrane, one group treated with bone graft alone, and another group treated with both bone graft and membrane. They used as membrane a non-resorbable material, and as bone graft they used hydroxyapatite ceramic material. The results showed that the best periodontal regeneration was assessed through the combination of both membrane and bone graft. The worst results were obtained in the control group, where no membrane nor graft were used. *Yoshikawa and co-workers*[8] compared the histological outcome of different types of membrane, non-resorbable ePTFE, resorbable PLGA, and resorbable collagen membrane, and found that the greatest amount of bone regenerated was achieved using non-resorbable membranes. Another group, comparing an open flap debridement with a bone allograft and a bone allograft with a collagen membrane in an animal study, showed similar results in terms of bone formation in all cases[9]. The studies mentioned above and many others, showed different results and suggest different approaches in order to achieve an excellent periodontal regeneration. However, case selection is very important to the success of regenerative technique, which might explain some of the inconsistencies in the literature[6]. Factors that affect success could be due to the specific patient, specific disease and healing categories. The success of a surgical procedure, which involves the use of bone graft or membrane or else a combination of both, depends on many factors such as good plaque control, compliance, non-

smoking, anti-infective therapy, and systemic health[10–12]. Furthermore, also during the surgical procedures, there are many variables which could affect the results of the regeneration process, for example, the possible infection of the implanted material, which could cause periprosthetic infection.

The thesis was aimed at the development of biologically active materials, with improved osteoconductive and antimicrobial properties, in order to control the re-infection of the defect site and, in parallel, promote new bone formation. In this work, we focused on the possible application of natural polysaccharides in combination with widely used bi-phasic calcium phosphates.

Specifically, we developed and characterized three different materials, designed to overcome the current drawbacks of available materials, i.e. unpredictable tissue response, re-infection of the implant site, difficult mouldability, uncontrolled cell response, and high complexity which reduces the possibility to reach the market.

Common approach to control the periprosthetic infection is to combine an osteoconductive material with a systemic antibiotic therapy[4,13–15]. This type of approach could cause side effects, since in order to be effective against bacteria with this therapy, the antibiotic should be provided in a very high dosage to ensure that a clinical amount can reach the defect site[16]. An *in situ* release is preferable to combat against bacteria and to prevent a re-infection of the defect wound[17–19]. On the other hand, it is important to provide a mechanically stable material such as an osteoconductive scaffold to promote bone formation. Usually, to reach a drug delivery in situ, hydrogel, micro-spheres, or injectable gels are used[20–24]. However, this approach makes necessary the use of another material, which acts as a scaffold to sustain and promote new tissue formation. An engineered scaffold was developed, using a bi-phasic calcium phosphate material for fabricating a three-dimensional porous scaffold, and a natural polyelectrolyte as coating material to encapsulate antibiotic drugs, in order to successfully combine in one biomaterial all the needed characteristics to achieve periodontal bone regeneration.

Effective antibacterial properties have been demonstrated through deep biological analysis. The material developed could inhibit bacterial growth by *in situ* drug release, promote osteoblast proliferation and mechanically sustain the defect site.

To treat orthopedic infection, there are several commercial available products, which claim to be antimicrobial bone graft substitutes, for example Osteoset-T ®, Perossal ®, BonAlive®, Herafill® beads, Cerament-G®[25]. However, a summary of clinical evidences or evidence-based guidelines for the application of these products to assist surgeons is not available. Furthermore, some are just impregnated in drugs, others are designed to adsorb liquids such as drugs, or with a burst release within the first 72 hours.

The biomaterials developed during this doctoral activity were specifically designed to achieve a sustained release with predictable responses in different environmental conditions, maintaining the mechanical strength and osteoconductive properties typical of calcium phosphate materials[26–28]. Furthermore, *in vivo* studies are needed to ultimately assess the clinical effectiveness of the engineered scaffolds and the non-toxicity of the drug released. Depth studies are ongoing in order to replace the vancomycin antibiotic with natural molecules with antimicrobial properties, to overcome the certification issue arising from the presence of a drug in the system.

A criticality of a rigid scaffold is the difficult mouldability to fill irregular defect[29–32]. An urgent need of professionals working in dentistry is the ability to customize the biomaterial for each patient. Furthermore, the osteointegration of dental implants and the consequent long-term success is guaranteed by the presence, in the extraction site, of a healthy and sufficient alveolar bone[33,34]. In these cases, placement of titanium implants is contraindicated until a vertical bone augmentation is obtained. To achieve this goal, in this thesis we developed a bone graft material which should simulate the extracellular matrix (ECM), aimed at promoting osteoblast proliferation and filling the void, maintaining the space without collapsing until the new bone is formed. For this purpose, a novel chitosan-pectin hydrogel reinforced with biphasic calcium phosphate particles within 100-300 μm has been designed and characterized. The polysaccharide nature of hydrogels simulates the ECM of natural bone, and the ceramic particles promote high osteoblast proliferation, assessed by in vitro cell studies[31,35–37]. Swelling properties allow water solution adsorption (up to 200 % of solution content) and the space to be filled without compromising the mechanical strength, in both static and cyclic conditions. The ability to adsorb water is necessary in order to promote the clot formation in the defect site[30,38]. Clot formation promotes the migration and proliferation of osteoblast cells which regenerate new alveolar bone[39,40]. The incorporation of ceramic particles makes the material stable at different pH and increases the compressive elastic modulus, toughness and ultimate tensile strength compared to the base polymer.

A chemical crosslinker is usually employed to obtain a stable polymer; in this work, the stability is given by a ionic interaction which makes the material stable and mechanically resistant enough, without any cytotoxic effect[41]. Only a few biomaterials like PCC are present on the market, and the gold standard could be considered the Geistlich Bio-oss Collagen® material, that consists of 90% Geistlich Bio-Oss® granules with the addition of 10% porcine collagen. Compared to Geistlich Bio-Oss Collagen®, PCC material possesses enhanced handling characteristics, and is more formable and easy to handle. Furthermore, PCC material showed higher swelling behavior, and the polymeric network made with pectin and chitosan makes the material more elastic, with a higher ability to recover the shape after a cyclic stress, compared to the Bio-Oss Collagen® material. The

absence of interactions between the granules and the collagen matrix, makes un-predictable the degradation of the Bio-Oss Collagen ® material, with a loss of cohesion in hydrated conditions. The interaction between chitosan and pectin and between pectin and calcium phosphate particles reduces the migration of the granules in hydrate environment, which could cause an inflammatory response[42,43]. The future study will concentrate on the possible anti-inflammatory properties of pectin polysaccharides. The preliminary study showed an increase in the expression of IL-10 gene, which is correlated with the anti-inflammatory cascade[44,45]. There are some research works that hypothesized an anti-inflammatory action of pectin[46], but few demonstrations are available[46–48]. Following the Melcher's hypothesis[49], in the research work we developed a guided tissue regeneration membrane, always based on the chemistry of pectin and chitosan, but with an antiadhesive coating made by hyaluronic acid on the surface, in order to exclude fibroblast cells from the wound defect. *Gottlow* was the first who founded the name "Guided Tissue Regeneration", in 1986[50]. Ten years later, in 1996 at the World Workshop in Periodontics the first definition of GTR was given: "procedures attempting to regenerate lost periodontal structures through differential tissue response. Barriers are employed in the hope of excluding epithelium and gingival corium from the root surface in the belief that they interfere with regeneration". The hypothesis formulated by Melcher[49], and histologically proven by *Karring et al.*[51], argues that certain cell populations in the periodontium have the potential to create new cementum, alveolar bone and periodontal ligament, when they have provided the opportunity to populate the periodontal wound. This opportunity is achieved if the gingival epithelial cells or fibroblasts are excluded from the wound space and periodontal ligament cells are allowed to migrate and populate the wound space[52,53]. This need led to the development of periodontal devices known as barriers or membranes for guided tissue regeneration. Nowadays, many types of membranes are commercially available, both non-resorbable and resorbable. Resorbable membranes are the most used and studied since they do not require a second surgery for their removal. Absorbable membranes are usually made from natural materials such as collagen, pericardium, dura mater, laminar bone, connective tissue or periosteum. These materials are completely biocompatible, but the process to purify them and to obtain these membranes is expensive and, in many cases, low repeatable, since it depends to the source of the native tissue. In our preliminary study, we assessed the mechanical and degradation *in vitro* of seven different absorbable membranes present on the market, derived from animal tissue, and the results showed a great variability in both properties. These results are translated in unpredictable outcomes during the periodontal regeneration. On the basis of stability demonstrated by the polyelectrolyte formed between pectin and chitosan, a novel guided tissue regeneration membrane has been developed, with an inner porous structure which allows nutrients transportation,

and an antiadhesive surface obtained through a functionalization with hyaluronic acid, a widely used molecule to obtain non-adhesive surface in biomedical field[54]. A cheapest, repeatable, and predictable production process was developed, using simple and economic natural material. Our concept was to avoid the adhesion of the cells to the membrane, and their infiltration in the structure enhancing the proliferation and growth in parallel to the material, guiding the gingiva regeneration. The results demonstrated that fibroblast cells do not adhere to the surface of the GTR membrane but any cytotoxic effect was detected, the cells tend to form aggregate, grow and proliferate on the surface of the material.

Three different materials were successfully developed and the characterization demonstrated that they could be used in periodontal tissue engineering with predictable and excellent outcomes. With this set of biomaterials it is possible to control or prevent possible bacterial growth, achieve the correct alveolar bone quantity and quality and guide the tissue regeneration.

Future studies will be conducted in the Research and Development department of Nobil Bio Ricerche srl and will focus on the development of biomaterials, which could meet the need of professionals, and could enhance the regeneration process. Biomimetic materials, based on natural molecules, coupled with synthetic controlled materials are the basis on which will be developed the future biomaterials.

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Resume of Giorgio Iviglia

Giorgio Iviglia was born in 1987 in Asti, Italy. He lives in Asti (AT), Italy, and is currently working as a Research and Development Engineer at Nobil Bio Ricerche srl, developing novel biomaterial for periodontal tissue engineering, and in particular his research focus is in the use of polyphenols extracted from winery by-products in combination with natural and synthetic materials for the treatment of periodontitis.

He received a BS degree (October 2009) and a MS degree (June 2012) in Biomedical Engineering from Politecnico di Torino. During his MS thesis, he worked in the Khademosseini Lab, Harvard-MIT, Health and Science Technology division, developing synthetic co-polymers for cardiovascular tissue engineering.

In 2012 he was qualified to practice the profession of Industrial Engineer. From October 2012 and March 2013 he worked at Nobil Bio Ricerche as Biomedical Engineer, developing an automatic biomolecular surface unit, for the production of the Synergoss® bone filler material.

From March 2013 to March 2016, he was a PhD student in Biomedical Engineering in the R&D department of Nobil Bio Ricerche in collaboration with the Department of Applied Science and Technology, Institute of Materials Physics and Engineering, Politecnico di Torino; under the supervision of Prof. Chiara Vitale-Brovarone and Dr. Marco Morra. His research work focused on the development of novel biomaterial combining natural and synthetic compounds for the regeneration of periodontal bone tissue.

During the doctoral period he has actively participated at the company projects, and he was involved in different European projects.

As a result of his research work he has authors of 8 proceedings in national and international conferences in the tissue engineering and biomaterial fields; 6 papers in international journal (4 published, 2 submitted) and his research has been recognized by some national and international awards.

List of Publications

"Engineered Porous scaffold for Periprosthetic infection prevention" **G. Iviglia**, D. Bollati, C. Cassinelli, F. Baino, E. Torre, M. Morra, C. Vitale-Brovarone. ***(Submitted to Material Science and Engineering C)***

" Novel bioceramic-reinforced hydrogel for alveolar bone regeneration" **G. Iviglia**, C. Cassinelli, F. Baino, E. Torre, M. Morra, C. Vitale-Brovarone ***(Submitted to Acta Biomaterialia)***

"Elastomeric nanocomposite scaffolds made from poly (glycerol sebacate) chemically crosslinked with carbon nanotubes" Akhilesh Gaharwar, Alpesh Patel, Alireza Dolatshahi-Pirouz, Zhang Hongbin, Kaushik Rangarajan, **G. Iviglia**, Su-Ryon Shin, Mohammad Asif Hussain, Ali Khademhosseini ***(Biomaterials Science****, Advance Article, DOI:* 10.1039/C4BM00222A*)* - ***2015***

"Nanoclay-Enriched Poly(e-caprolactone) Electrospun Scaffolds for Osteogenic Differentiation of Human Mesenchymal Stem Cells" A. Gaharwar, S. Mukundan, , E. Karaka, Alireza Dolatshahi-Pirouz, A. Patel, Kaushik Rangaraja, Silvia M. Mihaila, **G. Iviglia**, Hongbin Zhang, Ali Khademhosseini; (***Tissue Engineering Part A,*** DOI: 10.1089/ten.tea.2013.0281) - ***2014***

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"Higly elastomeric poly(glycerol sebacate)-co-poly(ethylene glycol) amphiphilic block copolymers"; **G. Iviglia§,** A. Patel§, A.K. Gaharwar§**,** S.M. Mihaila, H. Zhang, S. Mukundan, D. Demarchi, A. Khademhosseini; (***Biomaterials***, DOI: 10.1016/j.biomaterials.2013.01.045) – ***2013***

List of Proceedings

"Dreamer:an innovative Bone filler paste for treatment of periodontitis". G. Iviglia, D. Bollati, C. Cassinelli, M. Morra. ***Proceeding in Frontiers (In press) – (Oral Presentation)***

"Engineered Porous scaffold for Periprosthetic infection prevention" G. Iviglia, D. Bollati, C. Cassinelli, M. Morra, F. Baino, C. Vitale-Brovarone. ***WBC 2016****, Montreal, CA.* ***Proceeding in Frontiers (In press)***- **<u>WBC-SIB Travel Award 2016</u> – *(Poster Presentation)***

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"In vitro evaluation of osteogenic and antibacterial activity of a synthetic bone substitute modified with collagen-hyaluronic acid-vancomycin complexes" – G. Iviglia, D. Bollati, C. Cassinelli, M. Morra. ***Proceeding in European Cells and Materials Journal Vol.28.Suppl.5,2014 (page 42)***- **<u>Poster Prize GRIBOI 2014</u> – *(Poster Presentation)***

"Surface chemistry and effects on bone regeneration and inflammation of a novel biomimetic synthetic bone filler" – D. Bollati , M. Morra, C. Cassinelli, G. Iviglia, A. Ferrari, M. Fini, G. Giavaresi, A. Parilli, M. Sartori, R. Rodriguez Y Baena – ***Proceeding in European Cells and Materials Journal Vol.28.Suppl.5,2014 (page 42)- (Poster Presentation)***

"HA-βTCP porous scaffold filled with pectin as antibiotic release system for treatment of dental pathologies" – G. Iviglia, D. Bollati, C. Cassinelli, M. Morra, F. Baino, C. Vitale-Brovarone. ***Proceeding in Journal of Tissue Engineering and Regenerative Medicine (DOI:10.1002/term.1932) – (Poster Presentation)***

"*Higly elastomeric poly(glycerol sebacate)-co-poly(ethylene glycol) amphiphilic block copolymers",* G. Iviglia, Alpesh Patel, Akhilesh Gaharwar, Ali Khademhosseini – ***In Book of Abstract Congresso Società Italiana Biomateriali – 2013 – (Poster Presentation)***

Prizes and Awards

Lifebility Award 2013 (*Multilayer PU/PDMS_CHIT_ePTFE membrane for the treatment of burns*)

1st Place Poster Prize GRIBOI 2014 (*In vitro evaluation of osteogenic and antibacterial activity of a synthetic bone substitute modified with collagen-hyaluronic acid-vancomycin complexes)*

WBC-SIB Travel Award 2016 (*Engineered Porous scaffold for Periprosthetic infection prevention)*