Universidade do Minho
Escola de Engenharia

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Development of bacterial cellulose membranes coated with hydroxyapatite for bone regeneration

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Dissertation for the M.Sc. degree in Biomedical Engineering:
Clinical engineering

Supervisors:
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DECLARAÇÃO

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"Science never solves a problem without creating ten more."
George Bernard Shaw

"Somewhere, something incredible is waiting to be known."
Carl Sagan

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This is probably the most difficult part to write down on my thesis. Due to tiredness, due to inertia or due to difficulty in expose my feelings, but essentially due to the inherent difficulty at summing up all people and how they have contributed to my grow as person, as student and as scientist.

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Abstract

Bone, the major constituent of skeletal system, is susceptible to several pathological scenarios that could compromise its correct function. Advanced solutions to overcome these handicaps have always been a desire of the medical community, being guided bone regeneration (GBR) one of the approaches under development. Non-resorbable GBR membranes are mainly composed of synthetic materials. However, bacterial cellulose (BC) arises as an attractive natural origin, non-resorbable solution. In fact, this natural polysaccharide presents interesting properties for the medical field, which lead to increased research efforts into tailoring it with specific features to fit the requirements of the envisaged applications.

The development of BC membranes coated with hydroxyapatite (HA) to be used both as a barrier in GBR and as a promoter of bone regeneration was the main goal of the present work. Two different types of BC, a commercial one (cBC) and in-house produced (gxBC), in three drying states, named never dried, air dried and freeze-dried, where used. BC composites (BC-HA) were prepared following a biomimetic approach involving materials pre-rehydration and chemical modification with sodium hydroxide 3M (NaOH 3M), followed by immersion in specific simulated body fluid (SBF) solutions, NaCl-free SBF and complete SBF. The influence of BC origin, drying procedures and parameters associated with the mineralization protocol were evaluated and deeper characterization was performed on a set constituted by freeze dried cBC and gxBC membranes produced with the defined and optimized protocol, namely modification with NaOH 3M for 1 min, NaCl-free SBF for 1 day and SBF for 7 days.

Scanning electron microscopy (SEM) results denote that air-drying and freeze-drying promote modifications in BC microstructure, and higher porosity of gxBC freeze-dried samples, both before and after rehydration, is evident. This differentiating feature influences the mineralization of the samples, allowing internal formation of a ceramic filler, only observed on gxBC. On the other hand, in the particular case of cBC membranes, this ceramic layer is only observed on the surface of the membranes. Nevertheless, successful formation of a ceramic coating grants the effectiveness of the mineralization protocol. Elemental analysis (EDS) and x-ray diffraction (XRD) reveal the nature of this phase, identified as calcium deficient hydroxyapatite (CDHA). Material's microstructure and the alkali treatment also influence water uptake and degradation profiles, being the highest values associated with gxBC, especially to modified

samples. The effects of alkali treatment on mechanical performance were also more evident on gxBC and the presence of ceramic phase did not completely mitigate its effect. Finally, biological assays revealed promising results, with long-term cytocompatibility for all sets of BC membranes. At the end of 21 days, 3T3 and MC 3T3-E1 cells present higher metabolic active and higher viability levels when seeded in gxBC in the absence and presence of coating, respectively.

Therefore, material-related and methodological variables influence both the biomimetic mineralization and final behaviour of BC membranes. The herein presented results demonstrate an effective biomimetic mineralization, with formation of CDHA phase, resulting in a final material with interesting characteristics that can be considered for GBR applications.

Resumo

O osso, um dos principais constituintes do sistema esquelético, é uma estrutura suscetível a várias patologias que podem comprometer a sua função. Soluções avançadas capazes de solucionar estes problemas sempre foram um objetivo da comunidade médica, sendo a regeneração guiada de osso (RGO) uma das abordagens em desenvolvimento. As membranas não reabsorvíveis usadas em RGO são maioritariamente compostas por materiais sintéticos. Contudo, a celulose bacteriana (CB) também tem sido estudada, surgindo como uma solução atrativa, natural e não reabsorvível. De facto, este polissacarídeo de origem natural é dotado de características interessantes para a medicina, o que tem estimulado a sua investigação e interesse em adaptá-las para que melhor se enquadre na aplicação desejada.

O desenvolvimento de membranas de CB revestidas com hidroxiapatite (HA) para serem usadas em RGO, tanto como membrana barreira como material promotor de regeneração óssea, é o principal objetivo deste trabalho.

Neste trabalho foram usados dois tipos distintos de CB, comercial (cCB) e produzida em laboratório (gxCB), em três estados de secagem, nomeadamente nunca secas, secas ao ar e liofilizadas. Compósitos de CB (CB-HA) foram preparados seguindo uma abordagem biomimética que envolve pré-reidratação dos materiais, seguida de modificação química com hidróxido de sódio 3M (NaOH 3M) e imersão em soluções específicas que simulam os fluídos corporais, com e sem NaCl. A influência do tipo de CB, dos processos de secagem e dos parâmetros associados ao protocolo de mineralização foram avaliados. Um conjunto constituído por membranas cCB e gxCB liofilizadas e produzidas tendo em conta o protocolo de mineralização definido e otimizado, foi analisado mais profundamente.

Os resultados microscopia eletrónica de varrimento (SEM) demonstram que a secagem ao ar e a liofilização promovem modificações na microestrutura da CB e revelam a maior porosidade da gxCB liofilizada, tanto depois e antes da pre-rehidratação. Esta diferença influenciou a mineralização interna das amostras, apenas observada na gxCB. Contudo, uma camada de cerâmico foi também formada na superfície da cCB, indicando a efetividade do protocolo de mineralização. A análise elementar (EDS) e a difração de raios x (XRD) revelou a natureza desta fase, nomeadamente HA com défice de cálcio. A microestrutura assim como o tratamento químico

também influenciam a capacidade de inchamento e degradação das membranas, sendo que os valores mais elevados estão associados à gxCB, especialmente com as amostras modificadas. Os efeitos do tratamento alcalino na performance mecânica dos materiais também é mais evidente na gxCB, sendo que a presença da fase cerâmica não atenua completamente o seu efeito. Por último, os ensaios biológicos revelaram resultados promissores, evidenciando citocompatibilidade a longo prazo para todos os conjuntos de CB. Ao fim de 21 dias de cultura, as células 3T3 e MC 3T3-E1 apresentam maior atividade metabólica quando na presença de gxCB, com e sem recobrimento cerâmico, respetivamente.

Assim sendo, variáveis relacionadas com o material e com o próprio processo influenciam tanto a mineralização biomimética como o posterior comportamento da BC. Os resultados aqui apresentados demonstram a efetividade da mineralização, resultando num material final com propriedades interessantes para RGO.

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List of abbreviations

ATCC - American type culture collection

BC - Bacterial cellulose

BC-HA - Bacterial cellulose-hydroxyapatite composite

Ca-P - Calcium orthophosphates

cBC - Commercial BC

CDHA - Calcium deficient hydroxyapatite

cryo-SEM - Cryo-scanning electron microscopy

DP - Degree of polymerization

dH_2O - Distilled water

DMEM - Dulbecco's modified eagle's medium

EDS - Energy-dispersive x-ray spectroscopy

GBR - Guided bone regeneration

G. xylinus - *Gluconacetobacter xylinus*

gxBC - *Glucanocetobacter xylinus* ATCC 700178 BC

HS - Hestrin–Schramm

HA - Hydroxyapatite

HCl - Hydrochloric acid

NaOH - Sodium hydroxide

-OH - Hydroxyl groups

PC - Plant cellulose

PBS - Phosphate buffer saline solution

RT - Room temperature

SEM - Scanning electron microscopy

SBF - Simulated body fluid

XRD - X-ray diffraction

E - Young's modulus

ε_f - Strain-at-failure

σ_f - Fracture strength

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A. Introduction

C. Materials and methods

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E. Discussion

A. Introduction

Development of bacterial cellulose membranes coated with hydroxyapatite for bone regeneration

Introduction

A well-established fact in human biology comprehension is that human beings are not immune to disease nor trauma [1]. In times when average life expectancy was shorter, a common solution to overcome this setbacks was the removal of injured tissues in an attempt to marginally improve quality of life [2]. Nevertheless, since ancient times, Man tries to find solutions to deal with disease, transforming health care in an emerging area that awakens the interest of several scientific domains, including material science. As a result, medical devices where developed for several areas and found application in improving modern society health, now characterized by an high average life expectancy, also due to medical evolution in terms of antiseptics, antibiotics, hygiene and vaccination [2-4].

Biomaterials field emerged from the partnership between materials and biological sciences, outlining a set of "implantable materials that perform their function in contact with living tissues", as defined by M. Vallet-Regí [3].

Despite no material is fully inert, as all trigger a response by the physiological environment, the first generation of these materials, named bioinert, was intended to restore basic functions of tissues with minimal biological reaction, without toxicity and with a successful outcome [4]. However, these inert solutions are changing to more innovative ones, with desired bioactivity, taking into account the final application [7-9].

With an increasingly elderly population and/or with risk activities on daily routines, bone-related diseases and bone fractures increase and new and attractive solutions are of interest [6].

1. Bone

Skeletal system is one of the most important structures of vertebrate organisms as it provides support, protection, allows movements in conjunction with muscles, and is a reservoir of minerals and fats and a site of blood cells production. In its constitution is a specialized type of hard endoskeletal connective tissue, bone, associated cartilages, ligaments and joins [15-17].

Bone is the major constituent of this system and the human skeleton is composed of approximately 200 bones, in a total weight of almost 2 Kg [8]. Bone belongs to a large family of biological structures, all having in common mineralized collagen fibers in their structure [15-19]. Some of these are mineralized tendons, dentine and cementum (intern filler of the tooth and layer which connects the teeth roots to jaw, respectively) [9].

It is necessary to keep in mind that a clear distinction must be made between bone as a tissue and bone as an organ. The first, is generally composed of bone cells and mineralized extracellular matrix, while in the second case, in addition to bone tissue, also encompasses cartilage, fibrous tissue, marrow and blood vessels. All these variables relate together yielding four types of bone: long, short, flat and irregular [7][10-11].

1.1. Bone as a tissue

1.1.1. Constitution: bone cells and bone matrix

Bone cells are categorized in four groups, according to their function and origin: osteogenic precursor cells, osteoblasts, osteocytes and osteoclasts [12].

Osteogenic precursor cells, or osteochondral cells, have a broad distribution in bone tissue, being present in all nonresorptive bone surfaces and forming the deep layer of periosteum [12]. Furthermore they are mature and highly metabolic active cells and also the progenitor of osteoblasts [7][12].

These last play a unique role in osteogenesis as they are specialized cells capable of production of both organic and inorganic phase of bone: they are able to produce collagen and proteoglycans and to accumulate calcium and phosphate ions, as well as other enzymes [7][13].

Another type of bone cell are osteocytes that are mature cells, which represent 90-95% of totality of bone cells and are the result of the entrapment of osteoblasts by mineralized collagen matrix [7]. This cell type is less metabolically active when compared with its progenitor, being involved in the balance of calcium and phosphorus concentration, playing an important role in adaptive remodeling behavior in response to local environmental conditions [7][20-22].

Finally, osteoclasts are cells responsible for resorption of bone, having a crucial role in bone reorganisation [7]. This multinucleated cell entity has its origin in mononuclear precursors from red bone marrow, the same stem cells that are progenitor of monocyte (monocyte/macrophage lineage precursors), under strict regulation of osteoblasts, osteocytes and bone marrow stromal cells [7][13].

Bone matrix is responsible for the functional characteristics of bone. It is constituted by an organic and inorganic phases, that in a mature bone represent 35% and 65% of the bone weight, respectively [7]. Some physiological characteristics of this tissue are assigned to each part: organic phase grants flexible bone strength, while the inorganic phase ensures resistance to mechanical forces, hardness, strength and a protective effect on soft tissues [14-16].

The organic portion of bone matrix is essentially composed of collagen fibers and non-collagenous proteins [23-24].

Collagen fibers are the most representative part and are mainly collagen type I. Although little is known about the way these molecules are assembled, their internal organization appears to play an important role in bone mineralization. The arrangement and concentration of these proteins depends on the type of bone, showing higher levels in compact bone. In contrast, it is also in this same type of bone tissue that non-collagenous components appear in minimum concentration [23-24].

Non-collagenous proteins are a wide group comprised of two hundred or more elements, both permanent nature components, like proteoglycans, as well as cellular byproducts like alkaline phosphatase and growth factors, and other molecules normally present in circulating body fluids, including albumin and α2HS-glycoprotein. These components are considered crucial on the induction and regulation of biomineralization [23-24]. In addition, they vary both qualitatively, depending on the type of bone, and quantitatively, depending directly on bone formation rate and indirectly on collagen fibers density [15].

Historically, the description of inorganic phase composition has changed considerably. Nowadays there is a consensual claim that recognises hydroxyapatite as the mineral phase ($Ca_{10}(PO_4)_6(OH)_2$), being described by some authors as dahllite ($Ca_5(PO_4, CO_3)_3(OH)_2$), with about 5% carbonate and trace amounts of other ions, like sodium (0.9%) and magnesium (0.5%) [7][15-16]. However, bone hydroxyapatite differs from its nature counterpart and several studies have been carried out in order to unravel its true crystalline nature. Several factors, such as age and inorganic and organic matrix phases' correlation, influence both composition and structure of the mineral in

the biological environment. Polycrystallinity is considered the true crystalline nature of bone, undergoing changes according to the parameters mentioned above [23-24]. On the other hand, this polycrystalline nature is chiefly associated to the incorporation of the aforementioned ions and to the nonstoichiometry characteristic of this phase [16]. In addition, this biogenic mineral is also classified as a calcium-deficient apatite. In fact the Ca/P molar ratio is a distinctive factor between the two: in bone the ratio varies between 1.57 and 1.71 and in natural hydroxyapatite it is 1.67 [16][23-24].

1.1.2. Types of bone tissue

Besides the different types of mechanisms associated with bone formation (mentioned below), these always involve the formation of a less organized tissue that is replaced by a more mature one [11][14][16].

Primary bone formation is a rapid and unorganized process, resulting in woven bone, also known as immature, embryonic or primary bone. Its fibers are randomly aligned forming bundles of collagen with interfibrilar spaces containing non-collagenous proteins, resulting on calcification nodules or calcospherites formation [11][14-16]. Woven bone is replaced by lamellar bone due to the action of osteoclasts and osteoblasts during secondary bone formation [7][16]. Resultant tissue can also be called mature, secondary, parallel-fibered or osteonic and it is much stronger than the last one presented. Here, collagen fibers are clustered in structures designated as lamellae, in parallel arrangement to each other, alternating in longitudinal and transverse orientations [11][14-16].

Either woven or lamellar bone can be classified as compact and trabecular bone, based on structural organization, mechanical characteristics, amount of bone marrow, cellular function and metabolic activity [14].

Trabecular bone, also named cancellous or spongy bone, is considered, by some authors, as a primary type of bone [12]. It is essentially present in the core of short and flat bones and in epiphyseal ossification centres [7]. Trabeculae, formed by several lamellae, compose the network of this tissue, and its orientation is dependent of stress lines within bone, adaptable to slight changes [7][14].

Compact bone is denser and less porous than its counterpart and it is found in long bone diaphysis and in cortical shell of short and flat bones [7]. In fact, it is characterized by a dense organization where lamellae, in association with osteocytes, are arranged around a central channel called osteon

or haversian system. These structures are parallel to bone axis and contain loose connective tissue, nerves and blood vessels responsible for nutrition and depuration of bone cells. Feeding is performed via Volkmann's canals, which are perpendicular to bone axis [7][14].

1.2. Bone as an organ

As mentioned before, bones can be classified as long, short, flat or irregular [7]. Moreover, in the same bone there are often different types of bone tissue with well-known structural organization and composition characteristics [15-16] .

Long bones, like tibia, are one of the four types of bone, where three distinct parts may be defined: diaphysis or shaft, composed essentially by compact bone with a large space called medullary cavity filled with marrow; epiphysis or end of the bone, composed essentially by cancellous or spongy bone, filled also with marrow; and epiphyseal plate or growth plate, composed by hyaline cartilage, situated between epiphysis and diaphysis and where growth of bone in length takes place, being named of epiphyseal line, a calcificated area, when growth stops [7].

The other three categories of bone are usually devoid of diaphysis or epiphysis, having an interior framework of cancellous bone surrounded by compact bone. The main differences are based on the morphological features: short bones, including finger bones, are nearly cube-shaped or round and medullar cavity is not present; flat bones like skull and ribs for example, are thin, usually curved and with flattened shape; irregular bones, like vertebrae, present shapes diverse from the ones described above.

External and internal surfaces of bone are typically wrapped with periosteum and endosteum membranes, respectively. Periosteum is a connective tissue membrane constituted by two layers: the outer layer is dense and irregular containing essentially blood vessels and nerves, while the inner layer is composed of bone cells. Endosteum has a composition similar to the inner membrane of periosteum [7].

All bones have a complex hierarchical microstructure, where several levels of organization can be distinguished (Figure 1) [10][17].

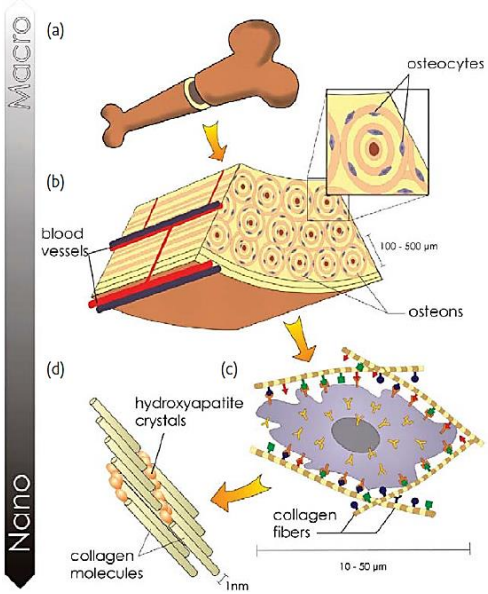


Figure 1. Hierarchical organization of bone over different length scales [17].

1.3. Bone dynamics: formation, growth, remodelling and repair

1.3.1. Formation, growth and remodelling

Two distinct developmental processes are involved in bone formation, occurring since embryological development: intramembranous and endochondral ossification [7].

Intramembranous ossification is the process involved in flat bone formation, like the majority of craniofacial complex bones and medial clavicles [7]. In this process, skeletal mesenchymal cells mediate bone formation, without the participation of a cartilage mold [11][18]. Around the fifth week of embryonic development, embryonic mesenchymal cells aggregate and form a highly vascularized membrane of connective tissue [7]. Cells in this membrane become osteochondral progenitor cells, which then differentiate into specialized osteoblasts ready to synthesize bone matrix and develop structures known as ossification centres. Here, connective tissue collagen fibers and cells are entrapped, culminating in the formation of woven bone that will be remodeled to trabecular bone. The process of bone formation continues, leading to trabeculae enlargement, with a decrease in the spacing between other similar structures, resulting in partial closing of vascular connective tissue, which is a precursor of hematopoietic tissue. The tissue becomes organized, forming layers, which are arranged in concentric rings around a blood vessel, known as haversian systems. The formation of the endosteum and periosteum occurs after condensation of outer and inner layers of connective tissue, being areas rich in osteoprogenitor cells [7][26-27].

In endochondral ossification, bone is formed by substitution of a skeletal cartilage template. This process is associated to appendicular skeleton, vertebral column, facial bones and pelvis bone formation [26-27]. This process involves mesenchymal cells differentiation into chondroblasts that are capable of cartilage synthesis. The cartilage template is normally enclosed in a capsule named perichondrium, whose invasion by blood vessels initiates the conversion of osteoprogenitor cells into osteoblasts and synthesis of compact bone by intramembranous ossification on the mold surface, forming the collarbone. At the same time occurs hypertrophy of cartilaginous mold and the hypertrophic chondrocytes (essentially in the middle zone of the structure) produce collagen type X (a hypertrophy indicator) and angiogenic factors such as vascular endothelial cell growth factor (VEGF). These features promote calcification of the template; cells became entrapped and eventually die, forming gaps. These enable the entrance of blood vessels in the calcified mold, carrying osteoprogenitor but also hematopoietic cells and culminating in the formation of trabecular bone on the mold surface and then of cancellous bone on the diaphysis of the structure. As the primary ossification centre is formed, the process of bone matrix deposition continues until all calcified cartilage is replaced by bone [7][18].

Long bones, in particular, present the development of secondary ossification centers in the epiphysis, where the ossification process occurs in a similar way to what happens in primary ossification center. The majority of cartilage in this area is replaced by cancellous bone, with the exception of epiphyseal growth plate and articular cartilage. Long bones and bone projections growth in length occurs in epiphyseal plate, involving interstitial cartilage growth followed by appositional bone formation, thus ensuring bone growth and maintenance of epiphyseal plate thickness. Articular cartilage is the place where epiphyses as well as bones without epiphyseal plate increase in size. The events are similar to those mentioned for epiphyseal line, with the difference that articular cartilage does not ossify. It is important to notice that bone grows both in width and thickness, phenomena associated with periosteum. Summarily appositional bone growth occurs under the periosteum, with varying rates, forming structures named osteons [7][18].

Finally, osteoclasts play an essential role in the diaphysis, resorbing bone in order to form the spinal canal, while the hematopoietic cells will form red bone marrow. Bone is fully formed when all the perichondrium is transformed in periosteum, the epiphyseal plate is mineralized and the process of bone remodelling is finished [7][18].

This last process is continuous, occurring through the entire life cycle. Once the maturity of the structure is reached, it plays a crucial role in the replacement of older bone by new one and in its

response to environmental conditions, like adjustment of bone to stress and regulation of calcium ions in the body [7][19].

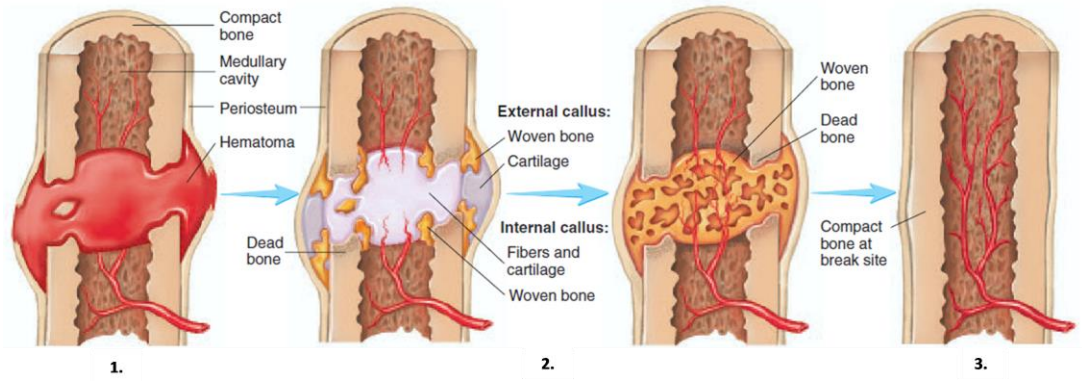
In a simple way, bone remodelling occurs through the action of osteoclasts that remove old bone, and of osteoblasts, which deposit new tissue, converting woven bone into lamellar bone [7][19]. Both cortical and trabecular bone remodelling are the two forms of this process. The first occurs within the osteon and it is associated with resorption of old haversian systems and formation of new ones. The last is associated with trabecular endosteal surface, being regarded as a surface process [18].

1.3.2. Repair

As seen before bone is a living and dynamic tissue where a series of biological events occur constantly: resorption, deposition and remodelling [7][11][26-28]. Another important process is bone repair or bone healing [7][20-21]. This complex process is synced in such a way that is effective in repair and restores bone functions. Induced by an immune response triggered by an injury, several entities participate in it, recapturing the processes of bone formation that occur during embryonic development [20].

Bone has the ability of restoring itself without forming a scar and during the last two decades, attention has been focused in understanding the fracture healing mechanism, unveiling the diverse pathways involved in it. Classically the process can be direct and indirect [30-31].

The majority of fractures heal by indirect bone healing, also named of secondary bone healing. This process is characterized by the use of intramedullary and external fixation and conservative treatment using a plaster cast. These solutions allow fracture stabilization, while maintaining a controlled motion between the fractured surfaces subjected to load, thus enhancing the process if the right stability is in fact achieved. This process combines both intramembranous and endochondral ossification in overlapping phases, with the involvement of a cartilaginous template: hematoma formation, cartilage callus formation and ossification and bone remodeling (Figure 2) [29-31].



1. Hematoma formation and inflammation

a) The damage of the surrounding tissues leads to the disruption of blood vessels and consequent formation of hematoma by the activation of coagulation mechanisms. This structure encloses the fracture area.

b) Acute inflammatory response: the avascular zones at the end of fractured bone lead to the appearance of apoptotic cells in that area, which produce chemotactic molecules (IL-1, IL-6, IL-11, IL-18 and TNF-α) responsible for the recruitment of other inflammatory cells, as well as mesenchymal cells. This increase in local vascularity is essential to the phagocytosis of necrotic material and the mesenchymal cells will differentiate into osteoprogenitor cells, essential to the repair of this tissue.

2. Cartilage callus formation and ossification

a) The hematoma organizes into granulation tissue, which results in the reduction of *in situ* strain to <10%, crucial to cartilage or soft callus formation. This phenomenon occurs initially in less stable areas in order to attenuate this factor (between the fracture ends and external to periosteal sites).

b) At the same time, intramembranous ossification occurs at periosteum and an external callus is formed, resulting in a semi-rigid structure able to support weight bearing.

c) The soft callus undergoes endochondral ossification becoming hard callus, essentially constituted by woven bone, a structure increasingly solid, rigid and with better mechanical properties. The formation of this structure can be followed by measurement of markers such as type I procollagen, osteocalcin, alkaline phosphatase and osteonectin.

3. Bone remodeling

a) In order to reach the right mechanical characteristics, woven bone is remodeled into lamellar bone and the vascular supply returns to normal levels. A perfect balance between bone resorption by osteoclasts, bone deposition by osteoblasts, biochemical mediators (IL-1, TNF-α, BMP), adequate blood supply and a gradual increase in mechanical stability is essential.

Figure 2. Indirect bone healing mechanism [7][29-31].

Direct fracture healing or primary bone healing occurs by direct contact between the cortical ends of fractured bones and depends on the action of functional units called "cutting cones" to re-establish the lamellar bone structure, without the involvement of a cartilage intermediate [11][21]. Furthermore, absolute stability is desired to ensure correct healing of the structure. In this process, a correct anatomic reduction of the fracture fragments is essential and a rigid internal fixation is achieved by compression, which promotes a reduction of interfragmentary force, elimination of any failure within the interfragmentary area and the need for an external callus or vessels and cells for the process to unfold [11][29-31]. The direct route can be divided in contact or gap healing, being the main difference between the two processes the fact that in the second case, bone union and haversian remodeling occurs separately in time [21].

1.4. Bone defects

There are several pathological scenarios, such as trauma and congenital malformation, fractures, surgery, periodontitis, osteoporose, osteomyelitis or tumors, which promote the destruction of essential components of bone tissue and/or compromise its correct function. In general, bone defects can be classified as critical-sized defects, calvarial defects or long bone or mandible segmental defects. If inappropriate osseous reconstruction occurs, it can result in impaired bone healing with the formation of fibrotic tissue, pseudoarthrosis or artificial joints, with no physiological significance [8][22][32-33].

Diverse factors influence both quantity and quality of bone matrix, including age and sex. In fact, the majority of bone defects can be correctly healed in younger people. Nevertheless and regardless of age, larger defects may need the use of advanced solutions [17][24]. On the other hand, men typically have denser bones than women as a result of their higher body mass and levels of testosterone. However, in both sexes, increasing age has deleterious effects on skeleton and bones become more brittle due to a decrease on collagen production and less dense due to changes in the equilibrium of bone resorption and formation. All this factors are favourable to the development of an osteoporosis scenario, where the probability of fracture is increased [7-8][19]. Another condition where bone becomes more fragile and there is a greater likelihood of fracture is osteomalacia. This results from organ decalcification and it is a case of attention during pregnancy when the need for calcium is higher for fetus development [7].

Bone fractures or defects can be the result of conditions that weakens and decreases the mechanical strength of bone, or of external aggressions, including trauma situations. Fractures are characterized by a break or discontinuity in the bone as a result of an applied force that exceeds the limits of tension or compression that the tissue is able to handle. These fractures may be complete or incomplete, displaced or undisplaced, comminuted or simple, open or closed [25].

Osteomyelitis and periodontitis are another example of conditions that affect bone integrity. Osteomyelitis is a bacterial infection that can lead to complete destruction of bone structure and is caused, in the majority of cases, by *Staphylococcus aureus* but also by *Mycobacterium tuberculosis*, known as bone tuberculosis [7][22]. Periodontitis is associated with the oral environment and the destruction of periodontal tooth supportive structures. These and other situations of chronical dental disease, either caused by inflammation or infections, may end in bone jaw resorption as well as reduction of jaw bone volume, which complicates implants placement [8][26].

Bone tumors and tooth extraction constitute other examples that can be associated with bone defects [7][26-27]. Although they do not directly originate bone defects, the removal process may lead to the formation of these gaps [26-27].

1.5. Solutions

The search for new advanced solutions for bone defects repair has been a defying goal for the medical community since ancient times. In fact, traces of the use of materials as bone extracts is dated as far as the Neolithic [5].

Presently, the availability of solutions for bone reconstruction include procedures that make use of bone grafts and bone graft substitutes [28]. These solutions must fulfil a set of properties that should be taken into consideration when choosing the most appropriate procedure to be used or in research of new solutions (Table 1) [7-9].

Table 1. Features to consider when choosing or designing bone replacement solutions [5][30]

Intended clinical application	Availability	Ethical issues	Biocompatibility
Defect size	Desired bioactivity	Durability	Sterilization
Biomechanical properties	Desired resorption rate	Associated side effects	Cost
Chemical composition	Handling characteristics		

Bone grafts are seen as the traditional solutions employed in bone defect treatment. These comprise autografts, allografts/alloimplants or xenografts/xenoimplants. However, some drawbacks associated to these solutions have fostered the growth of orthopaedic biomaterials market [17][27].

A broad range of materials are currently studied and employed either as bone grafts or for guided bone regeneration (GBR) or tissue engineering approaches. Commonly used materials include polymers, ceramics, metals and composites, either of natural or synthetic origin [17][29]. These are available in several forms, like chips and granules of different sizes, putty or injectable pastes, which is extremely important for surgeons in daily practice [30].

As research on novel solutions is moving from inert to bioactive materials, features like osteoconduction, osteoinduction and/or osteogenesis become crucial [11-14][31]:

- Osteoconduction: material's ability to ensure structural support for superficial and/or internal growth of blood capillaries and cells, promoting growth of new bone. This property may be associated with either viable or nonviable material and is heavily dependent on

structural properties of the material, such as porosity, pore size and interconnectivity and surface roughness.

- Osteoinduction: material's ability to induce recruitment of cells from the surrounding tissue into the material, thus stimulating bone regeneration. This ability is attributed to a wide range of molecules such as bone morphogenic proteins (BMPs), platelet-derived growth factors (PDGF), insulin-like growth factors I and II (IGF-I and IGF-II), fibroblast growth factors (FGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), retinoic acid, sulphated glycosaminoglycans and amino acids.
- Osteogenesis: material's ability to induce new bone formation due to the presence of osteoblasts and stem cells in the transplanted/implanted material.

1.5.1. Bone grafts

Autografts consist in transplantation of patient own bone tissue from a site to another within the organism, allowing the utilization of a bone solution with natural osteoconduction, osteoinduction and osteogenic characteristics. Considered the gold standard of bone defects treatment, it offers beyond inherent biocompatibility and minimal ethical issues, rapid healing and complete incorporation, lack of disease transmission and immunogenicity. On the other hand, increased operative time, limited availability of tissue, infection probability, inflammation, compromised biomechanical properties, donor site morbidity and pain are some of the disadvantages associated with this solution [2][8][11-14][17][30].

Donor site morbidity and availability of tissue are some of the drawbacks that may be resolved with the use of allogenic bone grafts and implants. These can be obtained from cadavers or living donors; however, the immunogenic potential and rejection rate associated with fresh, fresh frozen and freeze dried materials has to be considered. In order to try to minimize this, strategies based on demineralized freeze-dried allogenic bone have been proposed. Slow osteointegration, risk of disease transmission and chronic inflammation constitute another set of drawbacks [11-13][30][32].

Xenografs or xenoimplants are another option, which relies on living or non-living tissues from nonhuman species are used, more routinely bovine or porcine. Even when chemically processed, these solutions are far from what is considered ideal, presenting innumerous disadvantages mainly due to the lack of biocompatibility, pathogen transmission, immunogenic rejection, prolonged graft integration, and fracture. However, these solutions are utilized in diverse

situations as an end-line solution, including heart valve replacements (porcine) or bone substitutes (bovine), as ethical considerations have also to be considered [2][28].

1.5.2. Tissue Engineering

"An interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function" is Langer and Vacanti's definition of tissue engineering [37-38].

There are three main constituents that come into play in a tissue engineering strategy: cells, growth factors or signalling molecules and matrices or scaffolds. The combination of these elements depends on many factors, including patient age, gender, health, systemic conditions, habits and anatomical shape of the implant [37-38].

In the particular case of bone, cells that normally compose the tissue engineered construct should possess osteogenic potential. Periosteal cells, osteoblasts and mesenchymal stem cells (MSCs) are widely used, from which MSCs are the most often applied in bone tissue engineering [29][34].

In order to provide support for cells' expansion, differentiation and proliferation, but also for the load and subsequent release of growth and differentiation factors, scaffolds constitute an essential component in the overall tissue engineering approach. Pore size, surface and mechanical properties, osteoinductivity, biodegradability, biocompatibility and processability are some of the most important properties of a scaffold. In this sense, the appropriate choice of the materials to be used plays a critical role. However, there is a wide availability of materials that can be employed in tissue engineering research, ranging from metals, ceramics, polymers and composites, from natural and/or synthetic origin, and which can be used in solid preformed structures or injectable form [29][33].

Natural or synthetic hydroxyapatite, calcium phosphate solutions, like β-tricalcium phosphate and bioactive glass, are some of the ceramic materials employed in scaffolds manufacturing. Some advantages of these materials include osteoconductive and osteoinductive properties, being brittleness, low mechanical stability, difficulty to predict degradation/dissolution rates and poor reproducibility due to high variability associated to ceramics, some of the known drawbacks [29][33][35].

Polymers are seen as an attractive alternative to ceramics and can also be divided in natural and synthetic. Natural polymers widely studied for bone tissue engineering comprehend collagen, fibrinogen, chitosan, starch, hyaluronic acid and/or calcium alginate, which exhibit good

biocompatibility, low immunogenic potential, chemical versatility, osteoconductive properties and, in some cases, biodegradability and osteoinductivity [29][33][35]. On another hand, lack of mechanical stability and biochemical changes brought by sterilization techniques are described drawbacks [29][35]. Synthetic polymers are chemically more versatile and different physical and mechanical characteristics and degradation profiles may be obtained. Poly(α-hydroxy acids), poly(ε-caprolactone), poly(propylene fumarates), poly(carbonates), poly(phosfazenes), poly(lactic acid), poly(glycolic acid), poly(doxanonone), poly(anhydrides) associated copolymers are some of the materials included in this group [29][33][35].

Titanium and tantalum are two metallic materials that have been applied in metallic scaffolds' fabrication and as prosthetic implants coatings, with many different shapes and textures. These materials are biocompatible, resistant to corrosion and durable. Yet, they are bioinert and not biodegradable that together with their high stiffness when compared to bone are the main disadvantages of these metallic solutions [29].

In order to combine advantageous individual characteristics of different types of materials, composites have emerged as a new class. Some interesting combinations include polymeric-ceramic composites and metallic-ceramic composites, leading to improved properties as brittleness, drug delivery and mechanical properties in the first case and bioactivity in both [29].

The last main constituents of any tissue engineering approach are growth factors or signalling molecules. Particularly relevant for bone tissue engineering applications are cytokines, specifically BMPs, which are essential in the osteogenic differentiation process [38-39]. Infuse® Bone Graft/LT Cage® fusion device is a specific case of an existing commercial solution based on a bovine collagen matrix loaded with recombinant human BMP-2 (rhBMP-2) for the treatment of degenerative disc disease [35].

1.5.3. Guided Bone regeneration

Developed in 1980, based on the philosophy of guided tissue regeneration, GBR is another procedure applied in bone reconstruction, with particular emphasis on periodontics and implant dentistry [25][32].

The principle of this method relies on preventing the invasion of the defect by soft tissues (mainly composed of non-osteogenic cells), formation of fibrous connective tissue and both establishing and maintaining a propitious environment for recruitment, proliferation, differentiation and

maturation of osteoprogenitor cells on the isolated defect cavity. This is usually accomplished by the use of occlusive or barrier membranes [40-42].

GBR is influenced by a number of factors, including patient-related like smoking habits, diabetes or another limitative pathology; defect-related like morphology, length and angle; and membrane-related, including membrane fixation, passive flap tension, cortical penetration, excessive swelling and membrane composition [32].

A cellulose acetate filter (Millipore) was the first membrane used in this type of approach [32][37]. Nowadays, available solutions have evolved in order to meet the diverse requirements for the expected positive results. These include biocompatibility, space making ability, cell occlusion properties, prevention of bacterial infection integration by the host tissues and clinical manageability, adequate mechanical properties and ability to isolate and stabilize the defect and the fibrin clot or other materials of different origins, such as autogenous bone chips, allografts, xenografts or alloplasts (synthetic) that may be used to occupy the defect and facilitate bone ingrowth [32][41-43].

In general, GBR materials may be characterized as non-resorbable or resorbable materials and their properties are designed to optimize tissue regeneration. These materials may possess antimicrobial properties, be enriched with bioactive molecules or tailored to possess desirable physical or chemical characteristics [39][41-42].

Non-resorbable membranes are mostly synthetic and include expanded and high-density polytetrafluoreethylene (e-PTFE: Gore-tex®; and dPTFE: TefGen-FD®, respectively), titanium mesh (Ti-mesh) and titanium-reinforced ePTFE (Ti-e-PTFE) [32][38]. However, the natural polysaccharide bacterial cellulose, as Gengiflex from BioFill company, have also been studied with promising results [40-41].

Biocompatibility, predictable profile during the healing process, mechanical strength and easy handling are the main advantages that support the use of non-resorbable materials. However, there is the need for a second surgical intervention to remove these membranes, which represents increased costs and patient discomfort [36-37][39][42].

Resorbable solutions partly solve these problems, but immunogenicity, porosity, unpredictable duration of barrier function (degradation and resorption rates) and mechanical performance, when compared to the non-resorbable counterparts, represent some of the disadvantages of these solutions [39][40-41]. Used resorbable membranes may be from natural or synthetic origin. Poly(glycolic acid), poly(lactic acid), poly(ε-caprolactone), poly(urethane), poly(glactin 910),

poly(dioxanon) and their co-polymers represent the synthetic polymeric solutions (Guidor®, Resolute®, Epi-Guide®, Mempol®, Vicryl Periodontal Mesh®, Atrisorb®, Experimental Mempol®). Their natural equivalents include collagen derived from animal skin, tendon or intestines (BioGide®, BioMend®, Avitene®, Collistat®) [32][38-39][42].

2. Bacterial cellulose (BC)

Polymers are ubiquitous in the living world, forming the building blocks of life. On the other hand, polymers are extensively studied and used in diverse industrial areas, ranging from food, textiles, cosmetics, pharmacy and/or medicine, among others [47-48].

By definition a polymer (from the Greek *poly* – "many"; *meres* – "parts") is a macromolecule composed of small repeating units, named monomers, linked by covalent forces. These materials can be classified according to their origin, structure, polymerization mechanism, preparative techniques or thermal behaviour [43].

Focusing on polymer's nature, they can be classified as natural or synthetic [47-49]. Natural based polymers, or biopolymers, in particular, possess diverse properties that make them suitable to be applied in highly demanding areas such as the medical one. Similarity to biological macromolecules is one of the major advantages of these polymers, as compared to their synthetic counterparts, which enhance biological recognition, cell adhesion and metabolic acceptance and processing. Some disadvantages of this type of materials are related to batch-to-batch consistency; material source, which can lead to undesirable immune reactions due to, for example, the presence of impurities or endotoxins; and to processing technics that can impact polymer's final properties, including mechanical properties or biodegradability. However, the continuous research and development of novel methods for polymer production, purification and processing will allow an increasing control over material's properties and enhance its biofunctionality and contributing for the overall reduction of toxicity and chronic inflammation and immunological reactions [48-49].
Natural polymers are instinctively appealing and researchers are investing considerable efforts on their synthesis and production. In this context, biotechnological pathways arise as a strategic alternative, based on microorganisms' fermentation and enzymatic processes [45]. Considering their chemical structure, polysaccharides, proteins, polyesters, but also inorganic polyanhydrides, enclose the main classes of polymers produced by bacteria [49-50].

Polysaccharides are a group of macromolecules abundant in nature, composed of monosaccharides linked through O-glycosidic linkages. The variety of monosaccharides that can build them up, bonding, shapes and molecular weight greatly contribute for the diversity associated to this class of molecules. Any variation in any of these parameters will consequently result in a different structure with different surface, interfacial and physicochemical properties, including solubility, flow behaviour and gelling potential, and functionality [45-46].

Polysaccharides produced via microorganism's fermentation can be classified according to their morphological localization in intracellular polysaccharides, found within the cell or as a part of the cytoplasmic membrane (e.g. glycogen); capsular or cell wall polysaccharides, as a structural component of the capsule or cell wall (e.g. K30 antigen); and extracellular polysaccharides, located outside the cell wall, which can be synthesized or secreted by cell wall-anchored enzymes (e.g. xanthan, dextran, cellulose, hyaluronic acid) [50-52].

Cellulose is one of the most abundant biopolymer on earth and can be found in several natural reservoirs (Figure 3) [53-54].

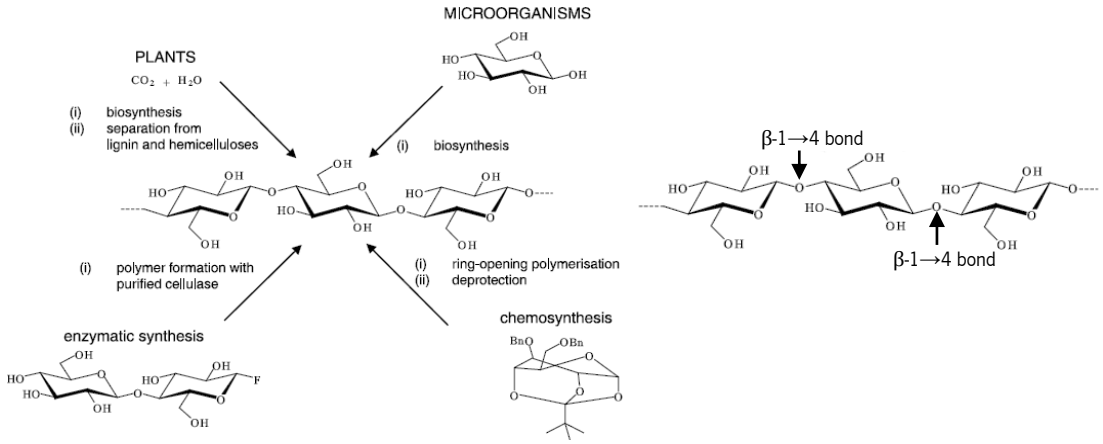


Figure 3. Principal pathways of cellulose production and molecular arrangement with identification of β-1→4 bond [55].

One of the best known and well established industrial sources of cellulose are plants, where cellulose is the major component of the vegetable biomass. Plant-origin cellulose constitutes the primary component of cell walls, naturally associated to other components like acidic polysaccharides, glycoproteins and waxy aromatic substances, including hemicelluloses, pectins and lignins. Cellulose is also naturally present in some fungi and algae, where it plays an important role in structural maintenance [50-52][55-56]. It is also a representative by-product associated with some bacteria, where it acts as an important reserve material, but also as a component responsible for structural protection, particularly relevant under unfavourable environmental conditions (Table 2) [57-58].

Table 2. Cellulose-producing bacteria [57-58]

Genus	Produced molecule	Biological Role
Acetobacter	Extracellular pellicle composed of ribbons	Keep aerobic environment
Achromobacter	Cellulose fibrils	Flocculation in wastewater
Aerobacter		
Alcaligenes		
Pseudomonas	No distinct fibrils	
Zoogloea	Not defined	
Agrobacterium	Short fibrils	Attached to plants
Rhizobium		
Sarcina	Amorphous celulose	Unknown

Bacterial cellulose (BC) is an exopolysaccharide produced by several species of bacteria, particularly gram-negative species, such as *Gluconacetobacter* (formerly known as *Acetobacter*), *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, *Pseudomonas, Alcaligenes* and *Salmonella*, but also by the gram-positive *Sarcina ventriculi*. The microorganism model used for cellulose studies is *Gluconacetobacter xylinus* (*G. xylinus*) due to its ability to produce this polymer in high quantities using a wide range of carbon and nitrogen sources as substrate [50-52][56-57].

Cellulose, with the molecular formula $(C_6H_{10}O_5)_n$, is a linear homopolysaccharide, composed of glucose (D-glucopyranose as D-glucose is present as a pyranosyl ring), linked through a β-1→4 glycosidic bond [46][52]. Assembly of the β-1→4 glucan chains will determine the crystallinity of the material (Figure 3) [48].

2.1. BC fermentation and biosynthesis

G. xylinus, also known as *Acetobacter xylinus*, is a rod-shaped gram-negative α-proteobacteria, found in diverse natural habitats, like vegetables, vinegar, alcoholic beverages, rotting fruits and niches where carbon is fixed in the form of sugar or alcohol [48]. This strictly aerobic and non-photosynthetic microorganism is characterized by its ability to convert several organic substrates, like glucose and glycerol, into cellulose. Despite being a non-flagellated cell, some strains show relative mobility, observed microscopically, during formation of cellulose ribbon [53-55].

BC production is dependent on several conditions, like environmental conditions, culture media and by-products [52-53].

Among different environmental conditions, pH, temperature, dissolved oxygen and culture agitation are the most widely studied for their effect on cellulose synthesis [52].

Culture media is typically composed of sources of carbon, nitrogen, phosphorus, sulphur, potassium and magnesium salts. Increased content of carbon, minimal content of nitrogen and supplementation with amino acids and vitamins are reported as adequate strategies to enhance BC's production [52]. There are several carbon sources employed in BC's production, ranging from monosaccharides to organic acids, where glucose is one of the most used, with the sole exception of arabitol and mannitol [48][53].

On a daily practice, the standard media used in *G. xylinus* liquid cultivation is Hestrin–Schramm (HS) culture medium, being the ideal pH between 4-7 and optimal growth temperature in the range of 25-30 °C. HS culture medium is essentially constituted by D-glucose, yeast extract, peptone, citric acid and disodium phosphate (the last two used as buffer systems) [52-53].

These liquid cultures can be performed in static or shaken conditions, with considerable influence over the BC structure and morphology [50]. In both cases, cellulose production tends to follow the liquid-gas interface, where oxygen availability is increased [49][52].

Static cultures result in a cellulose mat-like pellicle, formed on the liquid-gas interface [50]. On the other hand, in shaken cultures, bubble columns or airlift reactors, cellulose acquires the form of irregular granules, star-shaped structures or fibrous strands, well dispersed in the culture medium [48-50]. Both in static and shaken cultures, produced cellulose is obtained as a crude (impure) polysaccharide structure, typically contaminated with bacteria cells and medium residues. There are several approaches to purify this material and the most common ones involve treatment with alkali (sodium hydroxide or potassium hydroxide), organic acids (acetic acid) and washing with water. In addition, effective sterilization procedures may be executed for medical applications and can resort to methods such as gamma radiation, autoclaving and ethylene oxide [49][52].

The increased interest in BC for several industrial and medical applications has led to a subsequent investment on the production of this polymer with more attractive final properties, tailor made to fit the requirements of the envisaged applications [47].

The recent understanding of the diverse pathways of biosynthesis of BC and regulation mechanisms opens and widens the possibility to engineer bacteria to produce a "personalized" BC polymer. Although the structure of the polymer is characteristic of the producer bacterial strain, it is known that these pathways and mechanisms are generally conserved over the different species of cellulose-producing bacteria, being a precisely regulated multi-step process [47][50][52-54].

A single cell of *G. xylinus* is known to possess at least 100 pores responsible for cellulose extrusion to culture media (Figure 4) [49].

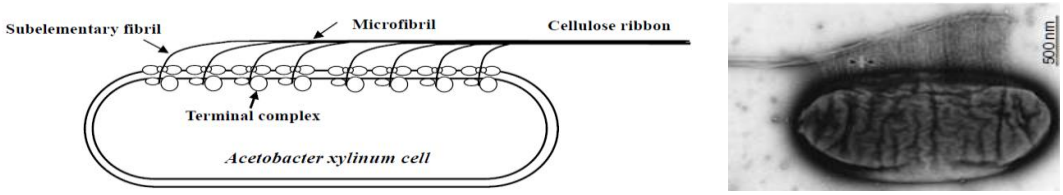


Figure 4. Mechanism and SEM micrograph of cellulose ribon formation by *G. Xylinus* [49][54].

During biosynthesis, each pore produces a cellulose chain, called sub-elementary fibril (approximately 1.5 nm in width), that groups with other fibrils to form microfibrils or an elementary fibril (approximately 3.5 nm in diameter). Microfibrils associate with each other, forming a cellulose ribbon (width ranging from 40 to 60 nm) [50][54]. Ribbons arrange themselves to form a larger unit, called a fiber, able to intersect with neighbouring fibers, leading to the formation of a reticular structure with a regular shape, stabilized by hydrogen bonds [49][55]. This multistep phenomenon describes the polymerization of cellulose, which is independent of the BC's biogenesis process [52][55].

In addition, BC crystallization is also an independent event, related with intra and inter molecular hydrogen bonds established between hydroxyl groups (-OH) that also provide structural coherence and rigidity to the material [49][52].

Cellulose can assume four crystalline forms (cellulose I, II, III, IV) being the most common in nature cellulose I and II [56]. Both are produced by *G. xylinus* and the main differences are essentially related with chain rearrangement after cellular extrusion [46][50]. Cellulose I is more abundant as compared with type II cellulose. The later can also be obtained after chemical treatment of cellulose I, through mercerization (an aqueous treatment with sodium hydroxide) or dissolution of the material followed by precipitation [60-61].

Cellulose I is considered a metastable polymer, produced by *G. xylinus* under static conditions [50]. Chains are arranged in a parallel fashion and the microcrystallinity of the material is related to the existence of well-ordered regions within the microfibril chains. -OH groups are distributed in a quasi-planar arrangement, allowing the formation of linear chains capable of binding to other chains, maintaining this kind of organization. These well-ordered regions alternate with paracrystalline and less ordered amorphous ones [49-50][52]. In this sense, cellulose I can be divided in two types of crystalline allomorphic phases, Iα and Iβ. Iα has a tricyclic structure found mainly in algae and bacteria, while Iβ has a monocyclic structure and is characteristic of plant cellulose [46][49-50][56].

Cellulose II is an amorphous polymer where the chains are arranged in an anti-parallel mode and it is obtained by culturing *G. xylinus* in shaken conditions. This form is more thermodynamically stable than cellulose I, mainly due to the existence of a larger number of hydrogen bonds (there is an additional bond of this type per glucose residue) [46][50][57].

2.2. BC characterization

The choice of a polymeric structure, to be applied as a biomaterial for medical applications, is regulated by a number of physicochemical requirements. In this context, cellulose is emerging as a quite promising material, particularly BC that has shown better properties when compared with its counterpart plant cellulose (PC). In fact, evolution of the scientific, fundamental and applied, knowledge on BC will make this material even more attractive for industrial applications (Table 3) [52][54][58].

Table 3. Comparison of plant and bacterial cellulose [49]

Properties	Plant origin cellulose (PC)	Bacterial origin cellulose (BC)
Fiber width	1.4-4.0 x 10^{-2} nm	40-60 nm
Degree of polymerization	13000-14000	2000-6000
Water content	60%	98.5%
Crystallinity	56-65%	65-79%
Young's modulus	5.5-12.6 GPa	15-30 GPa

Wound dressing, artificial skin, duraplasty, nerve anastomosis, artificial blood vessels or membranes such as barrier to bone defects are some of the medical fields where BC have been tested and refined [40][59].

2.2.1. Physical and chemical characteristics

BC originates from the aggregation of molecular chains, forming different units that are dependent on the source of the material. As a result, BC is characterized by an ultrafine network structure, as compared with PC, being the thickness of the cellulose fibrils about 100 times lower than the plant-origin ones [46][54-55][60].

BC is also distinguished from PC in terms of purity, DP and crystallinity.

As mentioned before, PC is the primary constituent of plant cell wall, naturally associated with cell wall components molecules, which become impurities difficult to remove, and not present in BC [49][61].

On the other hand, the degree of polymerization (DP) of BC is generally between 2000 and 6000 reaching up to 16000 and 20000, much higher than the one observed in the plant counterpart, which varies between 13000 and 20000 [50-52][60].

Both materials are highly crystalline: while PC crystallinity index is in the range of 56-65%, BC is higher, above 60% [49-50]. As this cellulose crystalline structure is dependent on hydrogen bonds formation between polymer sheets, the reported difference might be due to the different origins of cellulose and also due to different arrangements of glicosyl units within the unit cells of crystallites. Furthermore, the producer strain and the culture medium composition are parameters that will also influence the degree of crystallinity. The former will influence the ratio of $I\alpha/I\beta$ crystalline forms and the later will influence the chain size [49-52][60].

Water retention ability is also different for both materials. BC is characterized by higher water holding capacity when compared with its widespread plant-based counterpart (60%), both in never dried state. In fact, BC is far more hydrophilic than PC, being able to hold almost 100 times its weight in water [45][51][62]. This might be due to its network structure with well separated nano and microfibrils, porosity and presence tunnels and interstitial spaces that create an extensive surface area able to sustain large amounts of water. In fact, if preservation of this porous structure is intended, freeze-drying is reported as the ideal method for drying BC [55].

BC is a low-density material, with good heat resistance. This biopolymer has the ability to withstand temperatures up to 300 °C, at which it begins to degrade. It is worth noting that an alkaline treatment of the material makes it more thermostable, rising the tolerable temperatures to a range comprised between 343 °C and 370 °C [52].

Furthermore, BC is insoluble in the vast majority of solvents, exception made for concentrated acids, including sulphuric, hydrochloric or nitric acid and strong alkali, with or without the addition of urea [51-52].

On the other hand, this cannot be considered as a biodegradable material for medical applications, unless it is associated with cellulose-degrading enzymes [63].

Other important characteristics of BC are its simple and cost-effective production, transparency, *in situ* moldability, non-toxicity and biocompatibility, showing excellent biological affinity [45][60][68-69].

2.2.2. Mechanical characteristics

Mechanical performance is a critical feature in the success of a particular device or material envisaged for implantation in the human body. It is indeed important that the material is capable of withstanding all the efforts that will be subjected throughout its lifetime, while maintaining its properties [58].

As we have seen, intra and intermolecular hydrogen bonds established between cellulose fibrils' -OH groups are of major importance on chemical stability of the material (Figure 5). These same interactions are responsible for structure rigidity and the mechanical strength characteristic of this material [46][54-56].

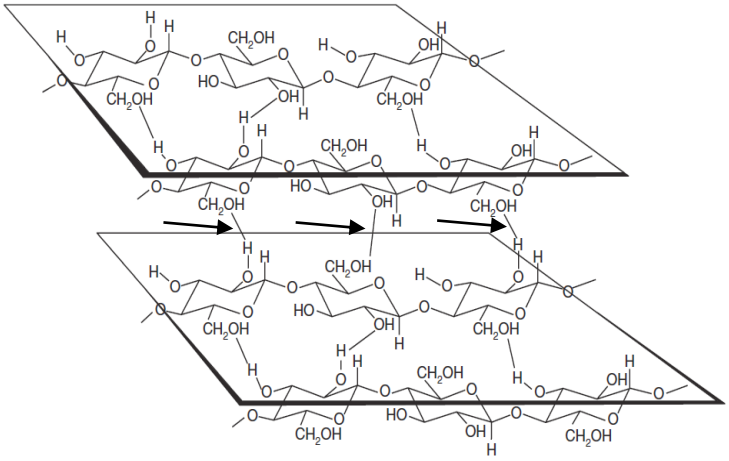


Figure 5. Molecular hydrogen bonds (indicated by arrows) established in cellulose [49].

It is reported that polysaccharides are considered materials with low strength and rigidity, which limit their applicability [58]. However, BC is characterized by an ultrafine network structure and this property alongside with its DP and crystallinity index, are also responsible for its characteristic mechanical strength [52].

Correlating all those variables, it is expected that BC presents good, if not better, mechanical results when compared to its plant homologue [49]. In fact, BC presents high mechanical properties in dry state. Although, this feature can also be tailored according to the intended application through BC-based composite materials, including the combination of polymeric and inorganic phases, which results in a material with higher stiffness and strength [49][52].

3. Biomimetic mineralization

Man tends to turn to nature for inspiration; the scientific community is no exception, studying natural phenomena in order to understand its fundamentals and apply them for the development of novel technologies. These innovative approaches, called biomimetic, are a result of the study of organization and behaviour of biological systems, aiming the improvement of methodologies, materials production and properties and create brand new solutions in order to solve technological gaps.

The search for new solutions, especially for bone regeneration, able to stimulate specific responses on tissues, have been one of the areas where biomimicry has had a strong impact, taking as inspiration mineralized tissues present in all five kingdoms of organisms [10][65].

3.1. Calcium orthophosphates

Calcium orthophosphates (Ca-P) are considered the main mineral components present in several organisms. For this reason, it has have aroused the interest of several cross-scientific fields such as geology, chemistry, biology and medicine [66].

These minerals are chemical compounds constituted essentially by calcium, phosphorus and oxygen [10]. However, they can also include hydrogen, as an acidic orthophosphate anion - HPO_4^{2-} or $H_2PO_4^-$; hydroxide, as in $Ca_{10}(PO_4)_6(OH)_2$ or water molecules, as in $CaHPO_4{\cdot}2H_2O$. On the other hand, different phosphate anions (ortho-, meta-, pyro- and poly-) as well as different numbers of hydrogen ions attached to the anion (mono-, di-, tri and tetra-) can be founded, providing a large diversity of calcium orthophosphates. According to their structural organization, calcium orthophosphates minerals can be divided into three groups, namely apatite type, glaserite type and $Ca–PO_4$ sheet-containing compounds. These are described in Table 4 [71-72].

An important feature of these minerals is their solubility, which conjugated with calcium/phosphate (Ca/P) molar ratios, allows the distinction of different mineral phases. In general, calcium orthophosphates minerals present low solubility in water, are quite soluble in acidic solutions and not soluble in alkaline solutions. In addition, there is an inverse relationship between Ca/P ratio and solubility: a low Ca/P ratio is reflected in a more acidic and more soluble phase [71-72].

A problem commonly associated with bone implants is the formation of fibrotic tissue encapsulating the implant, which hinders correct osseointegration [67]. Bioactivity, biocompatibility and osteoconductivity naturally associated to calcium orthophosphates, but also to other bioceramics

(bioactive glass, glass-ceramics), positions these materials as ideal for bone engineering and mimicry, as they are known to be able to induce migration, proliferation and differentiation of bone-forming cells and consequent formation of calcium phosphates, such as hydroxyapatite (HA), octacalcium phosphate (OCP) and dicalcium phosphate dehydrate (DCPD), in a physiological environment [6][65][68].

These compounds are available in several forms, namely porous or dense blocks, powders, granules and coatings [6].

Table 4. Different types of calcium orthophosphates [71-72]

Mineral classification	Compound/ Molecular formula	Ca/P molar ratio	Biological Occurrence Application in medicine
$Ca–PO_4$ sheet-containing compounds	Monocalcium phosphate monohydrate (MCPM) $Ca(H_2PO_4)_2·H_2O$	0.5	Never found in biological calcifications. Used in medicine as a component of self-hardening CA-P cements and added to toothpastes.
	Monocalcium phosphate anhydrous (MCPA or MCP) $Ca(H_2PO_4)_2$	0.5	Never found in biological calcifications. Not biocompatible (highly acidic). No application in medicine.
	Dicalcium phosphate dihydrate (DCPD) $CaHPO_4·2H_2O$	1.0	Found in pathological calcifications and some carious lesions. Used in Ca-P cements and added to toothpaste.
	Dicalcium phosphate anhydrous (DCPA or DCP) $CaHPO_4$	1.0	Never found in biological calcifications. Used in Ca-P cements and in toothpaste as polishing agent.
Glaserite type	α-Tricalcium phosphate (α-TCP) α-$Ca_3(PO_4)_2$	1.5	Used in Ca-P bone cements and added to some brands of toothpaste as a gentle polishing agent.
	β-Tricalcium phosphate (β-TCP) β-$Ca_3(PO_4)_2$	1.5	In pure state never occurs in biological calcifications (only the Mg-substituted is founded). Used in Ca-P cements and in toothpaste as polishing agent.
	Amorphous calcium phosphates (ACP) $Ca_xH_y(PO_4)_{z·n}·H_2O$, n = 3 - 4.5; 15 - 20% H_2O	1.2–2.2	Found in soft-tissue pathological calcifications. Used in Ca-P cements and as a filling material in dentistry.
Apatite type	Octacalcium phosphate (OCP) $Ca_8(HPO_4)_2(PO_4)_4·5H_2O$	1.33	Found in human dental and urinary calculi. Precursor phase to biological apatite. Used for implantation into bone defects.
	Calcium deficient hydroxyapatite (CDHA) $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ 0<x< 1	1.5–1.67	In pure state never occurs in biological systems (only substituted CDHA). Promising compound for industrial manufacturing of artificial bone substitutes.
	Hydroxyapatite (HA, HAp or OHAp) $Ca_{10}(PO_4)_6(OH)_2$	1.67	Pure state never occurs in biological systems. Used as a coating on orthopedic and dental implants and added to toothpaste.
	Fluorapatite (FA or FAp) $Ca_{10}(PO_4)_6F_2$	1.67	Pure state never occurs in biological systems. Minor amounts might be intentionally added to Ca-P biomaterials.
	Tetracalcium phosphate (TTCP or TetCP) $Ca_4(PO_4)_2O$	2.0	Never found in biological calcifications. Used for preparation of various self-setting Ca-P cements.

3.2. Calcium orthophosphates coatings

It is increasingly recognized the importance of an ideal balance between surface and bulk properties of an implant. Bioceramic coatings, such as those based on calcium orthophosphates, possess enormous potential for bone engineering applications and are being applied in traditional solutions used in tissue regeneration or replacement. Apart from their limited mechanical performance, chemical parameters such as composition, crystallinity, resorbability, association with drugs or cells, can be engineered to tailor the material for the final application [65].

Nowadays, numerous methods can be employed for coatings production based on calcium orthophosphates, including ion-beam sputter deposition or radiofrequency magnetron, sol-gel deposition, plasma spraying, sputter coating, dip coating sintering, chemical vapour deposition, laser deposition, laser cladding and electrochemical processes, namely electrophoretic deposition, electrocrystallization, anode oxidation and electroless coatings. Among all these, plasma spraying is one of the most popular methods [65][67][69].

However, these techniques have some drawbacks, such as high costs, use of high temperatures, limitations on the type of substrate to be coated, not effective in coating complex shapes, difficulty to control coating composition and resorbability, poor adhesion of the coating to the substrate and production of thin and non-uniforn coatings, with weak integrity [65][67].

In order to try to overcome some of these limitations, the so-called biomimetic approaches have emerged. Studied for various metals, such as alloys of titanium, tantalum and alumina, these approaches are viewed as a particularly attractive solution for heat-sensitive and polymeric materials [65][70].

These techniques are generally simple, low cost, enable the deposition of bioactive molecules onto and into porous or complex-shaped substrates for controlled release, allow control of properties such as composition, crystallinity and dissolution, as well as the deposition of coatings with good bioactivity [67][70].

In general, biomimetic routs rely on the introduction of surface functional groups, which promote the formation of an apatite layer (Figure 6) [65].

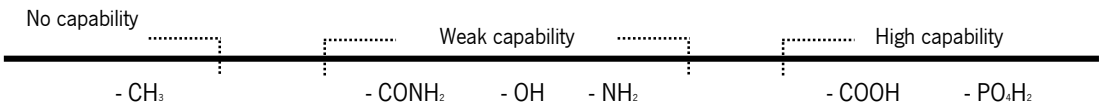


Figure 6. Hierarchy of nucleating ability provided by some functional groups [65].

Methods for chemical or physical surface modification or the addition of nucleating agents are some of the available approaches [65]. The subsequent immersion in supersaturated calcium and phosphate solutions appear to be an effective way to evaluate the effectiveness of these methods and to promote the formation of a calcium phosphate layer [65][67]. However spatial arrangement of the groups and surface morphology are important parameters that could influence the nucleation and growth of this layer [67].

The first record of biomimetic formation of a calcium orthophosphates coat involved the immersion of bioactive $CaO\text{-}SiO_2$-based glass particles in simulated body fluid (SBF). Kokubo and co-workers were responsible for the development of the SBF, a metastable solution that reproduces ion concentration, pH (≈ 7.40) and temperature (≈ 37.5°C) of human blood plasma [71]. Immersion conditions (time, temperature, agitation, renovation and solution ion concentration) influence the type of calcium phosphate formed on the substrate, being, β-tricalcium phosphate (β-TCP), calcium deficient hydroxyapatite (CDHA), OCP and HA the most common phases [67][70-71].

Besides SBF, which is the most used solution, others are also used, namely hanks' balanced salt solution (HBSS), phosphate buffer saline solution (PBS), α-modified eagle's medium (α-MEM), dulbecco's modified eagle's medium (DMEM), artificial saliva, synthetic urine and simulated milk ultrafiltrate (SMUF) [66].

3.3. BC biomimetic mineralization

Bone is a natural biocomposite, based on an organic collagen matrix reinforced with an inorganic phase of hydroxyapatite (HA) [7][16][23-24]. Following biomimetic principles, the development of biomaterials based on apatite-organic polymer composites for bone engineering or GBR is an evolving field of research [72][78-79].

For this propose several natural origin materials, such as collagen, silk fibroin, chitosan and cellulose, were studied [78-79]. In this context, BC presents itself as an auspicious and versatile biomaterial, with several advantages over its plant counterpart and other studied natural polymers [59][74-75]. However, BC's surface is rich in -OH groups, associated with weak nucleating power (Figure 6) [65]. Furthermore, some authors claim that the lack of osseointegration capability, which culminates in the non-promotion of a direct bond with bone, is one of the disadvantages of this material [59][75-76]. In order to overcome this limitation, several investigations have been and are being carried in order to introduce bioactivity on BC and induce the formation of BC and HA composites (BC-HA) tailored for bone applications [59][75]. In fact the

coating with HA is an essential characteristic to obtain a bioactive BC-based solution for bone engineering, specially GBR [81-82].

In order to produce BC-HA biocomposites, several strategies may be followed, among the available methods based on biomimetic approaches. In general, these involve surface modifications by phosphorylation or chemical treatment with calcium chloride ($CaCl_2$), strontium carbonate ($SrCO_3$), sodium hydroxide (NaOH) or polyvinylpyrrolidone (PVP) solutions, followed by treatment with SBF or modified SBF (SBF 1.5x or NaCl free SBF) and adequate drying process (freeze drying or air drying) [59][75][77-79]. Others are based on alternating cycles of contact with calcium and phosphate solutions or on the supplementation of BC pulp with HA and subsequent air drying of the mixture placed into molds [84-85]

4. Considerations

GBR is an innovative approach under development to restore correct function of bones. Non-resorbable membranes developed for this technique are traditionally synthetic. However, BC is a natural polysaccharide, which has been studied for GBR in critical and noncritical size bone defects and periodontal disease, due to its nanostructure and properties that stand out from other natural origin polymeric structures: higher mechanical properties when compared with collagen, chitosan, chitin and gelatin and null probability of cross-infection associated to animal-derived polymers [76-77][79].

Gengiflex (from BioFill company), is a BC-based membrane constituted by an internal nucleus of pure BC and a coating of alkali-modified BC which as shown promising results when applied in the treatment of class II furcation lesions (a type of periodontal disease) [80]. This BC-based membrane presented good results in *in vitro* studies. However, when compared with e-PTFE, *in vivo* evidences revealed that this was more effective in GBR applications [41][81].

An ideal balance between surface and bulk properties of an implant are imperative in its success under biological environment. Bioceramic coatings, such as those based on calcium orthophosphates, are considered ideal for bone engineering due to similarity to inorganic bone phase as well as bioactivity, biocompatibility and osteoconductivity [65]. These coatings could be produced applying several approaches, where biomimetic principles are included [65][67][74-75].

BC is characterized as a material with a lack on osseointegration capability and a coating with HA is mentioned as an essential characteristic to obtain a bioactive BC-based solution for bone engineering, especially GBR [59][76][81-82]**.** According to Saska ans co-workers, BC-HA composites are considered as attractive solutions for bone regeneration, demonstrating that on bone defects of rat tibiae this composite accelerated bone formation and bone defect correction [79].

In the present work, preparation and characterization of BC-HA composites through a biomimetic approach is proposed, involving introduction of surface functional groups via chemical modification and subsequent immersion in supersaturated calcium and phosphate solution, in order to develop a material with attractive characteristics for the envisaged GBR applications, able to act both as a barrier material and as a promoter of bone regeneration.

B. Aims

Development of bacterial cellulose membranes coated with hydroxyapatite for bone regeneration

Aims

In the present work, the main goal is fixed on the development of BC-based membranes, coated with HA, to be used as barrier membranes for GBR applications. BC membranes were modified to introduce and optimize bioactive properties, particularly as substrates for HA formation, following a biomimetic approach. In this sense, these membranes serve a dual objective: act both as a barrier material and as a promoter of bone regeneration.

To achieve this goal, the research work herein described was performed in three phases, each one with specific purposes (Figure 7):

- Phase 1: two types of BC membranes (a commercial solution and a membrane produced in-house) were prepared and subjected to three different drying processes. In this sense, this phase is focused on BC production and preparation of both materials for subsequent assays.
- Phase 2: Design and optimization of the biomimetic mineralization process. The two types of BC, the effect of different drying processes and changes in parameters associated with mineralization protocol were studied in order to assess their influence on the production of an HA coating on BC and select a range of parameters that indicate the production of optimized mineralized membranes.
- Phase 3: Involves further characterization of a selected set of membranes.

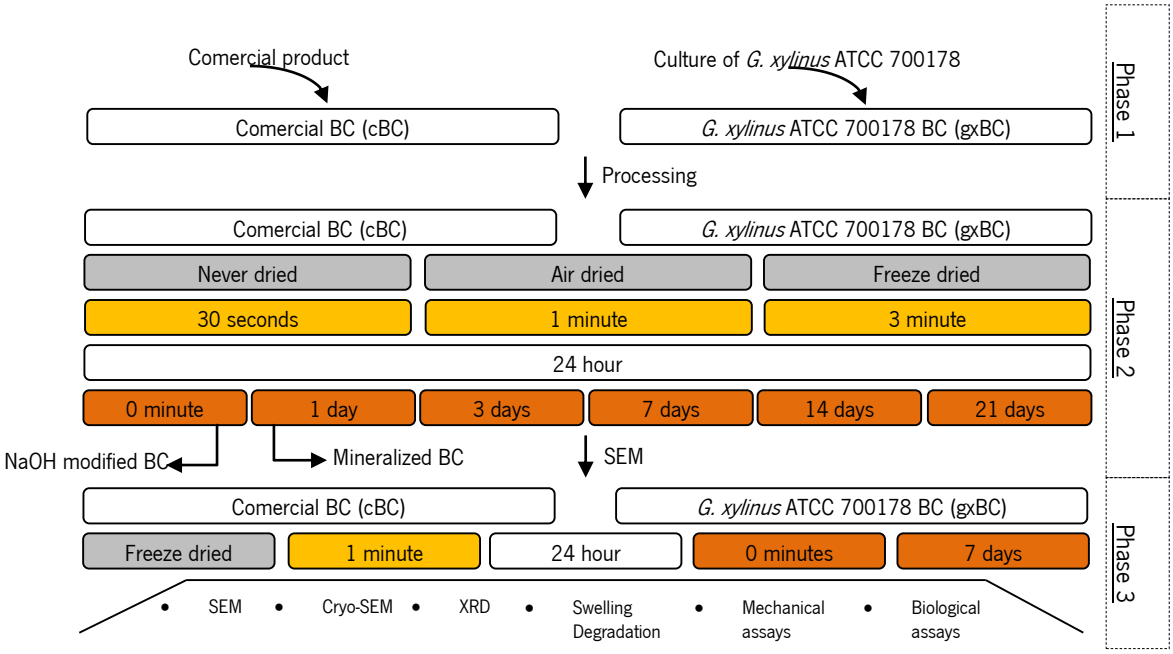


Figure 7. Work flow (colors identify the variables over which choices were made).

The presented work plan merges both fundamental and applied research. In fact, while BC membranes are produced aiming GBR applications, the influence of essential parameters will be assessed, including cellulose bacterial origin, drying methods prior to envisaged modifications and mineralization protocol.

C. Materials and methods

Development of bacterial cellulose membranes coated with hydroxyapatite for bone regeneration

Materials and methods

The present chapter aims to provide a description of both materials and methodologies employed to accomplish the proposed objectives.

1. Commercial BC

1.1. Commercial BC origin

Commercial BC (cBC) was imported as grade 2 raw *nata de coco* from HTK Food CO. Ltd (Vietnam).

1.2. Commercial BC purification

cBC was further purified following a laboratory routine protocol. In summary, BC sheets were sliced into thin membranes, which were subsequently washed. In a first step, membranes were subjected to alkali treatment with NaOH 1M (Fisher Chemical), for 1 day (renewed every 12 hour), under gentle stirring, at room temperature (RT). Maintaining environmental conditions of temperature and agitation, alkali was removed and substituted for distilled water (dH_2O). pH was monitored and several changes of water were made until the washing solution reached neutrality. Finally, membranes were sterilized by autoclaving at 121 °C, 1 bar for 20 minutes and stored in dH_2O with ethanol, 60% v/v, at RT until utilization.

2. *Glucanocetobacter xylinus* ATCC 700178 BC

2.1. Microorganism strain

In-house production of BC was performed using *G. xylinus* ATCC 700178 (gxBC).

2.2. Culture media

For gxBC production and bacteria maintenance, HS media was used in broth and solid state, respectively. The constitution of the medium is as follows: glucose (2% w/v, Fluka), peptone (0.5% w/v, Himedia), yeast extract (0.5% w/v, Himedia), sodium phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) (0.338% w/v, Sigma – Aldrich) and citric acid (0.15% w/v, Pronalab). For solid medium production, agar was added (2% w/v, Himedia) to the HS medium.

Both media were prepared in dH_2O, pH adjusted to 5.5 with hydrochloric acid (HCl 1M) or NaOH 1M and sterilized by autoclaving at 121 °C, 1 bar for 20 minute. The broth media was stored at RT and the solid one at 4 °C.

2.3. Microrganism mantainence

G. xylinus ATCC 700178 was maintained in HS solid medium and cultures were refreshed every month. A bacteria colony grown in solid HS medium was subcultured with the help of a loop into a new solid medium, followed by incubation at 30 °C and dark environment for two days for bacteria growth. Grown culture was then stored at 4 °C.

2.4. gxBC production

For preparation of *G. xylinus* ATCC 700178 inoculum for gxBC production, a preceding stage of pre-inoculum preparation was performed.

Under aseptic conditions, bacteria grown on HS solid medium was cultivated, with the help of a loop, in 40 mL of HS broth in 100 mL flasks, for 3 days, at 30 °C, under static conditions and dark environment. Then, and again under sterile conditions, 150 mL of fresh HS broth were transferred to a new 500 ml flask and 15 mL of pre-inoculum was added (1/10 dilution). The culture was maintained for 7 days, at 30 °C, under static conditions and dark environment.

2.5. gxBC purification

After 7 days of fermentation, the obtained BC pellicles formed at the air/liquid interface were harvested and purified.

The first step involved autoclaving at 121 °C, 1 bar for 20 minute. Afterwards, and as described for cBC, gxBC membranes were subjected to alkali treatment with NaOH 1M, for 1 day (renewed every 12h), under gentle stirring and at RT. Under the same conditions of temperature and agitation, alkali solution was replaced by dH_2O. pH was monitored and several changes of water were made until the washing solution reached neutrality. Finally, membranes were sterilized by autoclaving at 121 °C, 1 bar for 20 minutes and stored in dH_2O with ethanol, 60% v/v, at RT until utilization.

3. BC processing

In order to achieve the defined objectives, work plan was divided into three phases. For the last two phases, membranes were processed and cut into different geometries, according to the diverse envisaged characterization tests and submitted to different drying processes.

In phase 2, discs with 5 mm of diameter and approximately 100 µm of thickness (in highly hydrated state) were subjected to three different drying methods: never-dried, freeze dried and air-dried.

Never dried membranes, as the name implies, were used on their hydrated state, without drying. These samples were stored in dH_2O and ethanol 60% v/v until utilization.

Freeze-drying was performed on a Scanvac CoolSafe VP100 (Labogene), for 2 days, at 0.014 hPa and -100 °C. Freeze-dried membranes were stored in a desiccator until use.

Air-dried membranes were dried at 37 °C for 2 days and stored in a desiccator until use.

For phase 3, discs with 5 mm and 13 mm, and approximately 100 µm of thickness (in highly hydrated state), were used, in addition to rectangular strips with 12-20 mm in length, 3-7 mm in width and variable thicknesses (detailed in section 5.4. Mechanical assays). Samples studied during phase 3 included both types of cellulose (cBC and gxBC), freeze-dried and modified according to the selected method (detailed in section 4. BC mineralization).

Mineralization was performed on the material in its final form, being the strips the only exception (mineralization of BC sheets and cut after material's drying). In the case of disc membranes, they were cut before mineralization and drying procedures and washed with dH_2O to remove residues of the process.

4. BC mineralization

The mineralization protocol proposed by Kwak and co-workers, based on chemical surface modifications, was herein adopted with some modifications [77].

First, BC membranes subjected to drying procedures were pre-rehydrated with dH_2O, at RT, for 3 days and gentle stirring (60 rpm). These were placed in falcons with a ratio of 1 mL dH_2O/1 membrane with 5 mm, 5 mL dH_2O/1 membrane with 13 mm or 50 mL dH_2O/BC sheet. These same ratios were used for the following treatments.

Both hydrated and pre-rehydrated membranes were then subjected to the mineralization protocol. These were soaked in NaOH 3M solution for different time frames: 30 seconds, 1 and 3 minutes. Immediately after alkali treatment, membranes were immersed in modified NaCl-free SBF for 1 day, followed by immersion in SBF solution for 0, 1, 3, 7, 14 and 21 days. Conditions of immersion, both in NaCl-free SBF and SBF, were the same: 37 °C and 60 rpm. In the specific case of SBF, supersaturation conditions and solution's pH were ensured by periodic replacement with fresh

solution every 3 days. After the defined soaking time, samples were taken out from the solution, washed twice with dH_2O, and dried at 37 °C for 3 days.

For phase 3, the selected mineralization protocol was applied, with the following conditions: 1 minute of contact with NaOH 3M, 1 day immersion in NaCl-free SBF and 0 and 7 days of contact with SBF.

Mineralization of BC membranes resulted in three sample groups described as follows:

- Native BC: samples were air dried after pre-rehydration step and, consequently, never submitted to mineralization process.
- NaOH modified BC: membranes submitted to part of the mineralization protocol, stopped after contact with NaCl-free SBF and air-dried.
- Mineralized HA: membranes submitted to the complete mineralization protocol and air-dried.

For analyzes performed on phase 2 and 3, native BC and NaOH modified BC were used as controls for SEM, cryo-SEM, EDS, XRD, water uptake, degradation and mechanical assays, and native BC membrane cultured without cells was also used as additional control for cellular assays *in vitro*.

4.1. Production of SBF and NaCl-free SBF

SBF solutions are highly saturated with respect to apatite, so in order to prevent precipitation of this phase a careful preparation must be performed [71].

Both SBF and NaCl-free SBF were prepared following indications provided by Kokubo and Takadama [71]. Powder reagent grade chemicals and dH_2O were used and Table 5 lists all the reagents, respective amounts and order of addition. Quoted values refer to the preparation of 1 L of solution [71].

As solutions were prepared and stored in glass materials, care was made to use materials in perfect conditions to prevent unwanted formation of precipitates.

Table 5. Reagents for SBF and NaCl-free SBF preparation: order of addition and quantity [71]

Sequence	Reagent		Amount	
			SBF	NaCl-free SBF
1	NaCl	Chem-Lab (99.8%)	8.035 g	–
2	$NaHCO_3$	Sigma - Aldrich (99.7%)	0.355 g	0.355 g
3	KCl	Riedel - de Haën (99.5%)	0.225 g	0.225 g
4	$K_2HPO_3 \cdot 3H_2O$	Merck (99%)	0.231 g	0.231 g
5	$MgCl_2 \cdot 6H_2O$	Merck (99.0%)	0.311 g	0.311 g
6	HCl 1M	Sigma – Aldrich (37%)	37.5 mL	37.5 mL
7	$CaCl_2 \cdot 2H_2O$	Riedel - de Haën (99%)	0.388 g	0.388 g
8	Na_2SO_4	Pronalab (99%)	0.072 g	0.072 g
9	Tris	Sigma – Aldrich (99.8%)	6.118 g	6.118 g
10	HCl 1M	Sigma – Aldrich (37%)	0-5 mL	0-5 mL

SBF-based solutions were prepared at 36.5 °C±1.5 °C, with constant monitoring of the temperature, and gentle stirring. Monitoring of pH of the solutions begun before adding reagent 9. At this stage it should be approximately 2.0±1.0. During addition of reagent 9 this value should increase slowly and in a controlled way, so this reagent should be added carefully and slowly, making sure that previously added mass is completely dissolved and the pH stabilized before continuing adding this reagent. When pH is around 7.45±0.01, reagent 10 is added to decrease pH to 7.42±0.01 and to ensure complete dissolution of reagent 9. This step was repeated as many times as necessary, until the total mass of Tris had been added. Finally, pH is brought to pH 7.40 (with reagent 10), and volume adjusted to 1 L. Final solution is allowed to cool to RT and stored at 4 °C. Prepared solution is valid for 1 month.

5. Material characterization

5.1. Scanning electron microscopy and energy-dispersive x-ray spectroscopy

Scanning electron microscopy (SEM) is a technique that enables the study of surface morphology and microstructure of dried samples.

In phase 2, SEM was used to evaluate these parameters on the produced membranes and follow the formation of HA layer. The presence and morphology of the HA layers was the definite parameter to choose and define the mineralization conditions employed to produce BC-HA composites to be further characterized.

Discs with 5mm of diameter were cut in half, to allow a comparative analysis of both sides of the membranes, and sputtered with a thin layer of Au/Pd (60 seconds and 15 mA), using Polaron SC502 equipment (Fisons Instruments). Subsequently, samples were examined using a Leica Cambridge S360, at an accelerating voltage of 15 kV.

In phase 3, SEM analysis was complemented with energy-dispersive x-ray spectroscopy (EDS) technique, to determine the elementary composition of the prepared materials.

The exams were performed using a high resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis, Quanta 400 FEG ESEM/EDAX Genesis X4M, at an accelerating voltage of 15 kV. Membranes were also sputtered with a thin film of Au/Pd (40 seconds and 15 mA current), using the SPI Module Sputter Coater equipment (SPI supplies).

5.2. Cryo-scanning electron microscopy

Bulk morphology and microstructure of selected membranes were evaluated by cryo-scanning electron microscopy (cryo-SEM), using wet state 5mm discs (not submitted to the final drying process performed after the mineralization protocol). For this analysis, samples were stored at -20 °C until analysis and were allowed to thaw at RT for 1 hour prior to preparation and visualization.

Samples were frozen in slush nitrogen, fractured, sublimated ('etched') for 180 seconds at -90 °C, coated with Au/Pd by sputtering (40 seconds and 12 mA current) and then visualized at a JEOL JSM 6301F/Oxford INCA Energy 350/ Gatan Alto 2500 cryo-SEM, at a temperature of -150 °C and an accelerating voltage of 15 kV.

5.3. X-ray diffraction

Bulk characterization and crystalline phases identification were evaluated by x-ray diffraction (XRD).

XRD patterns were collected at RT using a Philips PW-1710 X-ray diffractometer system (Philips Analytical), attached to X'pert software (Philips Analytical). The operational conditions were monochromatized Kα radiation, at 20 mA and 40 kV, with a scan rate of 2 seconds/step and over a Bragg angle range of $0\ ^\circ<2\theta<58\ ^\circ$.

5.4. Mechanical assays

Young's modulus (E), fracture strength (σ_f) and strain-at-failure (ε_f) where the mechanical properties evaluated. For that, rectangular strips (12-20 mm in length, 3-7 mm in width) were used both in dry and wet state. Membranes tested in wet state were soaked in SBF immediately before the beginning of the assay.

Thickness of the strips was measured using a DUALSCOPE® MPOR (Fischer) and it varies depending on the type BC (cBC or gxBC), type of treatment (native BC, NaOH modified BC, mineralized BC) and drying state (dried or wet). Average values are presented in Table 6.

Table 6. Thickness average values of tested samples

	cBC		gxBC	
	Dry (µm)	Wet (µm)	Dry (µm)	Wet (µm)
Native BC	3.5±0.3	6.5±0.1	0.8±0.2	7.5±0.0
NaOH modified BC	7.8±2.5	11.4±0.1	1.8±0.6	8.4±0.3
Mineralized BC	8.9±2.3	22.3±0.2	1.5±0.3	9.2±0.7

Three specimens were tested for each condition (reported values are the average of those results). Tensile testing was performed on a SHIMADZU AG-IC (Shimadzu Universal Testing Machines), using a load cell of 50 N, medium gauge length of 15 mm and tensile force applied at an extension rate of 0.5 mm/minute. Values for force and stroke were recorded and used to calculate and define stress-strain patterns, using equation 1 (Eq. (1)) and equation 2 (Eq. (2)).

$$Eq.(1): Stess\ (\sigma) = \frac{force}{original\ area}$$

$$Eq.(2): Strain\ (\varepsilon) = \frac{final\ length - original\ length}{original\ length}$$

The materials can be ductile supporting large strains before failure or brittle, with low yield strength (Figure 8 (B)). On a standard curve, two types of behaviour/deformation can be present: elastic (comprehending the first linear zone of the plot) and plastic (when the elastic limit is exceeded) (Figure 8 (A)) [82]. These stress-strain curves are the basis for the determination of the envisaged mechanical parameters.

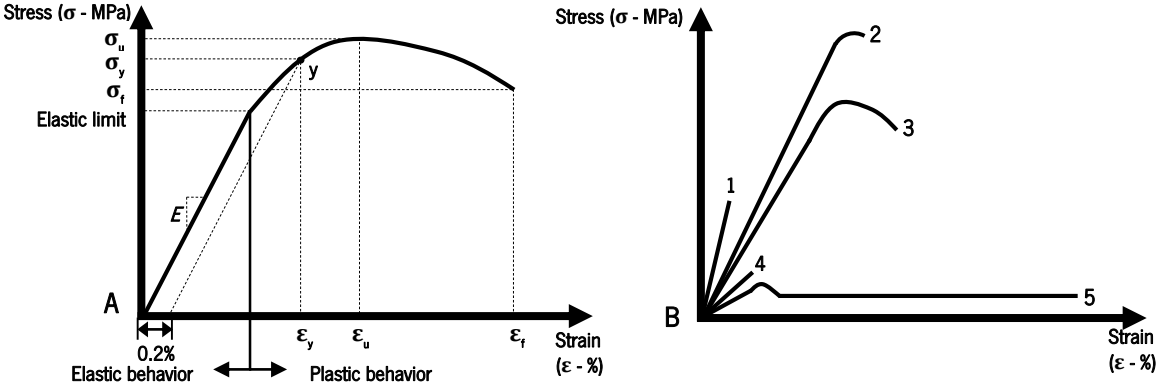


Figure 8. Schematic tensile stress-strain curve. A: Elastic and plastic behavior (zones) until fracture and main mechanical parameters. B: ductile vs brittle materials (1-brittle ceramic; 2- brittle metal; 3- ductile metal; 4- brittle polymer; 5- ductile polymer) [114-115].

5.5. Water uptake and degradation assay

Both water uptake and degradation can be easily accessed by mass based methodologies. Three specimens with 13 mm diameter were tested for each condition and weight measurements were performed in triplicate per sample (the values reported are the average of those results) using ae ADA 180 balance (ae ADAM).

At the beginning of the assay, all discs were weighted and results registered as W_i. Each sample was placed individually on the bottom of 24-well plates and 2.5 mL of PBS 1x (pH 7.40) was added to each well. The plates were incubated at 37 °C, under mild stirring (60 rpm) for different time points (1, 3, 7, 14 and 21 days). The solution was renewed every 3 days.

At each time point, samples were collected, excess liquid was gently removed with tracing paper and the wet weight was recorded immediately (W_w). These same discs were allowed to dry, at 37 °C for 2 days, and weighed once again (W_d).

The water uptake (or ratio of swelling) and the degradation (or rate of weight lost) were determined using equation 3 (Eq. (3)) and equation 4 (Eq. (4)) respectively:

$$Eq.(3): Water\ uptake\ (\%) = \frac{(W_w - W_i)}{W_w} x\ 100$$

$$Eq.(4): Weight\ loss\ (\%) = \frac{(W_i - W_d)}{W_i} x\ 100$$

5.6. Cellular assays *in vitro*

In order to evaluate the effect of a certain material or molecule over a specific target cell type, *in vitro* assays using cell lines are usually performed. In this work, cytotoxicity of materials through assessment of metabolic activity and cell viability were evaluated by MTS and Live/Dead assays, respectively.

5.6.1. Material preparation

In these assays, discs with 5 mm of diameter were used. The processing and mineralization procedures were performed as previously described (3. BC processing and 4. BC mineralization).

Samples were sterilized by ethylene oxide; sterilization was complimentary of Pronefro (Maia, Portugal).

Prior to cellular testing, samples were pre-rehydrated, in aseptic conditions, with PBS 1x (pH 7.40), for 7 days, at 37 °C, in a humidified tissue culture incubator with a 5% CO_2 atmosphere.

5.6.2. Cell lines

Two different continuous cell lines were used for these assays, namely 3T3 (ATCC ® CCL-164) mouse embryo fibroblasts and MC 3T3-E1 (C57BL/6) mouse pre-osteoblast cells. The MC 3T3-E1 cell line was kindly provided by Electroactive Smart Materials Research Group (University of Minho, Portugal).

5.6.3. Cell culture media

Cells were cultured in DMEM 4.5 g/L glucose (Sigma–Aldrich), varying added supplements according to each cell type. DMEM was supplemented with 10% bovine calf serum (CALF – Biochrom) for 3T3 culture and with 10% heat-inactivated fetal bovine serum (iFBS – Biochrom) for MC 3T3-E1 culture. In both cases, 1% of antibiotic-antimycotic solution (penicillin and streptomycin) (Gibco) was also added.

5.6.4. Culture conditions

3T3 and MC3T3-E1 cells were cultured *in vitro* in T75 tissue culture flasks containing 10 mL of culture media and incubated at 37 °C, in a humidified tissue culture incubator with a 5% CO_2 atmosphere. Medium was changed every 2-3 days.

Cells were seeded onto the prepared samples at a concentration of $15x10^3$ cells per well. In order to ensure the correct cell concentration, trypan blue exclusion test was employed (Trypan blue solution, Fluka analytical) – dead cells are coloured in blue while live cells remain clear. Plates were then incubated in the conditions previously described for different periods: 1, 3, 7, 14 and 21 days. Media were renewed every 2-3 days and cultures inspected on microscope periodically. At each time point, samples were collected and MTS and Live Dead assays were performed.

5.6.5. MTS cytotoxicity assay

In order to evaluate the metabolic activity of cells in contact with the proposed materials and subsequently ascertain on their cytotoxicity, MTS assay was performed. This test is based on a mitochondria-dependent reaction, where MTS compound (3-(4,5-dimethylthiazol-2-yl)-5(3 carboxymethoxyphenyl)-2(4-sulfofenyl)-2 H-tetrazolium) is reduced by cells into a brown formazan product, soluble in culture medium (Figure 9). This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes, only expressed by metabolically active cells.

The assay was performed using CellTiter 96® AQueous One Solution Reagent (Promega), following manufacturer's protocol. Five replicates were considered for each sample and triplicate culture experiments were performed. As MTS reagent is light sensitive, these assays were conducted in the dark.

After each time point, culture media and non-adherent cells were discarded and membranes were moved to clean wells. Following manufacturer's protocol, 100 µL of culture media and 20 µL of MTS reagent were added to each well and incubated for 3 hours at 37 °C. After the incubation time, 120 µL of the resultant solution were transferred to a new 96-well plate and optical density was measured at 490 nm using a microplate reader (Synergie HT, Bio-Tek).

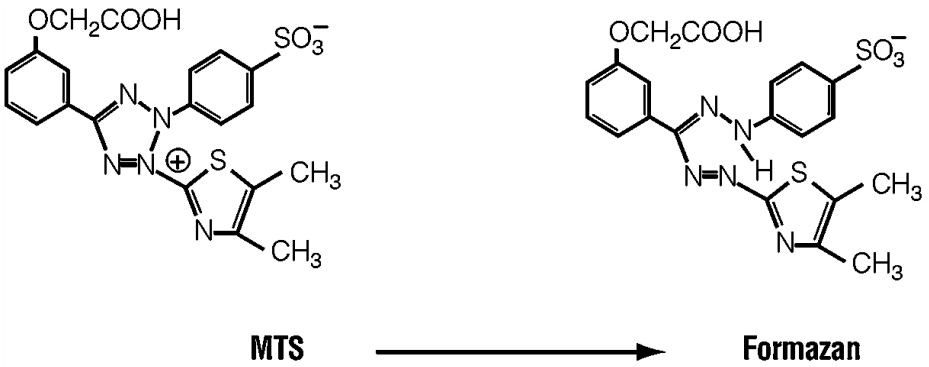


Figure 9. Reduction of MTS compound to a final formazan product (manufacturer's protocol).

5.6.6. Live/Dead assay

To determine cell viability, the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalinan Cells (Invitrogen) was used. The kit comprises two-color fluorescence probes, calcein AM and ethidium homodimer-1 (EthD-I), enabling the simultaneous determination of live and dead cells, respectively. This is achieved due to the differential physical and biochemical properties of cells, targeted by each probe: intracellular esterase activity (calcein AM) and plasma membrane integrity (EthD-I).
The assay was conducted following manufacturer's protocol and triplicate culture experiments were performed. As probes are light sensitive, tests were conducted in a dark environment.
After each time point, a fresh 1:1 mixture of calcein-AM (2 µmol/L) and ethidium homodimer-1 (2 µmol/L) was prepared. Culture media and non-adherent cells were discarded, each well was washed twice with PBS 1x (pH 7.40) and 100 µL of the prepared probe mixture were immediately added and incubated at RT for 30 minute.
Membranes were then placed on microscope slides and prepared for microscopic visualization. The materials were examined in the dark, in a fluorescence microscope (Olympus BX51) and images acquired with a colour camera Olympus DP71, using B-Cell software (Olympus).

6. Statistical treatment

GraphPad Prism statistic software (version 6.05 for MS Windows) was used to perform the statistical analysis of EDS, water uptake, weigh lost, mechanical assays and materials cytotoxicity (MTS) results.

D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov test with Dalla-Wilkinson-Lillie for P value normality tests were employed to ascertain on data normality. These tests have indicated that experimental data did not follow normal distributions and, as a result, non-parametric tests were employed for further analysis.
Non-parametric tests are characterized by high statistical robustness and by low statistical power. Although they are universally used, careful interpretation of results should be made, particularly due to the need of a large sample size in order to ensure the same degree of confidence achieved by parametric tests. In fact, the probability of to a false negative occurrence (or type 2 error) is higher: not rejection of null hypothesis when it should be disallowed [83].

Mann-Whitney and Kruskal-Wallis tests were employed and results were considered significant at $p<0.05$ (5% significance level).

Mann-Whitney test, or Wilcoxon rank sum test, is the non-parametric alternative of unpaired t-test, that is employed in comparisons between two independent groups and null hypothesis is fixed on identical distributions of both groups.[84] This test was applied for the study through direct comparisons between two groups of elemental composition between BC types, of Ca/P ratio between mineralized cBC and mineralized gxBC and of mechanical properties variations between BC type and drying states.

On another hand, statistical differences between three or more independent groups were evaluated through Kruskal-Wallis test and *a posteriori* comparisons were made through Dunn's test. This test is described as the non-parametric alternative to one-way ANOVA and an extension of Mann–Whitney test, where the null hypothesis states that samples' medians (ranks) are equal versus differences between at least two of them [84][85]. Both tests were used to study the effect of mineralization protocol on elemental compositions and mechanical properties, but also the effect of this mineralization protocol over materials water uptake, weight loss and cellular metabolic activity within each time point and over time.

D. Results

Development of bacterial cellulose membranes coated with hydroxyapatite for bone regeneration

Results

Different BC membranes - native BC, NaOH modified BC and mineralized BC - were produced, based on a simple mineralization methodology [77]. Mineralization protocol parameters, in addition to materials' pre-modification treatment (viz. drying state and/or applied drying method), were studied and optimized to obtain BC-based membranes, suitable for the envisaged applications.

Formation of a HA layer and its morphology defined the basis to select the appropriate pre-modification treatment and mineralization conditions. Prepared mineralized cellulose membranes were then further characterized. Obtained results are presented within this section.

1. Optimization of mineralization process

In order to optimize the production of mineralized BC, suitable for GBR applications, different procedural parameters were considered, assuming their importance and influence on the produced materials. In this sense, the influence of BC drying states (pre-modification treatments) and parameters associated with mineralization process (namely contact time with alkali solution and SBF) on the development of ceramic coatings on two different origins BC subtracts (cBC and gxBC) were assessed. Native BC and NaOH modified BC were used as controls.

The influence of three different drying methodologies (never dried, freeze-dried and air-dried) on the structure of both bacterial celluloses (cBC and gxBC) before and after pre-rehydration was assessed by SEM (Figure 10).

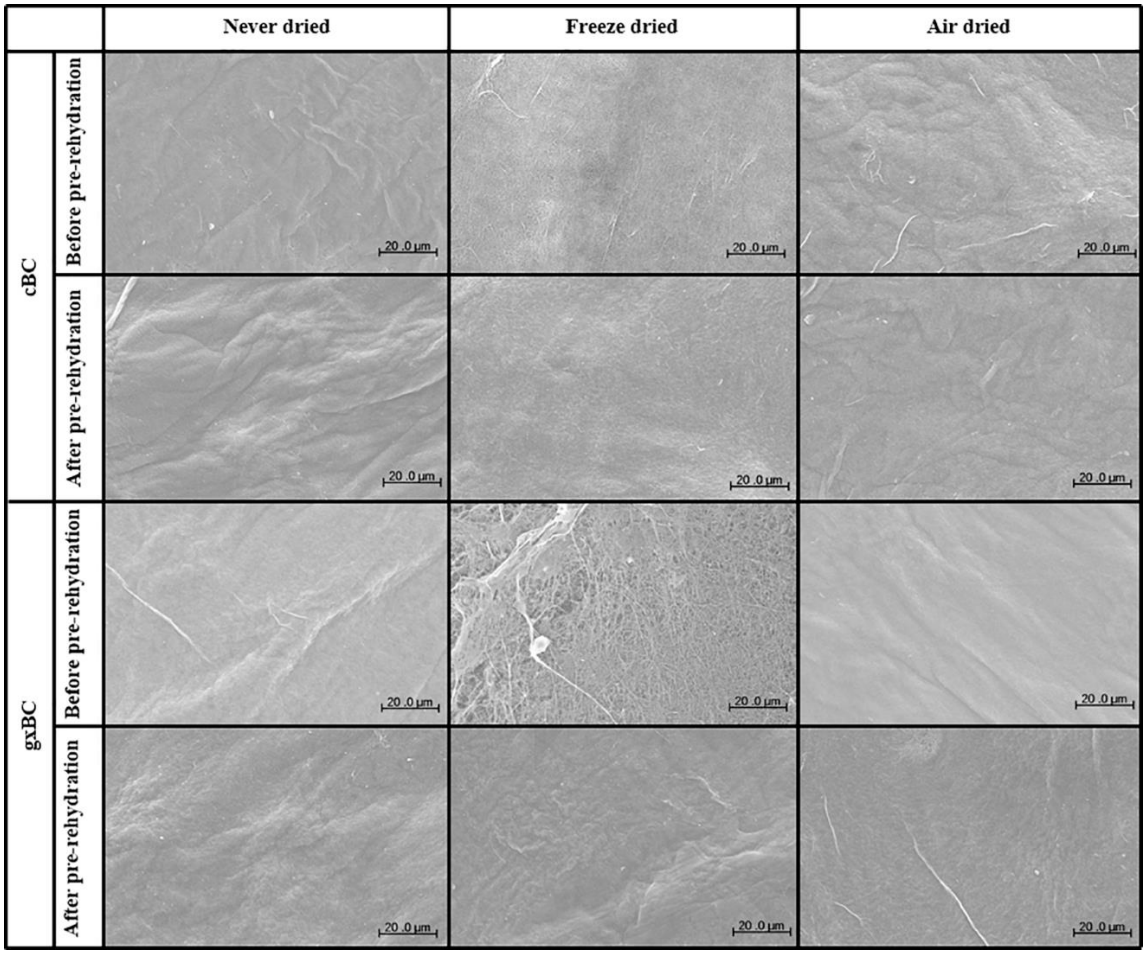


Figure 10. SEM images of native cBC and gxBC membranes submitted to different drying procedures, before and after pre-rehydration (Magnification 1.00 K x).

SEM micrographs reveal differences on membrane morphology following the diverse drying methodologies. These are particularly noticeable on freeze-dried structures, as compared to never dried and air-dried. In fact, these are quite similar.

Both cBC and gxBC air-dried membranes, prior to pre-rehydration, present a surface with slight roughness and uniformity, without distinguishable pores or fiber network. However, when freeze-drying was the employed technique, well-defined and continuous fibers, as well as a porous structure, could be observed on both BC samples. On the other hand, gxBC presented larger pores when compared to cBC.

After 3 days of pre-rehydration, observed differences appeared to be attenuated, both on cBC and gxBC. In fact, membranes appear to display the same characteristics presented by air-dried membranes prior to pre-rehydration.

Bioactivity of produced BC membranes, without chemical modification, was also studied by immersion on SBF for 21 days (Figures 11 and 12).

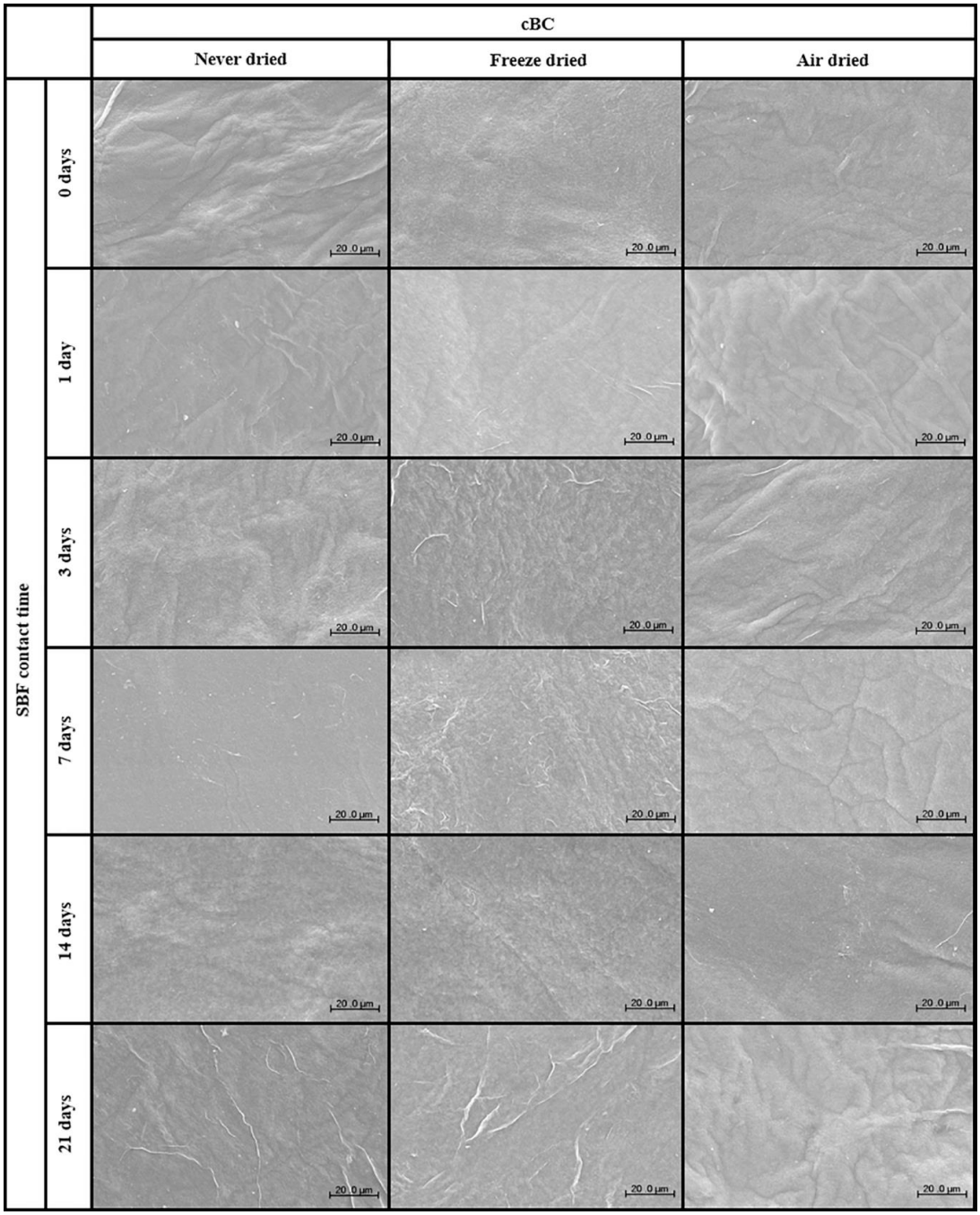


Figure 11. SEM images of native cBC membranes soaked in SBF for different time periods (magnification 1.00 K x).

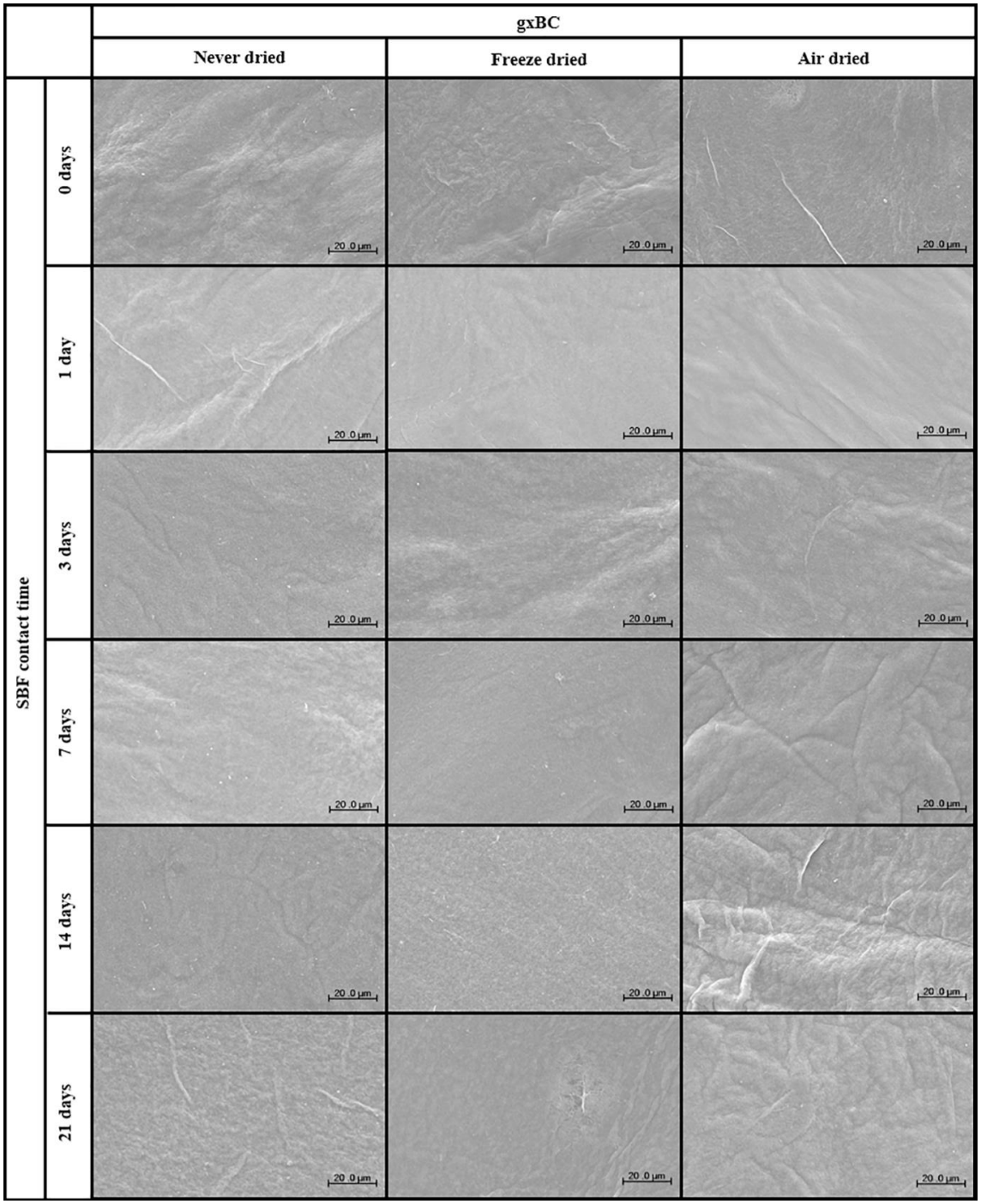


Figure 12. SEM images of native gxBC membranes soaked in SBF for different time periods (magnification 1.00 K x).

A clean surface, without formation of crystals or even a layer, was observed on produced samples, regardless BC origin or membrane drying procedure.

A different situation was noticed for BC membranes subjected to biomimetic mineralization approach (Figures 13 to 18). Furthermore, studying these membranes, it is also important to notice how BC origin, drying procedures and mineralization protocol parameters (alkali solution and SBF contact time) influence the formation of a ceramic coating on samples.

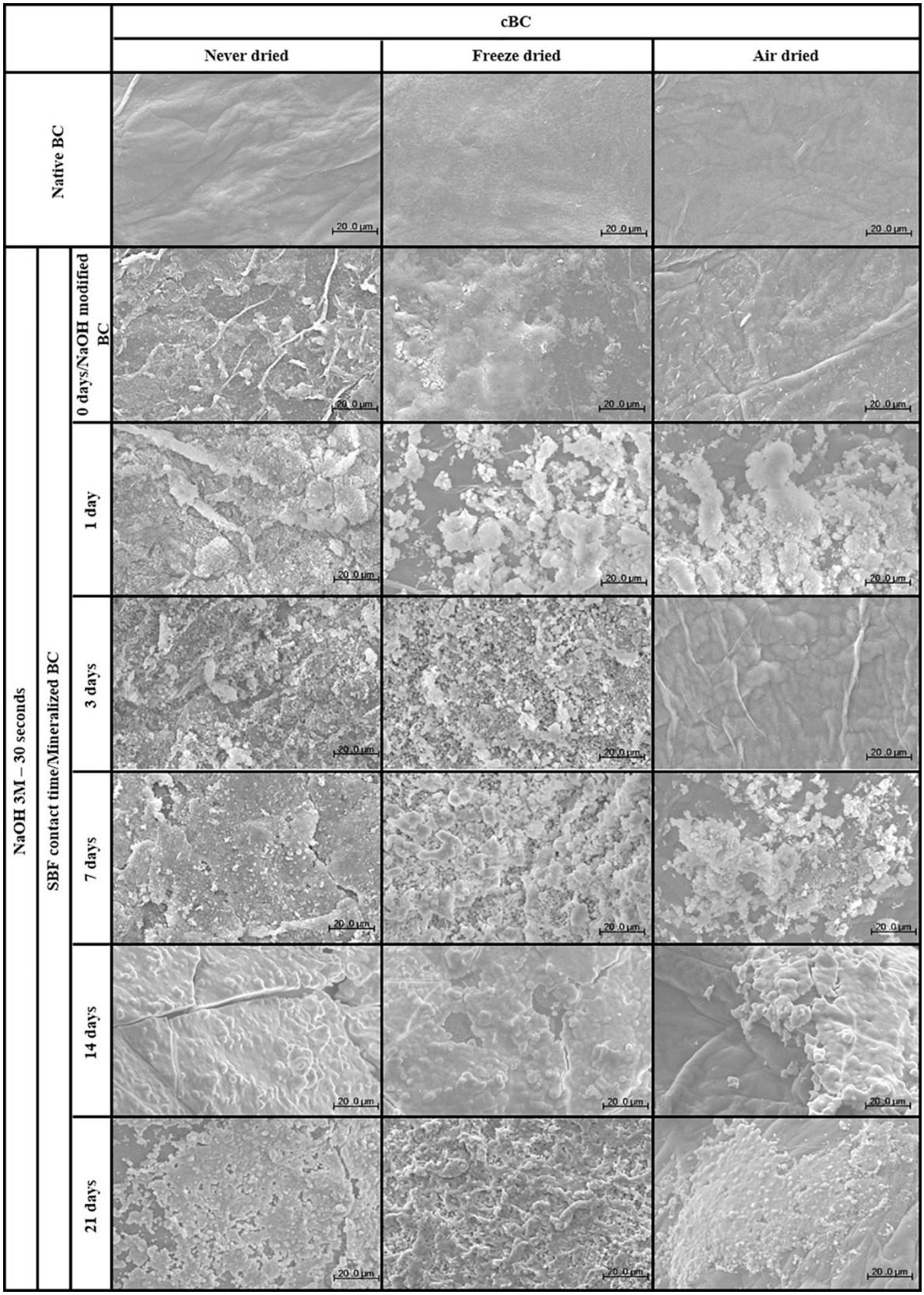


Figure 13. SEM images of NaOH modified cBC membranes (30 seconds NaOH 3M, 1 day NaCl free SBF) soaked in SBF for different time periods. Non-modified samples were only subjected to pre-rehydration and air-drying (magnification 1.00 K x).

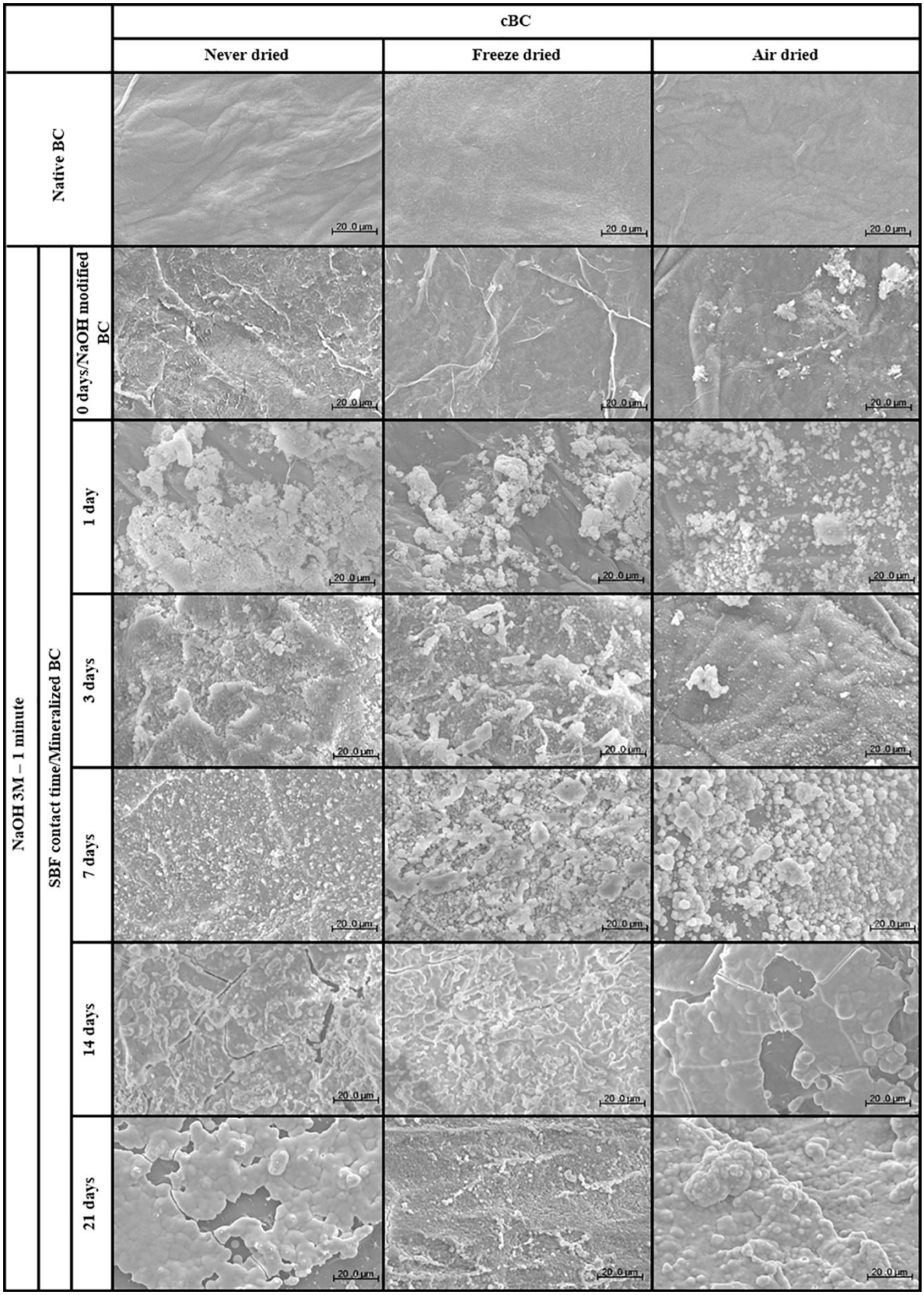


Figure 14. SEM images of NaOH modified cBC membranes (1 minute NaOH 3M, 1 day NaCl free SBF) soaked in SBF for different time periods. Non-modified samples were only subjected to pre-rehydration and air-drying (magnification 1.00 K).

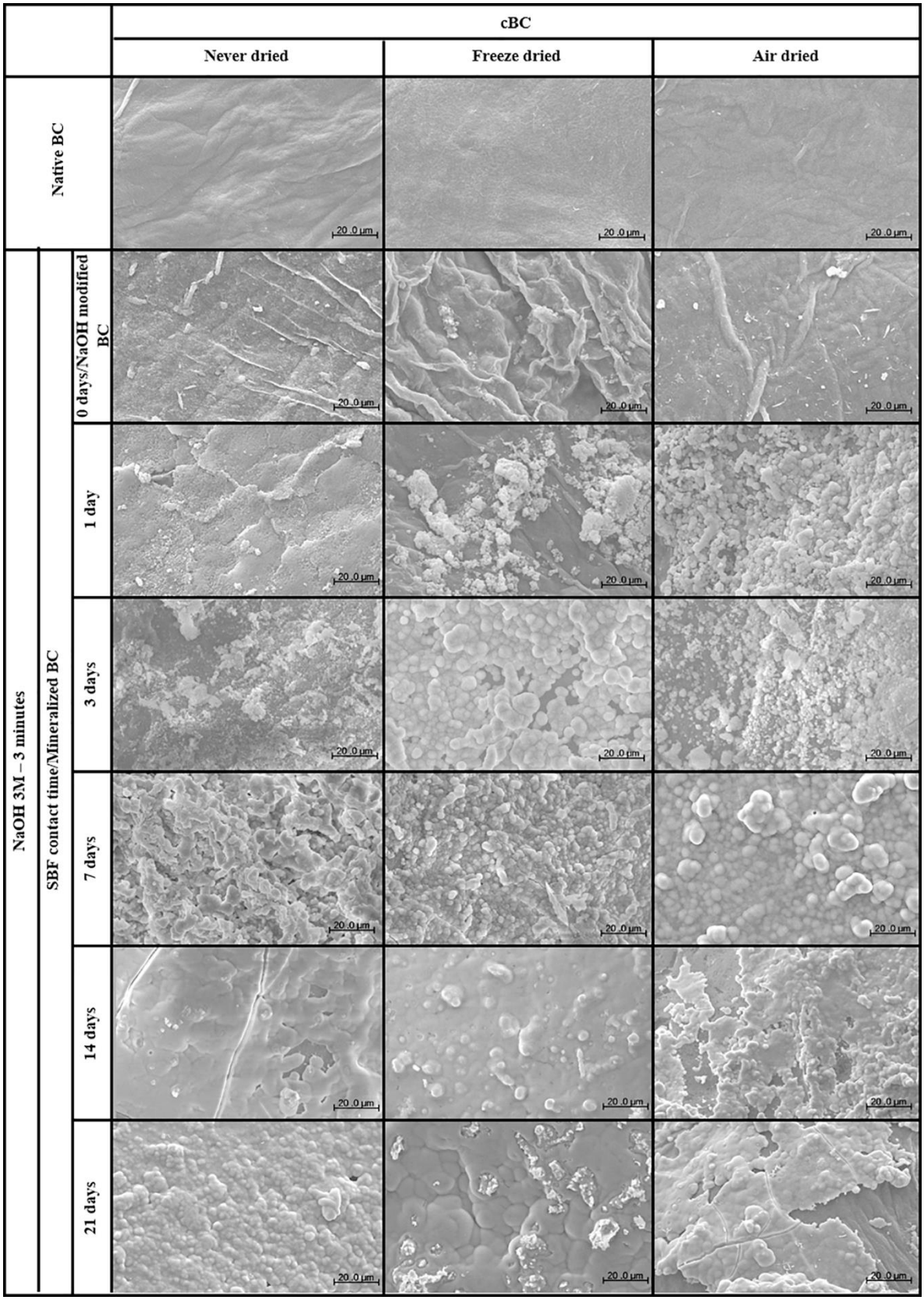


Figure 15. SEM images of NaOH modified cBC membranes (3 minute NaOH 3M, 1 day NaCl free SBF) soaked in SBF for different time periods. Native samples were only subjected to pre-rehydration and air-drying (magnification 1.00 K x).

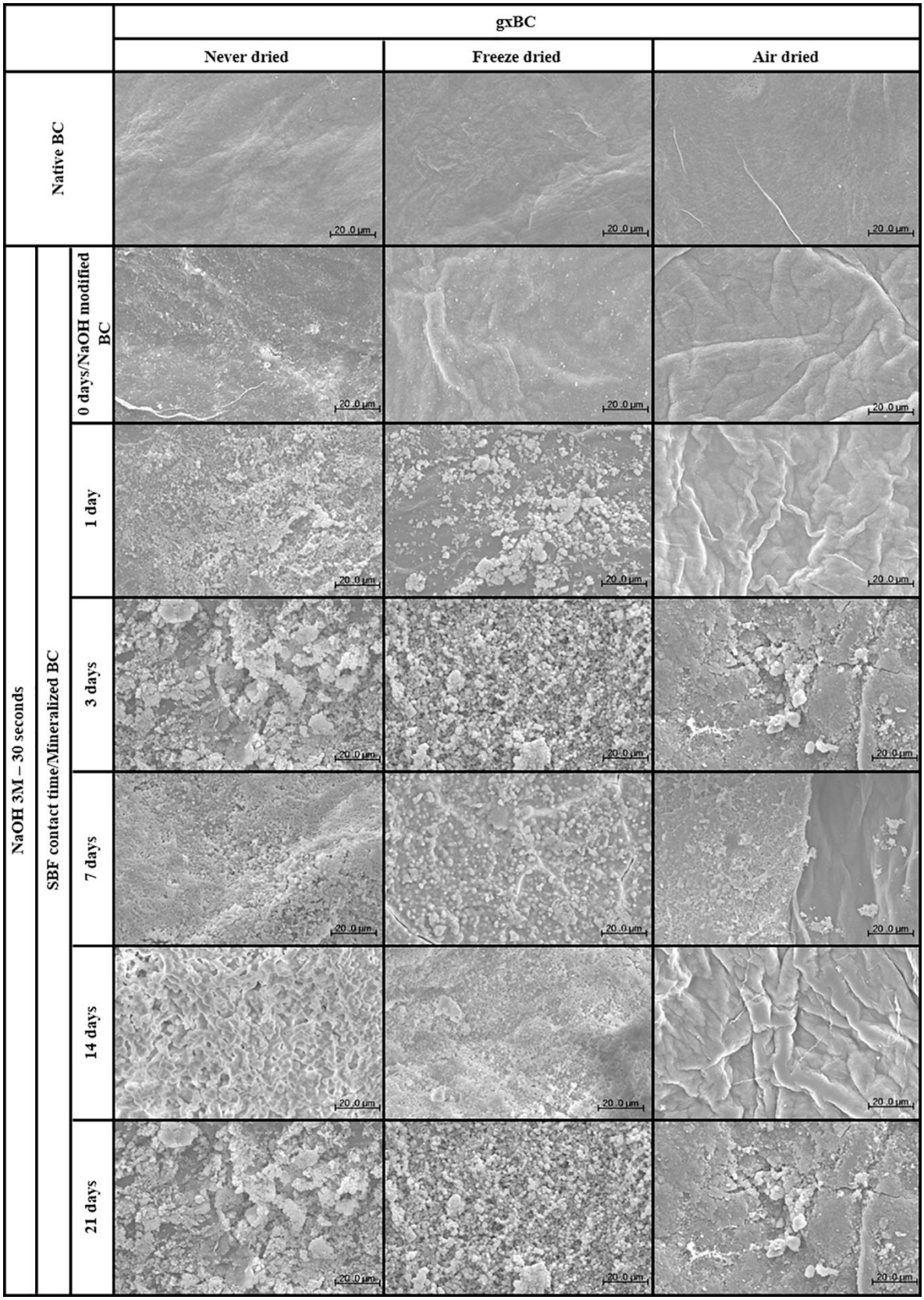


Figure 16. SEM images of NaOH modified gxBC membranes (30 seconds NaOH 3M, 1 day NaCl free SBF) soaked in SBF for different time periods. Native samples were only subjected to pre-rehydration and air-drying (magnification 1.00 K x).

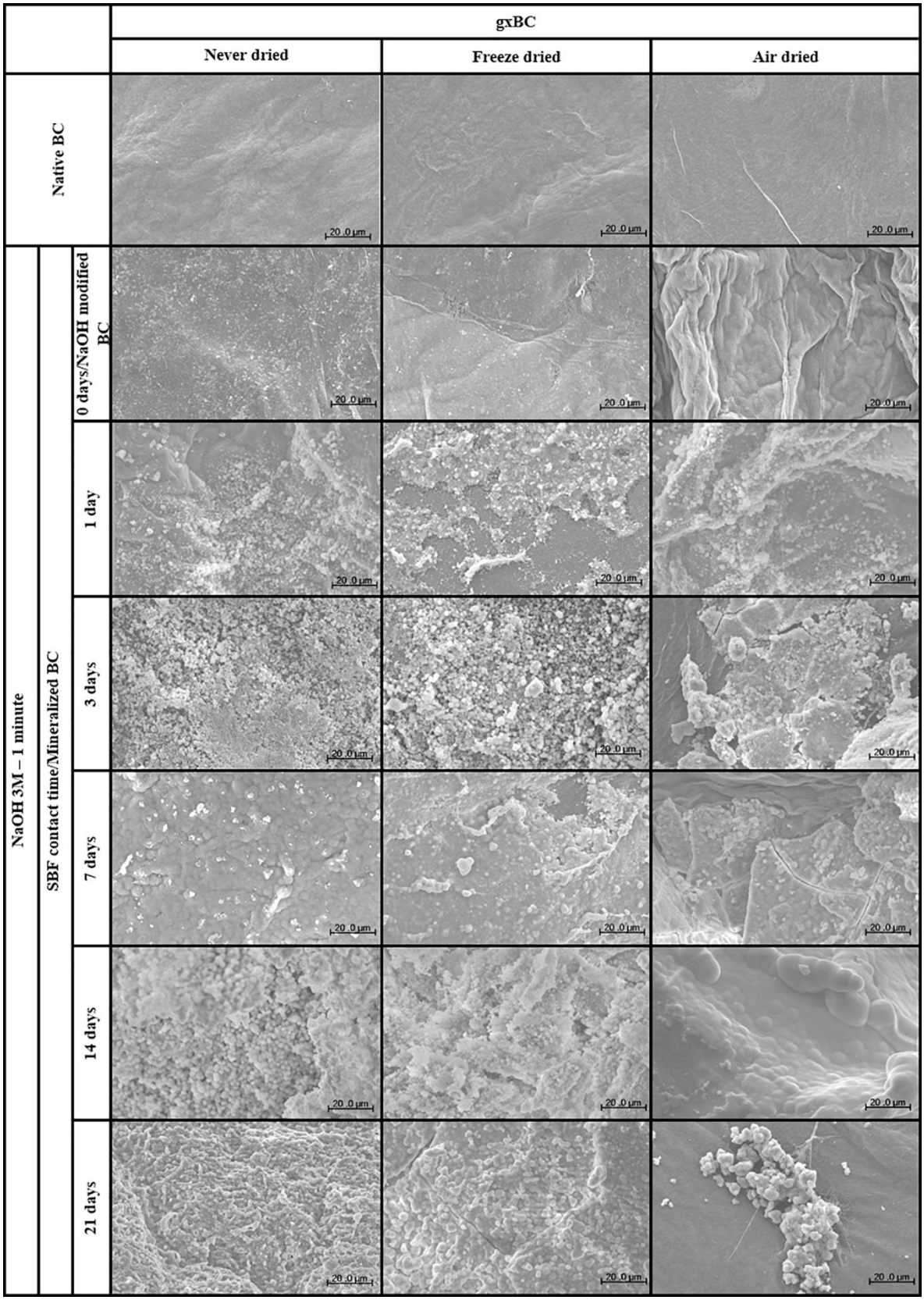


Figure 17. SEM images of NaOH modified gxBC membranes (1 minute NaOH 3M, 1 day NaCl free SBF) soaked in SBF for different time periods. Native samples were only subjected to pre-rehydration and air-drying (magnification 1.00 K x).

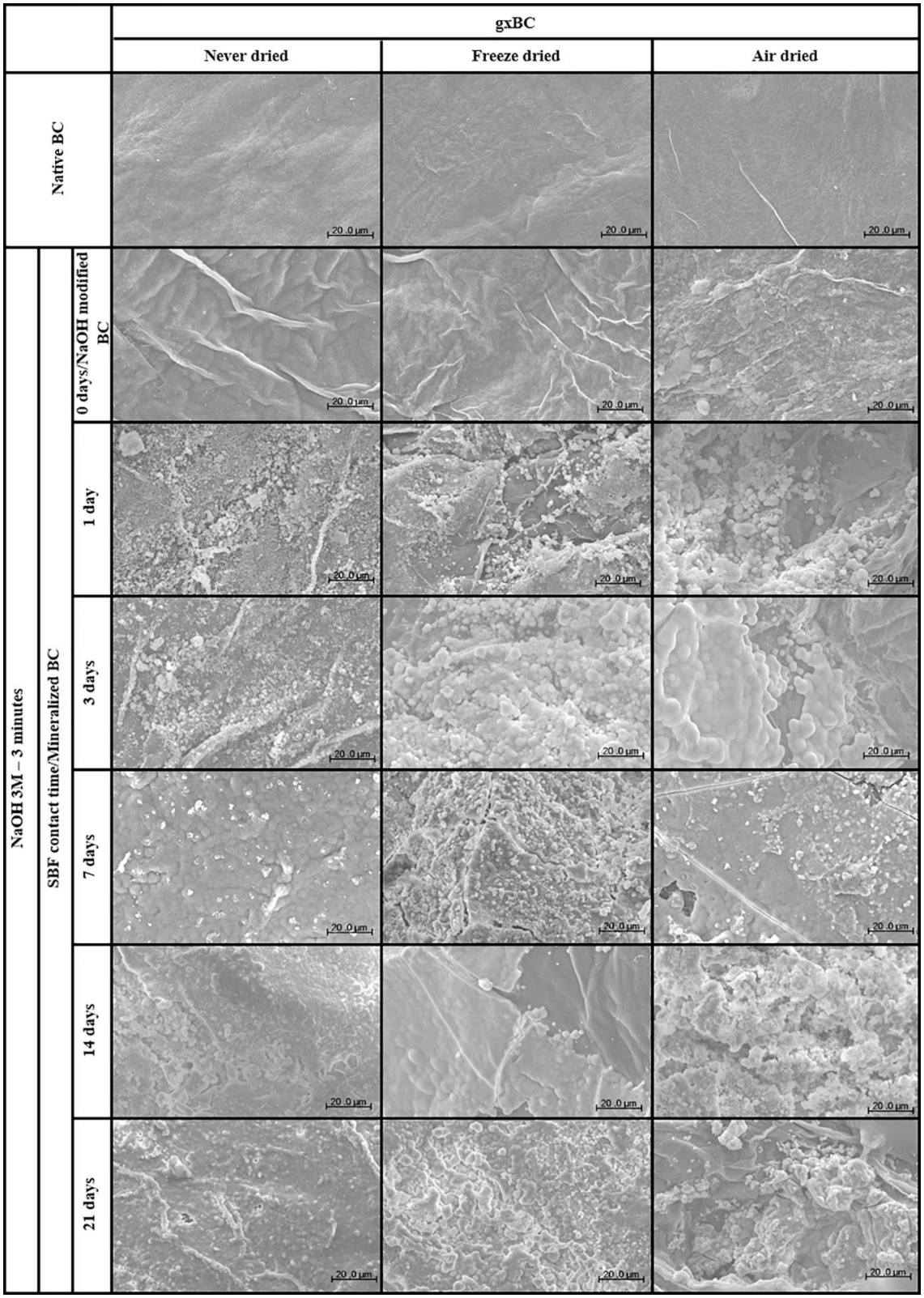


Figure 18. SEM images of NaOH modified gxBC membranes (3 minute NaOH 3M, 1 day NaCl free SBF) soaked in SBF for different time periods. Native samples were only subjected to pre-rehydration and air-drying (magnification 1.00 K x).

In some cases, it is possible to observe the presence of small particles on NaOH modified BC membranes surface, result of the BC treatment with NaOH and immersion on NaCl-free SBF.

Cauliflower-shaped crystals start to appear on the surface of the different produced BC membranes (diverse BC origin, drying procedure and alkali solution contact time) just after 1 day of immersion on SBF solution. This crystal coating increases and its structure changes with increasing SBF soaking time. After 3 days of contact with this solution, more crystals are scattered on samples' surface, which tend to gradually grow together. In some cases, a cohesive and dense layer starts to form that covers completely the surface of the BC membrane, which can be observed after 7, 14 and 21 days. As SBF contact time increases, cracks may start to appear.

The mineralization profile, over time, as well as the coating structure, varies with the different drying methodology employed to produce the BC membranes and with BC origin. On the other hand, increasing alkali contact time appears to be favourable for mineralization to occur. This is particularly evident for air-dried samples.

Sample manipulation during and after the mineralization process is an important characteristic as well.

During mineralization, cBC membranes appeared to present higher stiffness and consequently were easier to handle, when compared to gxBC. Considering drying procedures, air-dried samples were the most difficult to manipulate, followed by never dried and freeze-dried.

At the end of mineralization procedure and after final samples' air drying, membranes were more "resistant" than in never dried state and less malleable, keeping the same trend with respect to drying processes. In both situations, a longer contact time with SBF facilitated manipulation, as samples got harder. There appear to be no differences between diverse contact times with the alkali solution.

Results herein obtained allowed the definition of an optimized protocol to produce mineralized BC membranes to be proposed for GBR applications. For now on, membranes to be further characterized are produced and subjected to a mineralization protocol under the following conditions: freeze-dried membranes (cBC and gxBC), 1 minute of contact with NaOH 3M, 1 day of contact with NaCl-free SBF and 7 days of contact with SBF. Native BC, just subjected to pre-rehydration, and NaOH modified BC are used as controls. Prevalent factors involved on the selection of parameters for production of mineralized BC included morphology and homogeneity of mineral coating, methodological aspects and sample manipulation.

2. Physicochemical and biological characterization of mineralized BC membranes

Having defined a protocol to produce mineralized membranes, as specified above (freeze-dried membranes (cBC and gxBC), 1 minute of contact with NaOH 3M, 1 day of contact with NaCl-free SBF and 7 days of contact with SBF), these were further characterized.

Comparative SEM micrographs of these membranes are presented in Figure 19, highlighting their differences, particularly obvious with increasing magnification.

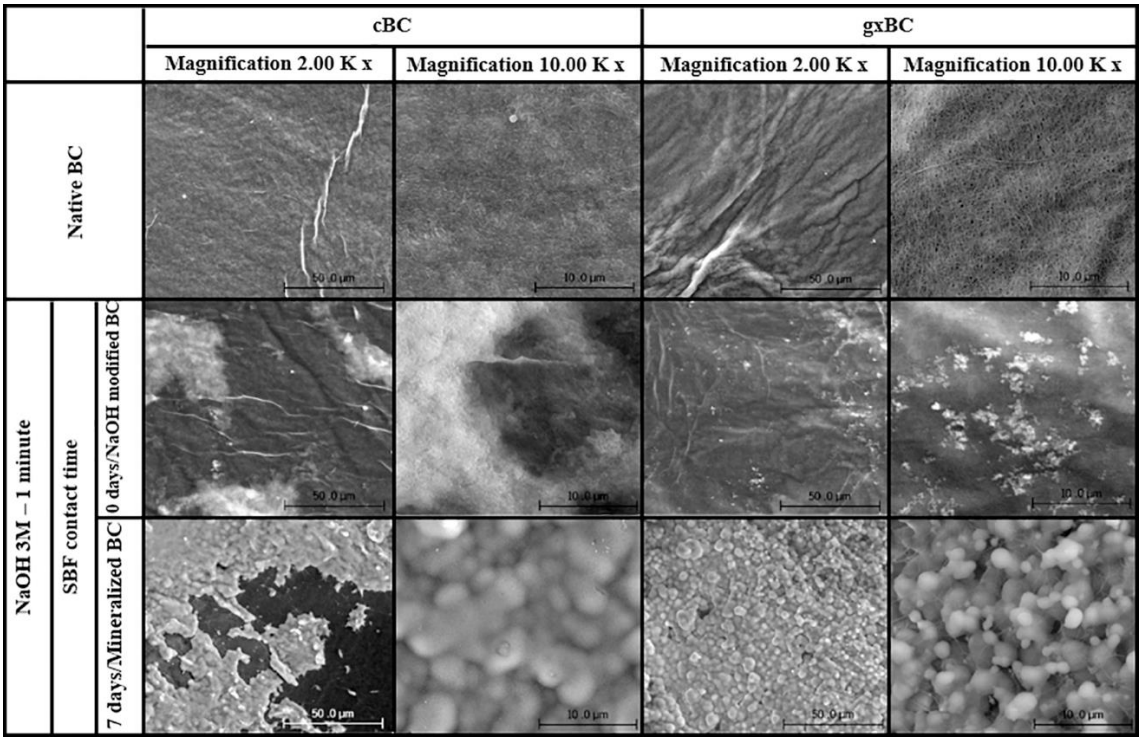


Figure 19. SEM micrographs of BC membranes produced taking into consideration optimized parameters: cBC and gxBC freeze-dried native, NaOH modified and mineralized.

Different bacterial cellulose origins result in diverse membrane surface topography; therefore, their modification is expected to result also in membranes with diverse characteristics.

In fact, gxBC demonstrates a fibrillated and porous morphology, as compared to cBC. Chemical modification appears to affect this structure and subsequent mineralization of both cBC and gxBC membranes results in different surface structures.

In order to access intern morphology and microstructure, as well as confirm if bulk mineralization occurred on the produced membranes, cryo-SEM was performed. This is an effective imaging technique employed at the study of highly hydrated samples, enabling the acquisition of morphological accurate images of materials on their almost natural state.[86] Cross-section micrographs are presented in Figure 20.

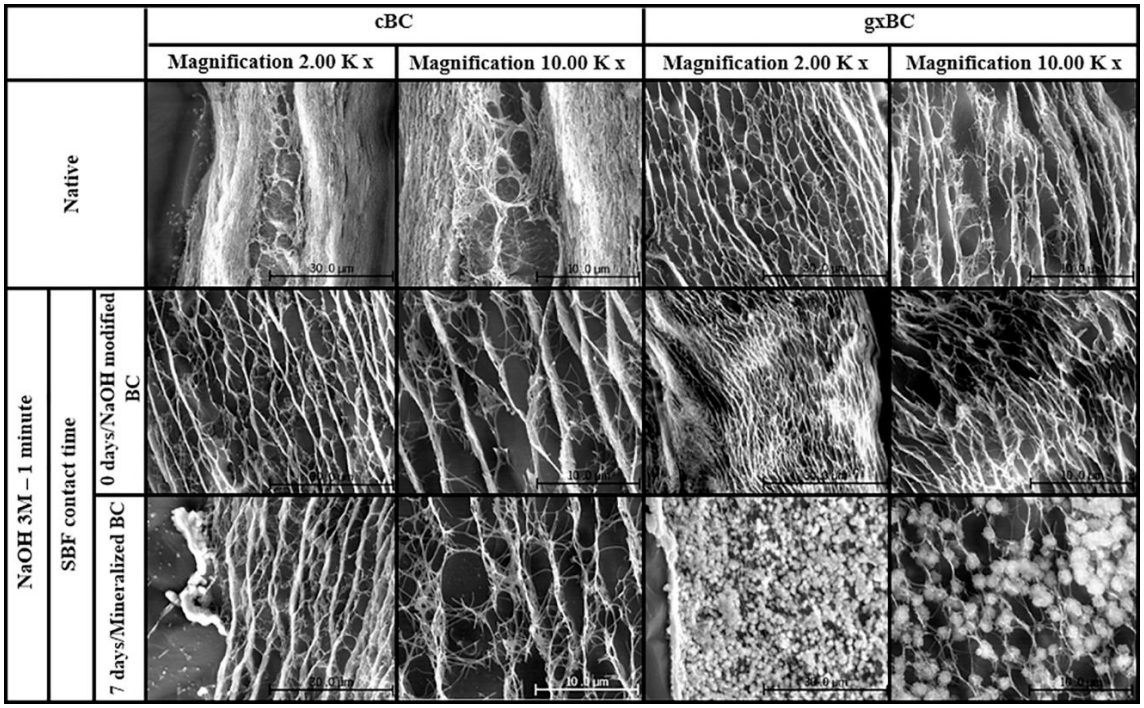


Figure 20. Cryo-SEM cross section images revealing internal structure of produced membranes: cBC and gxBC freeze-dried native, NaOH modified and mineralized.

These results further confirmed the dissimilarities observed between the two different origin BC membranes. gxBC presents a homogeneous bulk fibrillated porous structure, as compared to cBC. The later demonstrates a more compact outer layer surrounding a porous core. These bulk differences may account for the different surface morphologies observed in SEM micrographs.

Chemical modification had a significant impact on the inner structure of the membranes, particularly cBC membranes. Despite the apparent increase of bulk porosity, mineralization of cBC membranes appears to have occurred only on the surface of the membrane. gxBC membranes, on the contrary, demonstrate complete mineralization of the core of the membrane. In this specific case, mineralization throughout sample's length occurred and crystals appear well bound to gxBC fibers. In order to confirm chemical composition of the ceramic phase formed on the produced BC substrates, EDS elemental analysis was performed (Table 7).

Table 7. Elemental analysis of produced BC membranes (Wt – mass fraction). Data is represented as mean±SD (n=3) and was analysed by non-parametric Kruskal-Wallis test (P<0.05): (A) denotes significant differences compared with the native cBC; (B) denotes significant differences compared with the native gxBC; (C) denotes significant differences compared with the NaOH modified gxBC. Differences between BC types were analysed by Mann-Whitney U test (P<0.05)

		Wt% C	Wt% O	Wt% Ca	Wt% P	Wt% Mg
cBC	Native BC	53.74±0.63	46.02±0.69	0.02±0.03	0.02±0.03	0.03±0.06
	NaOH modified BC	24.71±6.84	40.34±3.08	21.27±2.90	9.00±0.063	1.93±1.00[A]
	Mineralized BC	9.37±0.64[A]	38.97±4.86	32.72±5.02[A]	15.96±0.0 9[A]	0.95±0.12
gxBC	Native BC	58.26±1.06	41.52±0.90	0.00±0.00	0.03±0.03	0.08±0.07
	NaOH modified BC	38.71±6.26	38.45±2.66	14.21±3.14	7.03±1.43	0.53±0.07
	Mineralized BC	8.35±0.40[B]	40.72±2.66	32.12±2.02[B]	16.88±0.63[B]	0.9±0.07[B]

		Wt% Na	Wt% Cl	Wt% Al	Wt% S	Wt% Si
cBC	Native BC	0.11±0.09	0.03±0.06	0.03±0.02	0.00±0.00	0.00±0.00
	NaOH modified BC	1.59±0.86	0.63±0.54	0.32±0.17[A]	0.15±0.025	0.07±0.13
	Mineralized BC	0.69±0.61	0.84±0.09[A]	0.12±0.07	0.00±0.00	0.00±0.00
gxBC	Native BC	0.05±0.04	0.02±0.03	0.04±0.04	0.00±0.00	0.00±0.00
	NaOH modified BC	0.83±0.10[B]	0.00±0.00	0.20±0.05[B]	0.05±0.09	0.00±0.00
	Mineralized BC	0.71±0.08	0.26±0.03[C]	0.06±0.06	0.00±0.00	0.00±0.00

Comparing both cBC and gxBC membranes, it is possible to observe a similar trend for both samples. In general, native structures are mainly composed of carbon and oxygen and the presence of other elements was almost negligible on these membranes.

Chemical modification introduced differences on the elementary composition of the samples. On one hand, reduction on the amount of carbon is observed; on the other hand, calcium, phosphorus, magnesium, sodium and chlorine are now present on these membranes.

The surface of mineralized cellulose membranes is characterized by a significant decrease on amount of carbon, as compared to native membranes (p=0.0146, for both cBC and gxBC), and denotes the presence of calcium, phosphorus, magnesium, sodium and chlorine. The increase on calcium and phosphorus was significant when comparing native BC with mineralized BC, for both BC types (for Ca, p=0.0141 and p=0.0127 and for P, p= 0.0141 and p=0.0146, regarding cBC and gxBC respectively).

To establish the Ca/P ratio on mineralized samples, molar concentrations were determined and are presented in Table 8.

Table 8. Ca and P concentration (% and g/mol) on mineralized BC samples. Ca/P ratio. Data is represented as mean±SD (n=3) and was analysed with Mann-Whitney U test (P>0.05)

		Ca concentration		P concentration		Ca/P ratio
		%	g/mol	%	g/mol	
cBC	**Mineralized BC**	32.72±5.02	0.82±0.13	15.96±0.09	0.52±0.00	1.58±0.25
gxBC	**Mineralized BC**	32.12±2.02	0.80±0.05	16.88±0.63	0.55±0.02	1.47±0.25

This ratio is higher for cBC membranes, when compared to gxBC membranes. However, this difference is not statistically significant.

Additional characterization of BC membranes and their ceramic coating was performed through XRD. This is a non-contact and non-destructive analytical technique usually employed on identification and structural characterization of materials' crystalline phases, based on the concept that each material has its own x-ray pattern. Phase identification is one of the major applications of this method, made by comparing the obtained diffractograms with catalogued known patterns [87].

Figures 21 to 23 show individual comparisons between native cBC *vs* native gxBC, NaOH modified cBC *vs* NaOH modified gxBC and mineralized cBC *vs* mineralized gxBC, and a compilation of XRD patterns for cBC and gxBC sets.

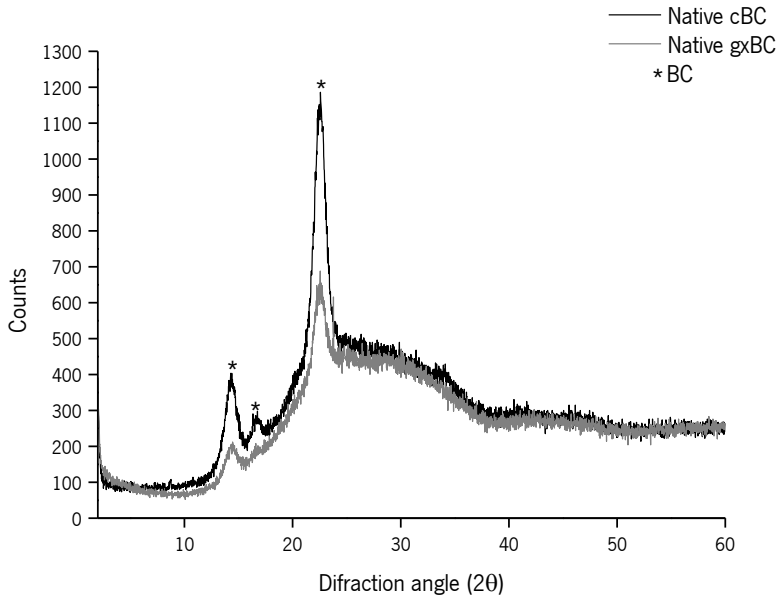


Figure 21. XRD diffraction pattern of native cBC and gxBC (* identifies BC characteristic peaks).

Observing patterns of native cBC and native gxBC (Figure 21), it is possible to notice that they are coincident. Well-defined peaks characteristic of BC are observed at 14.6º, 16.5º, and 22.6º on both samples. These signals are stronger on cBC diffractogram and the 16.5º peak is weaker and almost imperceptible in gxBC diffractogram.

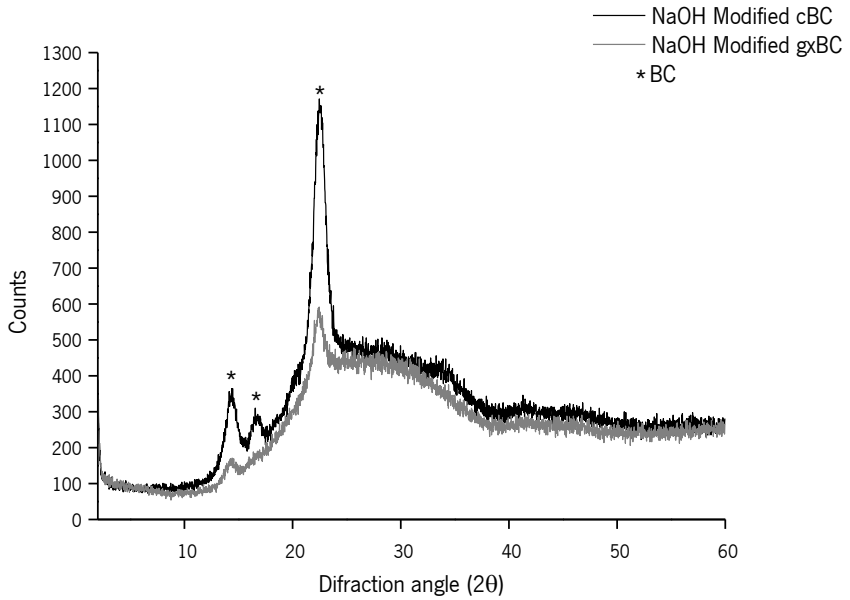


Figure 22. XRD diffraction pattern of NaOH modified cBC and NaOH modified gxBC (* identifies BC characteristic peaks).

XRD pattern of NaOH modified membranes (Figure 22) is quite similar to the one observed for native samples. Nevertheless, a general lowering of intensity of gxBC peaks and absence of 16.5º peak on its pattern is observed.

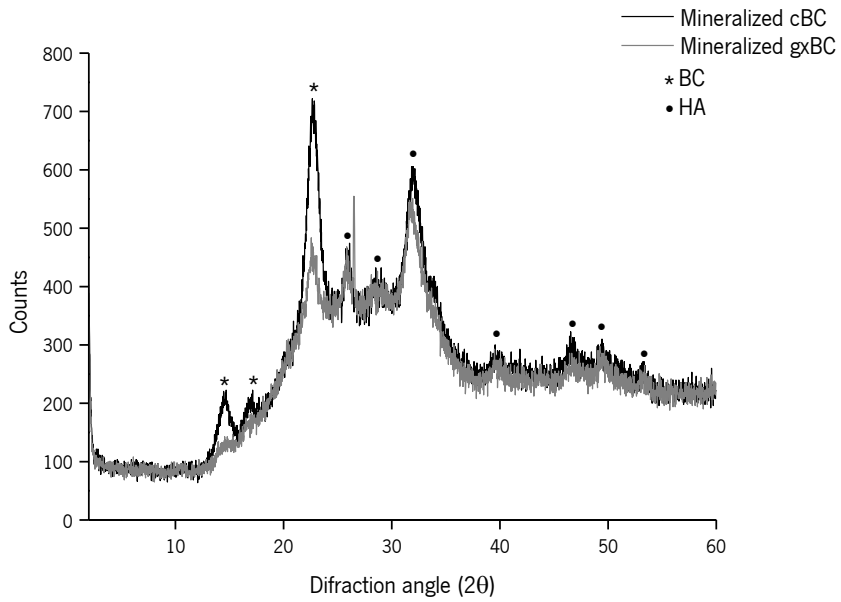


Figure 23. XRD diffraction pattern of mineralized cBC and mineralized gxBC (* identifies BC characteristic peaks and • HA characteristic peaks).

Diffractograms of mineralized samples (mineralized cBC and mineralized gxBC) are given in Figure 23. These reveal the presence of the characteristic BC peaks, already observed on non-mineralized samples, but also extra peaks, rising with similar intensity and width in both types of BC (cBC and gxBC) at 25.8°, 28.3°, 31.9°, 39.5°, 46.6°, 49.4° and 53.2°. These are believed to be related with the calcium orthophosphate layer formed on BC membranes.

When a material is placed on a biological environment, interactions with the surrounding tissues could lead to its swelling and/or degradation. Evaluation of these parameters becomes crucial, in order to understand and try to predict the behaviour of these membranes. In this context, swelling and degradation tests were performed and results are shown on Figures 24 to 27.

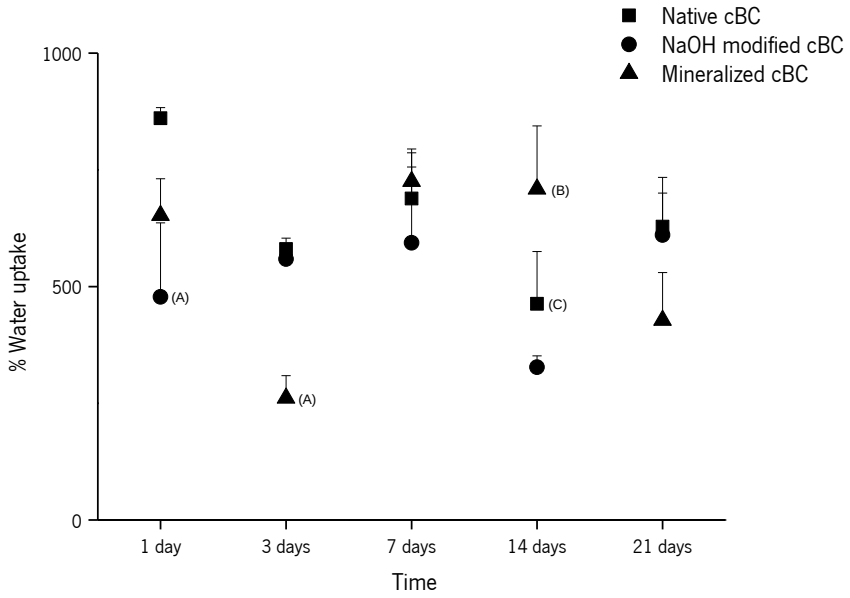


Figure 24. Water uptake profile for native cBC, NaOH modified cBC and mineralized cBC over time. Data is represented as mean±SD (n=3) and was analysed by non-parametric Kruskal-Wallis test ($P<0.05$): (A) denotes significant differences compared with the native cBC, within the same time point; (B) denotes significant differences compared with the NaOH modified cBC, within the same time point; (C) denotes long term significant differences compared with the native cBC after 1 day of immersion.

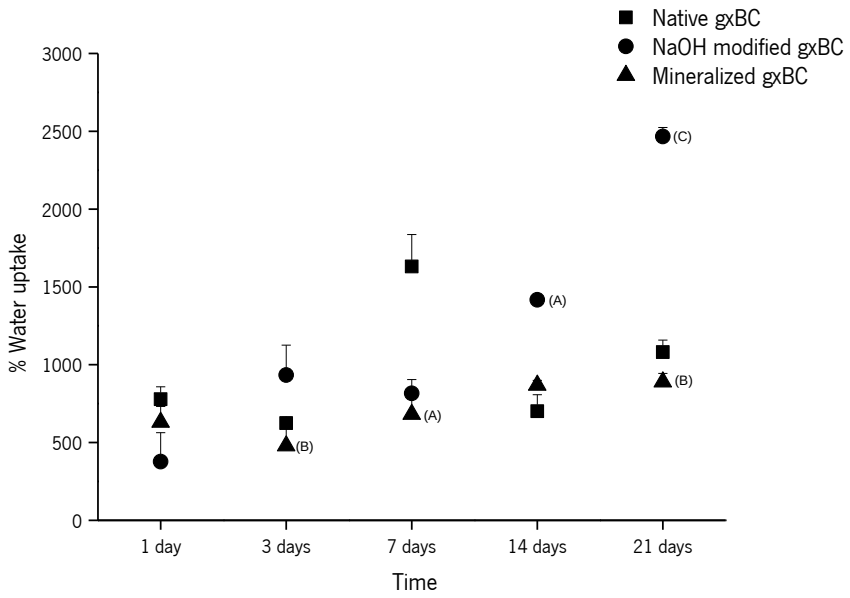


Figure 25. Water uptake profile for native gxBC, NaOH modified gxBC and mineralized gxBC over time. Data is represented as mean±SD (n=3) and was analysed by non-parametric Kruskal-Wallis test ($P<0.05$): (A) denotes significant differences compared with the native gxBC, within the same time point; (B) denotes significant differences compared with the NaOH modified gxBC, within the same time point; (C) denotes long term significant differences compared with the NaOH modified gxBC after 1 day of immersion.

In general, both BC types display high water uptake capability with values at least 100% greater than their initial weight, after one day of immersion on an aqueous saline solution (Figures 24 and 25).

Within each time point and regarding cBC membranes (Figure 24), variations were reported at the end of 1 and 3 days of immersion on PBS. Native cBC membranes present significantly higher values than NaOH modified cBC membranes, after 1 day of immersion ($p=0.0341$) and than mineralized cBC after 3 days ($p=0.0341$). From day 7 on, the overall swelling pattern was maintained and no significant differences were observed between the three cBC sets, except at the end of 14 days, where significant differences were noticed between NaOH modified cBC and mineralized cBC ($p=0225$). At the end of the assay, there are no significant differences on reported values of water uptake after 21 days of immersion, when compared to the ones reported after 1 day of immersion.

For gxBC membranes (Figure 25), no significant differences between native, NaOH modified and mineralized gxBC are noticed at the end of 1 day. However, NaOH modified gxBC membranes present significantly higher values than mineralized gxBC at the end of 3 days ($p=0.0146$) and 21 days ($p=0.0141$) of immersion and than native gxBC at the end of 14 days ($p=0.0146$). Native gxBC and mineralized gxBC displayed a similar swelling profile, except at day 7 where a peak on native gxBC water uptake was registered, significantly different when compared with mineralized gxBC within the same time point ($p=0.0341$). At the end of the assay, after 21 days of immersion in PBS, gxBC membranes subjected to chemical modification demonstrated a significant increase on water uptake when compared to the values reported after 1 day of immersion ($p=0.0040$).

For both types of BC, after 21 days of immersion, the lowest values of water uptake are attributed to mineralized BC. Higher values are reported for gxBC, particularly samples subjected to chemical modification.

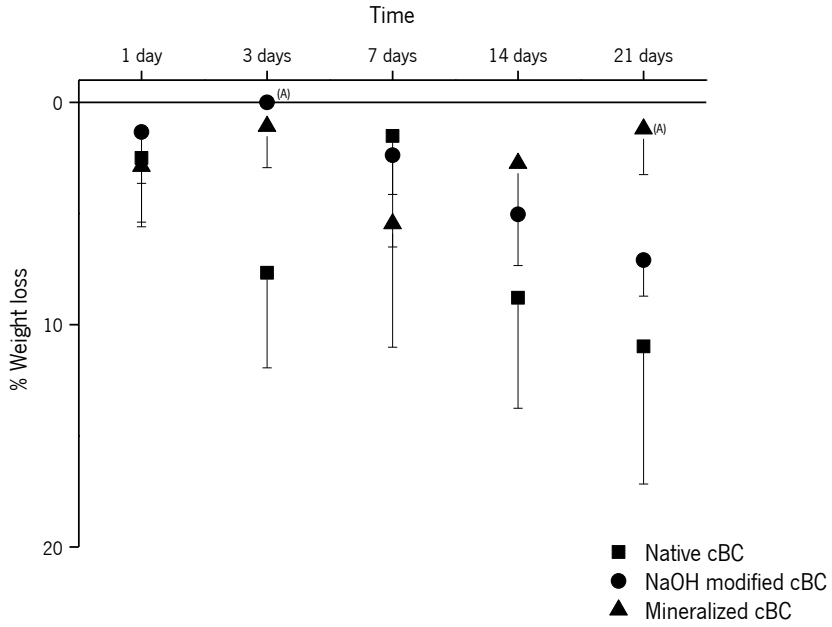


Figure 26. Weight loss profile of native cBC, NaOH modified cBC and mineralized cBC over time. Data is represented as mean±SD (n=3) and was analysed by non-parametric Kruskal-Wallis test (P<0.05): (A) denotes significant differences compared with the native cBC, within the same time point.

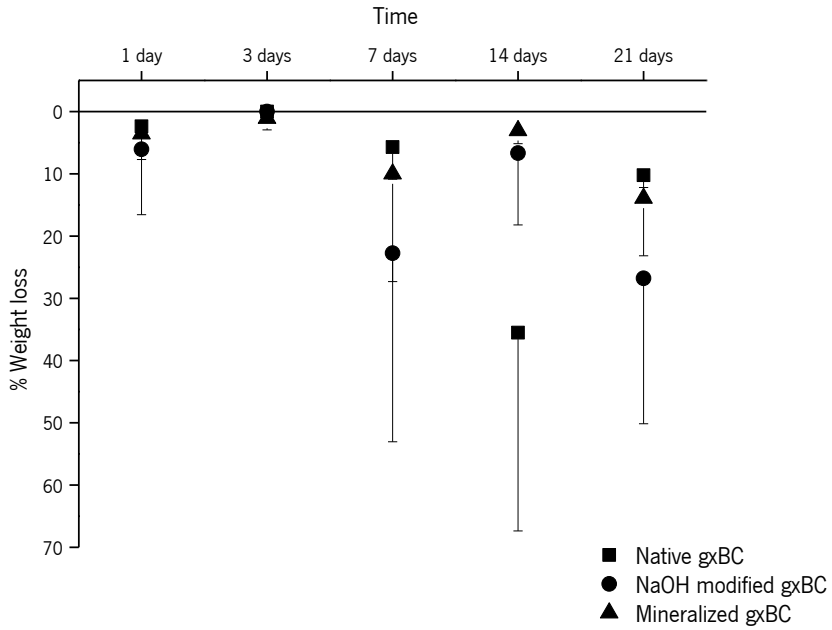


Figure 27. Weight loss profile of native gxBC, NaOH modified gxBC and mineralized gxBC over time. Data is represented as mean±SD (n=3) and was analysed by non-parametric Kruskal-Wallis test (P<0.05).

Degradation, evaluated as weight loss over time (Figures 26 and 27), revealed that gxBC membranes in general demonstrate higher values of degradation. Native cBC and NaOH modified gxBC membranes, in particular, appeared to be the sets more susceptible to this phenomena, as

highest values of weight loss were recorded for these membranes, over time. Mineralized cBC and native gxBC appear to be the most stable membranes. Nevertheless, after 21 days of immersion in PBS, reported values are not significantly different from the ones reported for 1 day of assay, for the three sets of each BC type. Within each time point, no statistical differences were noticed between native, NaOH modified and mineralized gxBC. However, native cBC registered a significant weight loss in relation to NaOH modified cBC (P=0.0396) and to mineralized cBC (P=0.0396) at the end of 3 and 21 days of immersion on PBS, respectively.

In the present work, uniaxial tension or tensile testing was also performed and tensile stress-strain patterns for each produced material were obtained, in order to assess BC membranes mechanical performance.

Analysing the stress-strain patterns for each produce membrane, all specimens in both drying states (dried and wet) exhibited similar plots, where stress augmented proportionally with respect to strain. In addition, membranes presented just one of the two characteristic deformation zones, the elastic one, revealing a brittle nature. At the end of the elastic zone, an abrupt decrease of strength discloses materials' fracture. Due to this brittle behaviour and absence of a plastic deformation zone, tensile strength parameter was not evaluated [82]. In this context, young modulus, fracture strength and strain-at-failure were determined and obtained results are presented in Table 9 to 11.

Table 9. Young modulus (MPa). Data is represented as mean±SD (n=3) and was analysed by non-parametric Kruskal-Wallis test (P<0.05): (A) denotes significant differences compared with the native cBC tested in wet conditions; (B) denotes significant differences compared with the native gxBC tested in wet conditions. Differences between BC types and testing conditions were analysed by Mann-Whitney U test (P<0.05)

	cBC			gxBC		
	Native	NaOH modified BC	Mineralized BC	Native	NaOH modified BC	Mineralized BC
Dry	18.23±3.94	6.09±1.28	6.25±3.10	11.17±5.04	2.13±0.97	10.76±4.69
Wet	1.76±0.92	0.58±0.32	0.20±0.04$^{(A)}$	0.71±0.09	0.06±0.02$^{(B)}$	0.59±0.15

Regarding young modulus results (Table 9), higher values were registered for dried samples, particularly non-modified samples. The presence of a ceramic phase appears to improve membranes' properties when compared with the ones chemically modified. Exception made for cBC modified samples, which presented higher values as compared to the mineralized ones. On the other hand, direct comparison between both types of bacterial cellulose (cBC and gxBC sets) revealed no significant differences.

Immersion of the samples in SBF prior to tensile testing resulted on a significant decrease of the elastic modulus of mineralized cBC (p=0.0146) and chemically modified gxBC (p=0.0341), as compared to NaOH modified cBC and native gxBC, respectively. Between BC types, higher values were reported for native cBC, followed by mineralized gxBC and NaOH modified cBC.

Table 10. Fracture strength (MPa). Data is represented as mean±SD (n=3) and was analysed by non-parametric Kruskal-Wallis test (P<0.05): (A) denotes significant differences compared with the native gxBC tested in dry conditions; (B) denotes significant differences compared with the native gxBC tested in wet conditions. Differences between BC types and testing conditions were analysed by Mann-Whitney U test (P<0.05)

	cBC			gxBC		
	Native	NaOH modified BC	Mineralized BC	Native	NaOH modified BC	Mineralized BC
Dry	38.82±5.81	49.31±14.51	49.30±15.13	157.74±17.24	7.09±1.45$^{(A)}$	25.11±8.79
Wet	21.30±6.15	13.19±3.80	25.26±5.66	17.19±3.56	0.42±0.12$^{(B)}$	5.35±1.31

Analysing fracture strength (Table 10), immersion of membranes in SBF solution resulted in an overall decrease of these values; chemically modified wet membranes revealed the lowest values. In general, chemical modification resulted in a decrease of this value, significant when compared NaOH modified gxBC with native samples, both in dry (p=0.0146) and wet (p=0.0146) state. In the particular case of cBC membranes, when tested dry, NaOH modified samples demonstrate better mechanical behaviour and the presence of the ceramic phase also beneficiate the material mechanical performance. When tested wet, alkali treatment results in lower values. The presence of the apatite layer promotes an increase in these values. However, differences reported for these cBC membranes (dried and wet) are not statistically significant. Comparing both BC types within each set, the highest values are reported for native gxBC, followed by mineralized cBC and NaOH modified cBC when tested in dry state and to native cBC, mineralized cBC and NaOH modified cBC, when tested in wet conditions.

Table 11. Strain-at-failure (%). Data is represented as mean±SD (n=3) and was analysed by non-parametric Kruskal-Wallis test (P<0.05): (A) denotes significant differences compared with the native gxBC tested in dry conditions; (B) denotes significant differences compared with the native cBC tested in wet conditions; (C) denotes significant differences compared with the native gxBC tested in wet conditions. Differences between BC types and testing conditions were analysed by Mann-Whitney U test (P<0.05)

	cBC			gxBC		
	Native	NaOH modified BC	Mineralized BC	Native	NaOH modified BC	Mineralized BC
Dry	2.77±0.46	7.27±1.22	8.40±2.66	13.99±8.01	3.84±1.21	2.80±1.05$^{(A)}$
Wet	19.20±3.57	10.90±2.98$^{(B)}$	15.39±2.57	28.84±4.45	7.47±2.66$^{(C)}$	19.98±2.81

Concerning strain at failure, materials' performance was better when these were tested in wet conditions (Table 11). In general, higher values are reported for native samples, except for dried cBC membranes, where the best value is related with the mineralized membranes (although without significant differences). gxBC membranes demonstrated the best results in terms of elongation capability, registering a significant decrease in mineralized gxBC (p=0.0341) and NaOH modified gxBC (p=0.0146) in relation to native gxBC, when tested in dry and wet conditions, respectively. cBC subjected to tension in wet state also revealed a significant decrease for NaOH modified cBC (p=0.0270) when compared with native cBC. Comparing both BC types, highest values were associated with native gxBC, followed by mineralized gxBC and NaOH modified cBC when tested in wet conditions and to native gxBC, mineralized cBC and NaOH modified cBC when tested dried.

The long-term effect of produced BC membranes on metabolic activity of 3T3 and MC 3T3-E1 cells was evaluated by MTS assay, for 21 days. The results are presented in Figures 28 to 31.

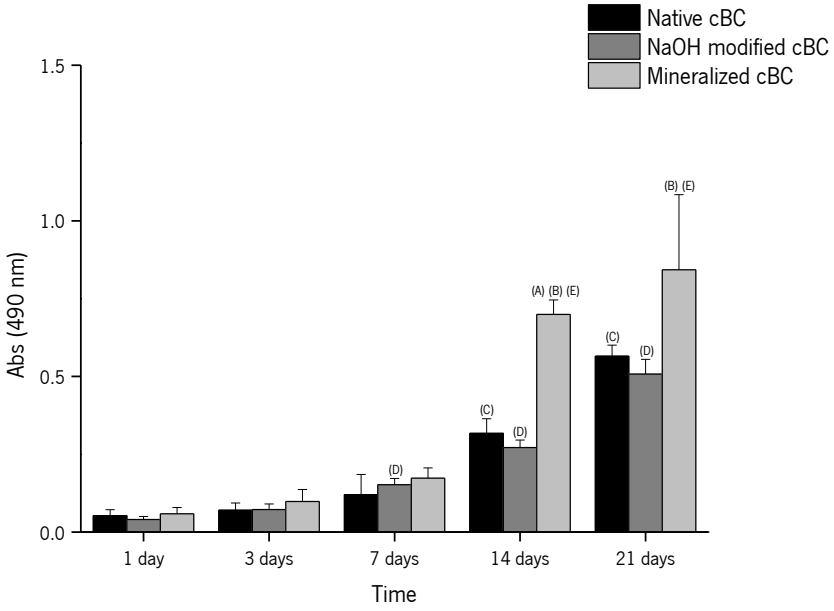


Figure 28. Metabolic activity of 3T3 cells cultured onto native cBC, NaOH modified cBC and mineralized cBC samples. Data is represented as mean±SD (n=15) and was analysed by non-parametric Kruskal-Wallis test (P<0.05): (A) denotes significant differences compared with the native cBC, within the same time point; (B) denotes significant differences compared with the NaOH modified cBC, within the same time point; (C) denotes long term significant differences compared with the native cBC after 1 day of incubation; (D) denotes long term significant differences compared with the NaOH modified cBC after 1 day of incubation; (E) denotes long term significant differences compared with the mineralized cBC after 1 day of incubation.

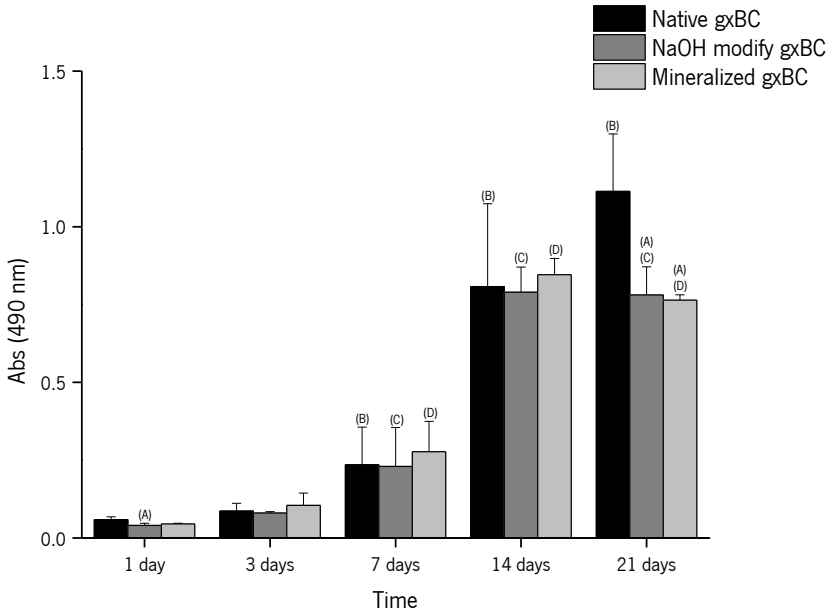


Figure 29. Metabolic activity of 3T3 cells cultured onto native gxBC, NaOH modified gxBC and mineralized gxBC samples. Data is represented as mean±SD (n=15) and was analysed by non-parametric Kruskal-Wallis test (P<0.05): (A) denotes significant differences compared with the native gxBC, within the same time point; (B) denotes long term significant differences compared with the native gxBC after 1 day of incubation; (C) denotes long term significant differences compared with the NaOH modified gxBC after 1 day of incubation; (D) denotes long term significant differences compared with the mineralized gxBC after 1 day of incubation.

After 1 day of contact, 3T3 cells revealed decreased metabolic activity for NaOH modified cBC when compared with values reported for mineralized cBC (p=0.0389) and cells in contact with native gxBC reveal higher values than NaOH modified gxBC (p=0.0052) (Figure 28 and 29).

Differences appeared again at the end of day 14 for cBC (Figure 28), where the metabolic activity of cells in contact with native and NaOH modified cBC membranes presented values significantly lower than mineralized cBC (p=0.0230 and p=0.0003, respectively). This trend is maintained after 21 days, but only when comparing NaOH modified cBC with mineralized cBC (p=0.0256).

For gxBC (Figure 29), differences between samples within the same time point, were noticed just at the end of 21 days of assay, corresponding to significantly lower values for NaOH modified gxBC and mineralized gxBC in relation to the native set (p=0.0082 and p=0.0089, respectively).

When comparing 3T3 cells' behaviour over time with values registered after 1 day of incubation for each set of materials, a gradual increase in metabolic activity was registered being significant from day 7 on, particularly for native gxBC (p=0.0219 at 7 days, p<0.0001 at 14 and 21 days), NaOH modified gxBC (p=0.0160 at 7 days, p<0.0001 at 14 and 21 days), mineralized gxBC (p=0.0169 at 7 days, p<0.0001 at 14 and 21 days) and NaOH modified cBC (p=0.0070 at 7 days, p<0.0001 at 14 and 21 days). For native cBC and mineralized cBC these differences were only significant

after 14 days (p=0.0003 at 14 days, p<0.0001 at 21 days and p<0.0001 at 14 days and 21 days, respectively). The highest values were reported for native gxBC followed by mineralized cBC.

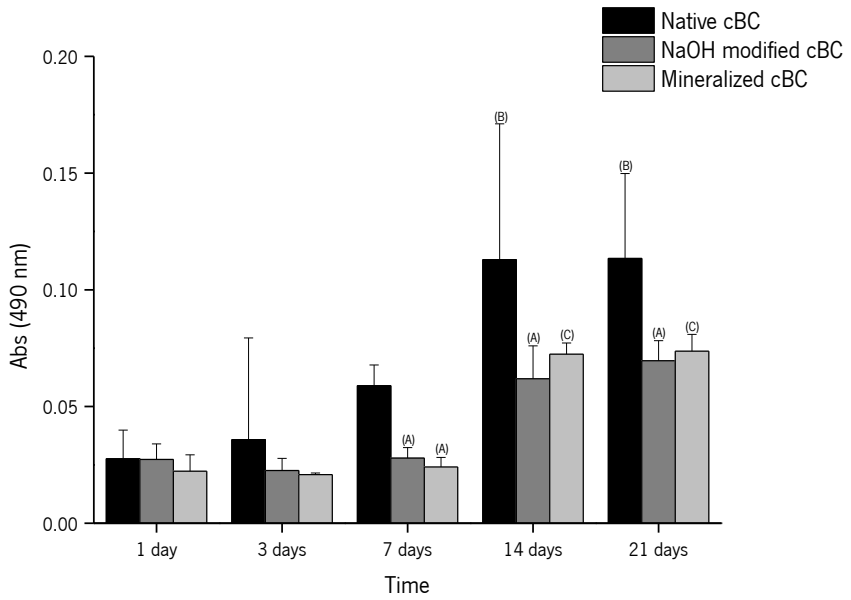


Figure 30. Metabolic activity of MC 3T3-E1 cells cultured onto native cBC, NaOH modified cBC and mineralized cBC samples. Data is represented as mean±SD (n=15) and was analysed by non-parametric Kruskal-Wallis test (P<0.05): (A) denotes significant differences compared with the native cBC, within the same time point; (B) denotes long term significant differences compared with the native cBC after 1 day of incubation; (C) denotes long term significant differences compared with the mineralized cBC after 1 day of incubation.

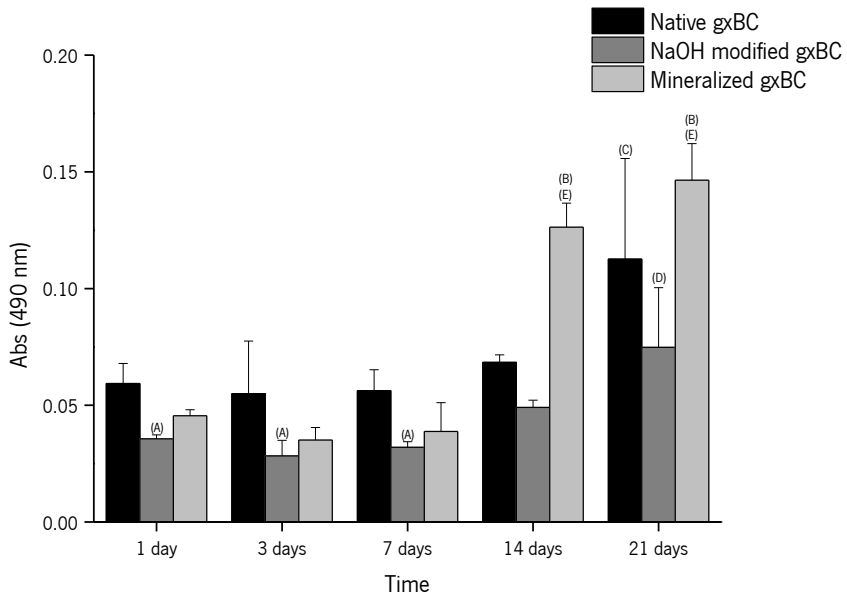


Figure 31. Metabolic activity of MC 3T3-E1 cells cultured onto native gxBC, NaOH modified gxBC and mineralized gxBC samples. Data is represented as mean±SD (n=15) and was analysed by non-parametric Kruskal-Wallis test ($P<0.05$): (A) denotes significant differences compared with the native gxBC, within the same time point; (B) denotes significant differences compared with the NaOH modified gxBC, within the same time point; (C) denotes long term significant differences compared with the native gxBC after 1 day of incubation; (D) denotes long term significant differences compared with the NaOH modified gxBC after 1 day of incubation; (E) denotes long term significant differences compared with the mineralized gxBC after 1 day of incubation.

Regarding results for MC 3T3-E1 cells, presented in Figures 30 and 31, the overall reported values appear lower than the ones recorded for 3T3 cells.

For cBC (Figure 30), differences between samples, within each time point were just revealed after 7 days of culture, where values reported for native cBC samples were higher than for NaOH modified cBC and mineralized cBC ($p=0.0003$ and $p=0.0004$, respectively). From this point on, an analogous situation was observed and a significant increase was registered in native cBC membranes in relation to NaOH modified samples ($p=0.0334$ and $p=0.0016$ for 14 days and 21 days, respectively). After 14 days of culture, despite the higher values for native cBC samples, no significant differences appeared when comparing it with mineralized cBC membranes.

In the case of gxBC (Figure 31), significant differences are already reported at initial time points. At the end of 7 days, metabolic activity of cells cultured on native samples revealed higher values than those reported for NaOH modified membranes ($p=0.0003$, $p=0.0060$, $p=0.0063$ for day 1, 3 and 7 respectively). On 14th and 21st day, the same trend was recorded when comparing mineralized gxBC with NaOH modified gxBC ($p<0.0001$). At the end of 21 days of culture, mineralized gxBC presented higher values, followed by native gxBC; however these differences are not statistically significant.

Regarding the metabolic activity of these cells over time, and comparing results with values registered after 1 day of culture, there is a significant increase after 14 days. At this point, cellular metabolic activity increased significantly on native cBC ($p=0.0003$), mineralized cBC ($p=0.0008$) and mineralized gxBC ($p=0.0255$). After 21 days, the same behaviour was also recorded for native cBC ($p=<0.0001$), mineralized cBC ($p=0.0005$) and mineralized gxBC ($p=0.0348$), but also for native gxBC ($p=0.0188$) and NaOH modified gxBC ($p=0.0076$). No significant differences are registered for NaOH modified cBC membranes. The highest values were reported for mineralized gxBC, followed by native cBC.

Analysing the results herein obtained, after 21 days of culture in contact with the produced membranes, the highest reported values for 3T3 and MC 3T3-E1 cellular metabolic activity are associated with native gxBC and mineralized gxBC membranes, respectively.

Based on physical and biochemical cellular properties, live/dead assays enable the discrimination between viable and unviable cells through the use of two fluorescent probes, which dye dead cells in red and the live ones in green. The results are presented in Figures 32 to 35. In these studies, a native BC membrane cultured without cells was used as a control of the experiment.

Differences are noticed in terms of cells' preferences towards the diverse substrates since early incubation periods. In fact, cells appear to prefer gxBC-based membranes to cBC-based membranes. In addition, 3T3 cells appear in a higher number when compared to MC3T3-E1.

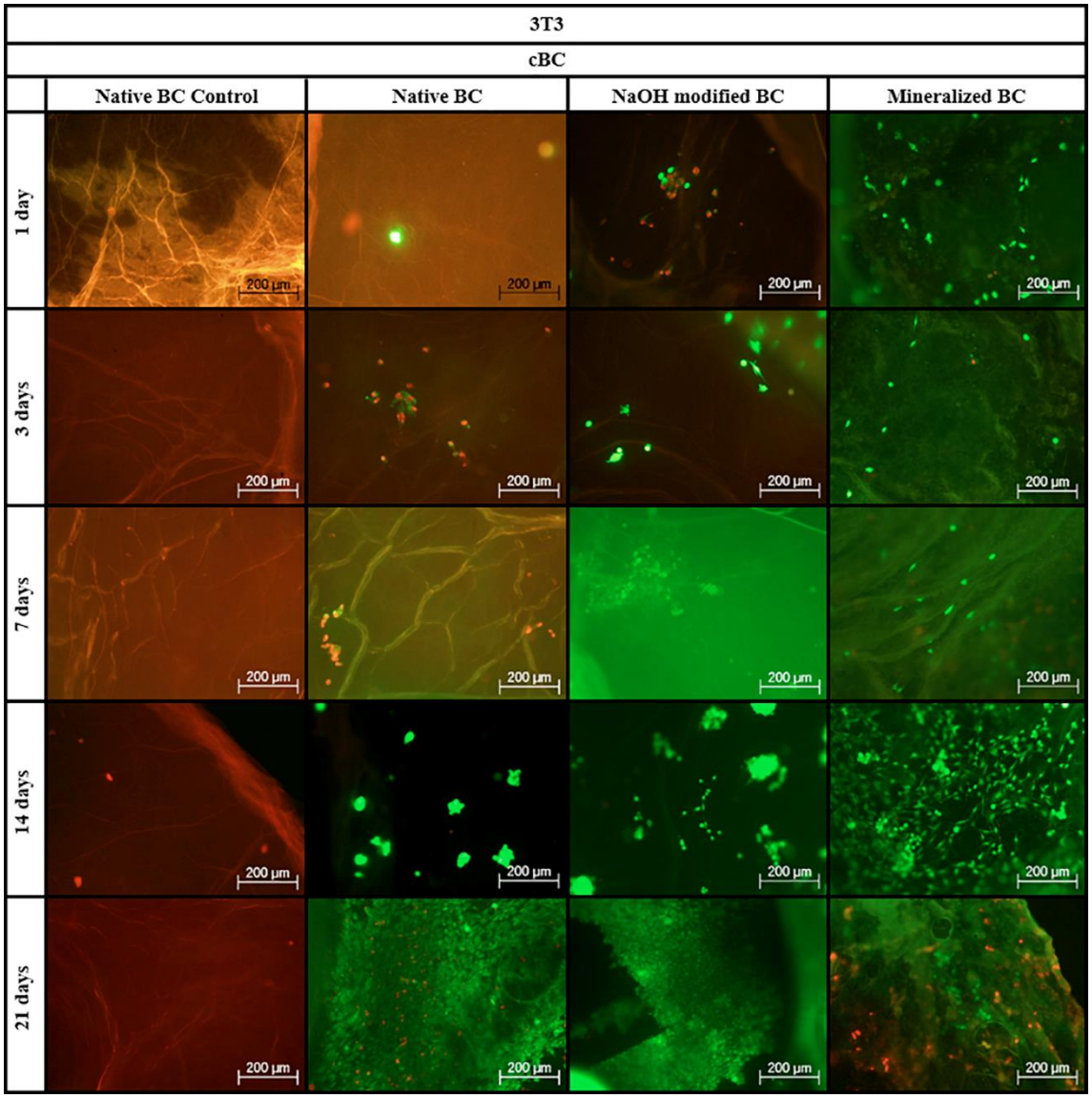


Figure 32. Fluorescence photographs of live and dead stained 3T3 cells after 1, 3, 7, 14, 21 days of incubation on native, NaOH modified and mineralized cBC. Live cells are stained in green and dead cells are stained in red.

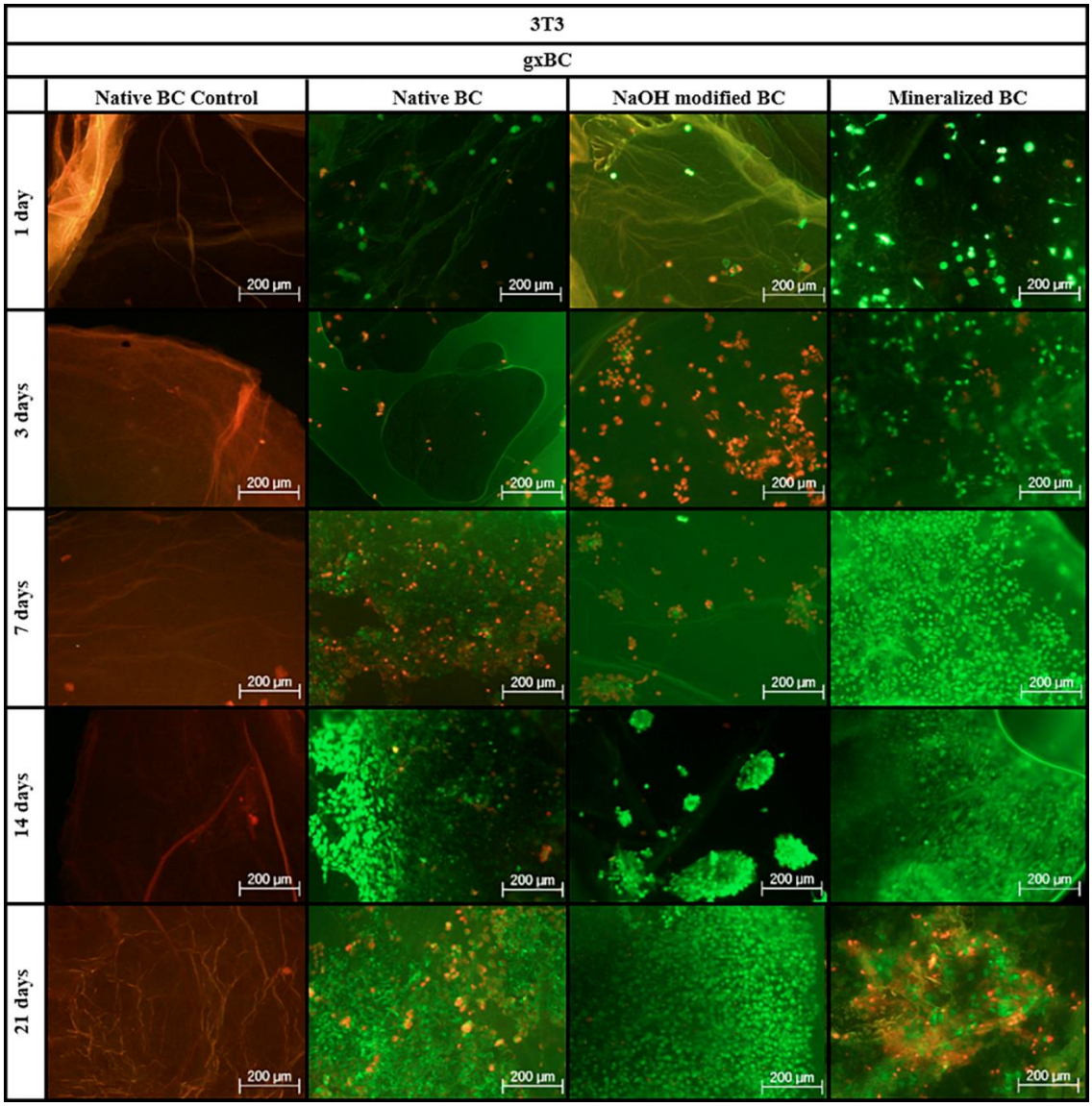


Figure 33. Fluorescence photographs of live and dead stained 3T3 cells after 1, 3, 7, 14, 21 days of incubation on native, NaOH modified and mineralized gxBC. Live cells are stained in green and dead cells are stained in red.

Colonization of cBC membranes by 3T3 cells (Figure 32) appears as a slow process, taking time to occur. In fact, it is only after 14 days that cells show signs of colonization of the membranes; native and NaOH modified cBC membranes denote the presence of cellular clusters, as mineralized membranes already demonstrate signs of an almost complete colonization of the membrane by these cells. Despite these early differences, all membranes are completely covered by 3T3 cells after 21 days of culture. At this point, mineralized membranes already demonstrate the presence of dead cells, probably associated to the fact that a cellular confluent layer was reached earlier.

In comparison, gxBC membranes (Figure 33) appear to be a better substrate to sustain 3T3 cells, particularly native and mineralized membranes, as colonization of these membranes becomes

evident since early time points. Colonization of chemically modified membranes occurs at a slower rate, with a first appearance of cellular clusters, particularly evident after 14 days of culture. Nevertheless, after 21 days, all gxBC membranes are completely covered by 3T3 cells. As observed before, membranes with early and faster colonization by cells start to present dead cells after 21 days of culture.

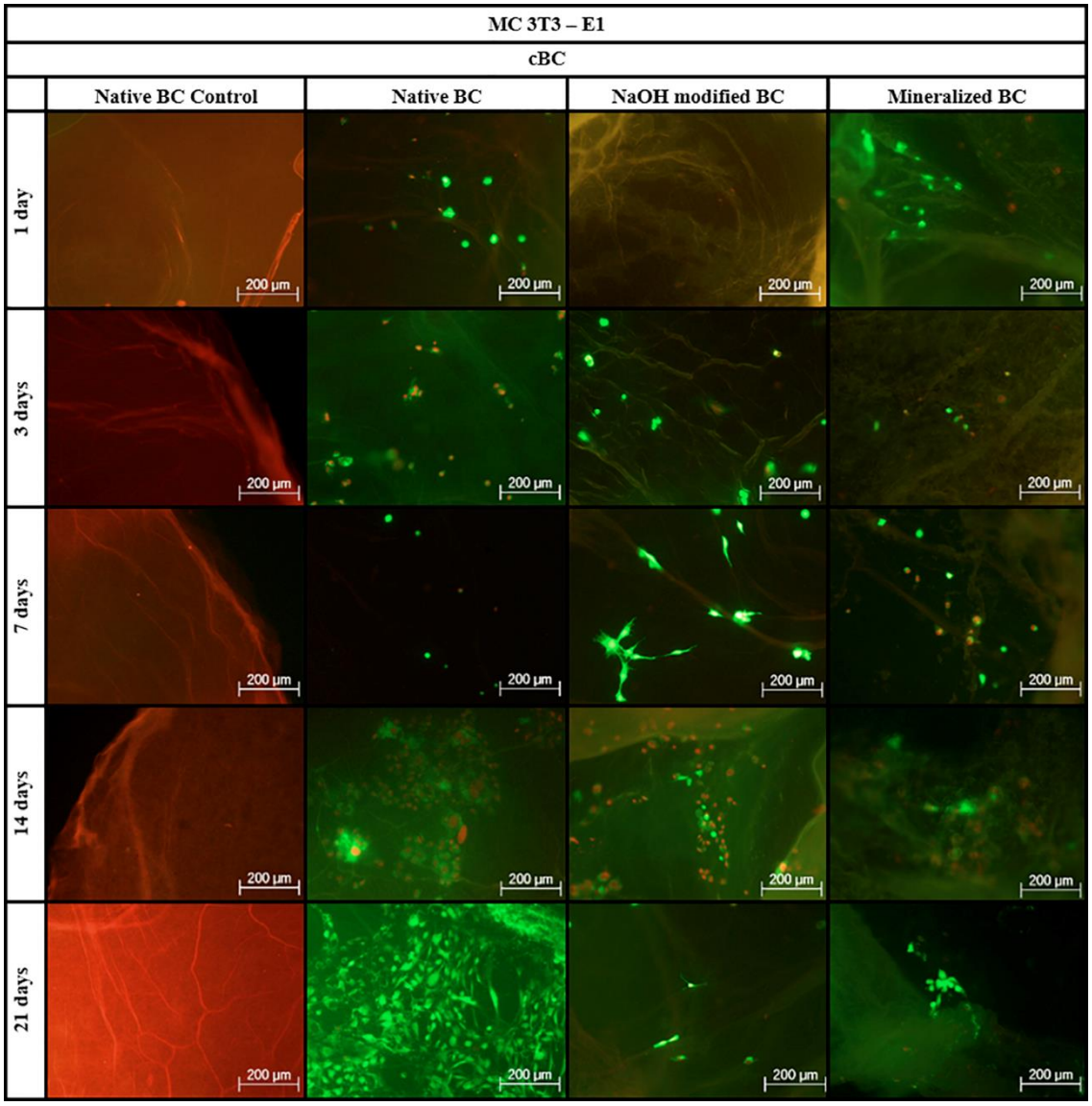


Figure 34. Fluorescence photographs of live and dead stained MC 3T3-E1 cells after 1, 3, 7, 14, 21 days of incubation on native, NaOH modified and mineralized cBC. Live cells are stained in green and dead cells are stained in red.

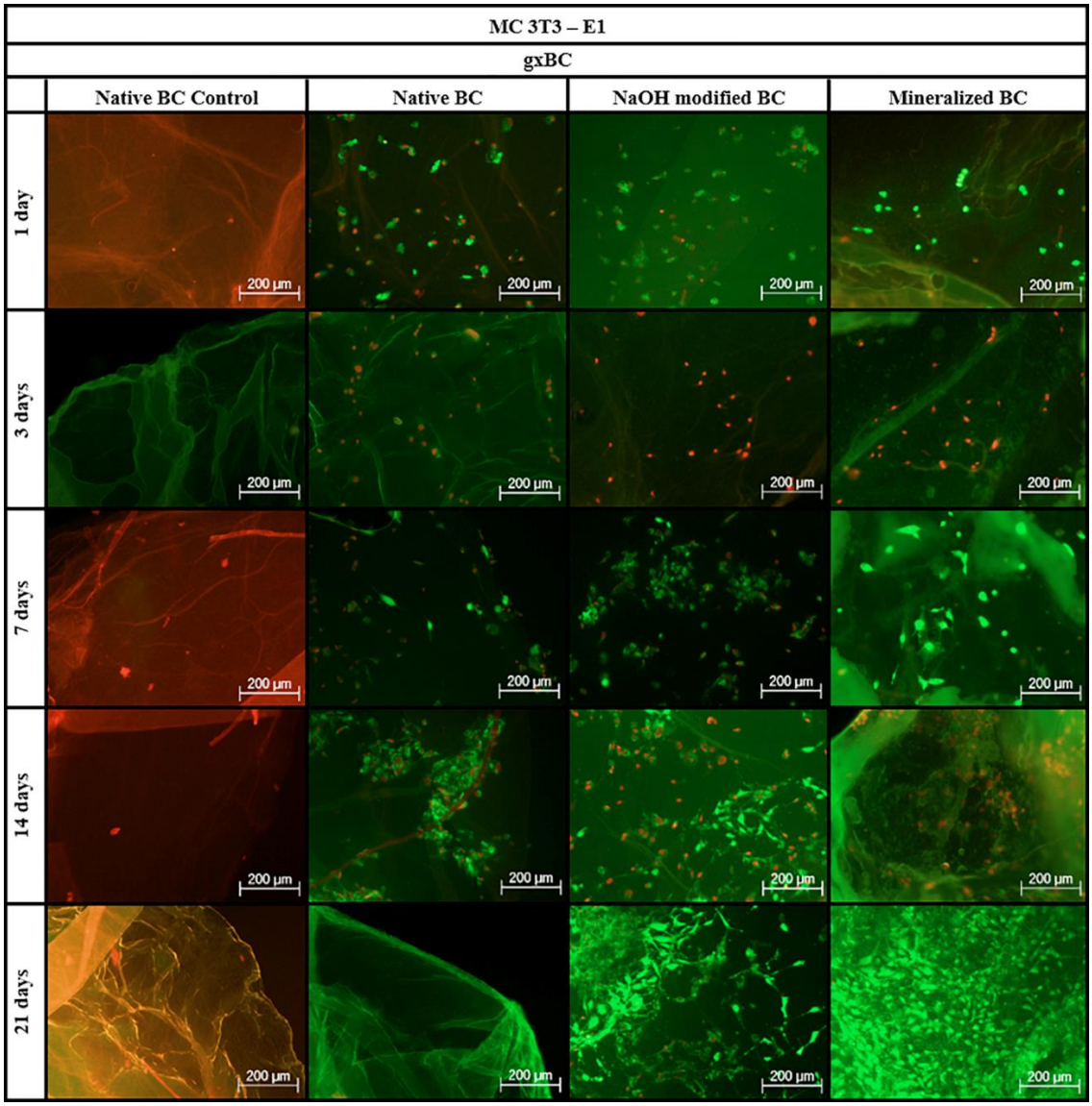


Figure 35. Fluorescence photographs of live and dead stained MC 3T3-E1 cells after 1, 3, 7, 14, 21 days of incubation on non- native, NaOH modified and mineralized gxBC. Live cells are stained in green and dead cells are stained in red.

When compared to 3T3 cells, MC 3T3 E1 cellular response to produced membranes is quite different. These cells demonstrate a delayed response towards cBC membranes (Figure 34) and they appear to denote a preference for native cBC materials. In fact, after 21 days of culture, this is the only substrate that reveals the presence of a cellular layer well spread over these membranes. These cells denote a more consistent response towards gxBC membranes (Figure 35), which demonstrate earlier cellular colonization, with a complete cellular layer formed after 21 days of culture. This is particularly evident for modified and mineralized gxBC membrane.

E. Discussion

Development of bacterial cellulose membranes coated with hydroxyapatite for bone regeneration

Discussion

GBR, a specific type of guided tissue regeneration, is an example of an innovative medical solution for bone defects' treatment [5][17][29]. The use of occlusive or barrier membranes to create and maintain an ideal environment for osteogenic cells' activity and prevention of defect invasion by soft tissues, is the underlying principle of this technique [40-42].

In this work, development of mineralized BC membranes for GBR capable of acting both as barrier material and promoter of bone regeneration was the main goal.

The biomimetic approach proposed by Kwak and co-workers for BC mineralization, which involves the treatment of the polymer with a strong alkali, followed by immersion in specific SBF solutions, was herein applied [77]. The choice of this protocol is strengthened by the successful chemical modification of materials' surfaces with alkaline treatments, which have shown good results on diverse polymeric or metallic substrates, such as polyether ether ketone (PEEK), high-density polyethylene (HDPE), ultra-high molecular weight polyethylene (UHMWPE), poly (ε-caprolactone) (PCL), poly(ethyl methacrylate-*co*hydroxyethyl acrylate) (P(EMA-*co*-HEA)) and silicone or tantalum, titanium and zirconium [95-101].

BC is a highly hygroscopic material able to establish strong hydrogen bonds between its –OH groups and surrounding water molecules. Taking into consideration the mechanism involved on apatite formation speculated by Shian and co-workers, the alkaline treatment of BC, namely with NaOH, results in several structural modifications [89].

NaOH is a polar solvent that can form sufficiently strong linkages with BC –OH groups. Consequently, polymer's inter- and intra-molecular interactions may be broken, leading to the formation of alkali BC, or BC–ONa [90]. After this alkali treatment, the placement of the modified material on NaCl-free SBF could lead to the release of BC-bond sodium ions, from BC-ONa, via ion-exchange with calcium ions from this particular solution, forming apatite nuclei. The establishment of electrostatic interactions with free –OH groups of BC, which have not reacted with NaOH, could also lead to absorption of calcium to polymer fibers. Subsequent immersion in complete SBF solution allows the desired biomimetic ceramic deposition. In fact, interactions between calcium ions and the surrounding solution, rich in CO_3^{2-}/PO_4^{3-}, are promoted and the mineralization process occurs. This reaction continues until SBF solution is depleted from these essential ions (Figure 36) [92][100-102].

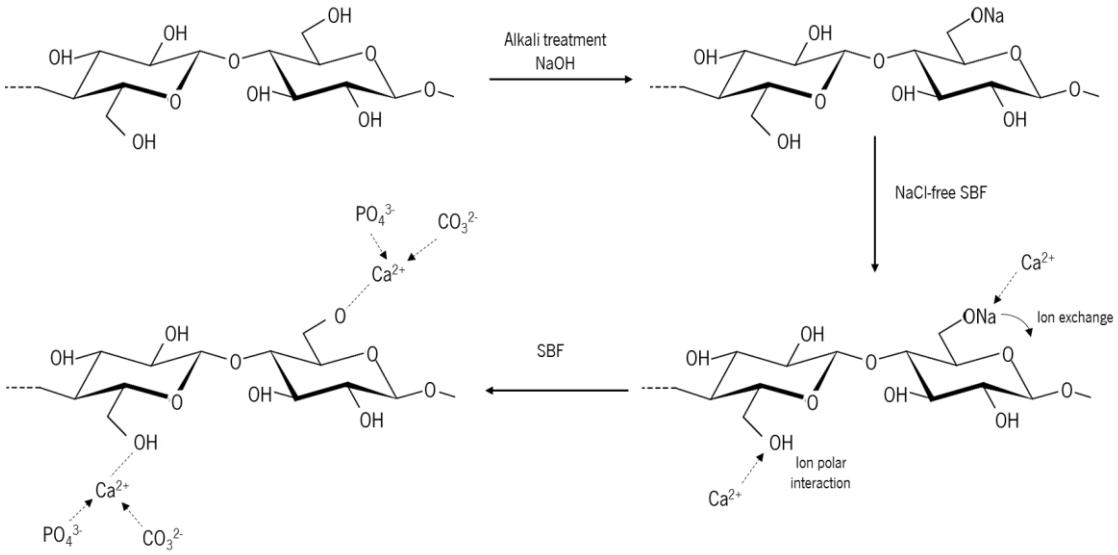


Figure 36. Schematic representation of the mineralization mechanism proposed for BC. Adapted from [89].

Based on these principles, mineralized BC membranes were produced. Despite of the importance of the methodology used to perform materials' modification and stimulate its mineralization, materials own properties also play a crucial role throughout the overall process. Having this in mind, two parameters susceptible to impact BC membranes' final properties were considered: membranes pre-treatment prior to chemical modification, namely membranes drying, and membranes origin.

Literature is consensual in reporting that drying or water extraction methods influence BC microstructure [74][105-107]. This fact becomes evident by the analysis of the results herein presented, both on the influence of polymer's structure, but also with putative impact on the modification and mineralization of the membrane. In this study, three different conditions were evaluated: never dried, air-dried and freeze-dried membranes. Differences in membranes topography, prior to pre-rehydration, are observed by SEM analysis: defined and continuous fibers, with increased porosity, are characteristic of freeze-dried structures, while these features tend to be absent on air-dried samples [105-106].

In 2006, Clasen and co-workers argued that simple imaging techniques and observation is not enough to perform a correct comparison over BC network, recommending oscillatory shear rheological experiments and IR-spectroscopy as complementary analysis methods [93]. These allowed them to conclude that air-drying promotes complete aggregation of polymer fibers, resulting in the absence of network meshes, while in freeze drying, a partial aggregation of free fibers occurs, with an increase of network meshes and retention of porous structure. These

differences impacted the material's water uptake ability, with an attenuated influence on swelling for freeze-dried samples, which decreases by a factor of 5, or a more pronounced decrease by a factor of 50 when the material is air-dried [93].

After pre-rehydration, followed by air-drying, differences between freeze-dried and air-dried samples are maintained, although these are less obvious, visible in microscopic observations at higher magnifications. Less differentiated fibers and smaller pores, associated to freeze-dried membranes, may be related with the final air-drying step performed after pre-rehydration and modification/mineralization protocols.

Differences are also evident when comparing two diverse origin BC membranes, particularly when comparing freeze-dried samples. In-house produced membranes (gxBC) appear to present higher porosity, both before and after pre-rehydration, when compared with the commercial membrane. In addition to drying methodologies, factors including fermentation conditions, such as bacteria species and strain, cultivation time, carbon sources or inoculation volume, as well as post-treatments, including physicochemical modifications, can influence materials' structure, especially porosity [40][94].

This multifactorial contribution to the final properties of the produced structures have to be considered for an optimized production and in order to propose the best material possible for the envisaged applications.

Within this work plan, mineralized BC membranes are desired. Native bacterial celluloses are not bioactive for themselves, as demonstrated by the herein presented results. In fact, immersion of these membranes in SBF solution, for as long as 21 days, was ineffective on apatite nucleation, for both BC types. These results demonstrate that despite their abundance, BC –OH groups are not sufficiently reactive to promote apatite nucleation from SBF. From all –OH groups present in BC, only the (C6) primary –OH groups are accessible to interact with the surrounding environment. BC is extremely hygroscopic and, when possible, these –OH groups tend to interact with surrounding water molecules through hydrogen bonds. These bonds are stronger than ion polar interactions, through which these functional groups would interact with Ca^{2+} ions, and breaking these strong bonds to form weaker ones does not pose an energetic advantage [59][76][108-109].

To favour and stimulate apatite nucleation by BC, a mineralization protocol based on alkaline treatment was chosen and revealed effective. This is visible by the formation of small particles scattered through membranes' surface or of an apatite layer covering the membranes.

NaOH modified BC already revealed the deposition of small particles that, according to the speculated mineralization mechanism previously proposed, could be due to calcium nucleation. The formation of these nuclei (BC-OCa) is mandatory for calcium phosphate phase nucleation [73]. In fact, in its work, Kwak and co-workers demonstrated that BC immersion in SBF right after treatment with strong alkali and without contact with a NaCl-free SBF solution resulted in cubic-shaped particles on polymer fibers, identified as sodium chloride [77].

When NaOH modified samples were immersed on a SBF solution, apatite particles appeared immediately after 1 day of contact with this solution, with their typical form, as cauliflower-like round shape particles [59][78-79][96]. These particles increased as the necessary ions were supplied by SBF solution, over time. This results in a layer that completely covers the produced membranes just after 7 days, for some samples. This mineralization profile is normally related to the high surface energy and density of HA particles [76]. After formation of an apatite nuclei, a continuous supply of Ca^{2+} and CO_3^{2-}/PO_4^{3-} should be maintained [73][97]. If SBF solution is depleted of these ions, apatite nuclei formation and crystal growth stops [76][89].

By varying drying methodologies, chemical modification protocols and BC origin, it was possible to produce mineralized membranes with diverse properties. In order to select an optimized methodology to obtain membranes suitable to be proposed for GBR applications, different factors were considered and their holistic contribution evaluated. These include the formation of a consistent ceramic layer in the shortest period of time possible, ceramic layer morphology, processing conditions and sample handling. Selected membranes were further characterized, still maintaing the comparison between membranes obtained from diverse origins. Native samples and NaOH modified membranes are maintained and analysed as controls.

Diverse origin bacterial cellulose membranes originate diverse mineralized materials. These differences are somewhat attenuated in SEM visualization. However, they are revealed as a more in-depth analysis is performed with cryo-SEM. Higher porosity of native gxBC over native cBC membranes, after pre-rehydration, reported by SEM images was also revealed by cryo-SEM cross section images. However, chemical treatment with strong alkali introduced structural modifications, particularly noticeable in cBC membranes, possibly due to the break of inter- and/or intra-molecular BC interactions [90]. These membranes appear with increased porosity. Nevertheless, their mineralization is constricted to the surface of the membrane. gxBC membranes, on the other hand, appear less affected by the alkali treatment, as visualized in cryo-SEM images. However, the most striking difference when comparing these membranes with the commercial ones is inner

mineralization. In fact, each fiber presents crystal growth, demonstrating the effectiveness of the optimized protocol for these membranes in particular.

Observed differences may be related with the initial morphology of each membrane and the appropriate diffusion of NaOH, NaCl-free SBF and SBF ions through the membranes [73]. Although the contact time of cBC membranes with the strong alkali solution was enough to open the membrane structure, it seems that it was not effective in assuring mineralization of the inner individual BC fibers. As the surface ceramic layer is formed and evolving, it can physically prevent ions' migration into BC inner network [59]. This can also explain the thicker ceramic layer formed on the surface of gxBC membranes, as compared with its bulk individual fibers.

The effectiveness of the mineralization process and characterization of the ceramic phase formed was confirmed by EDS and XRD. The main elemental components are carbon and oxygen, particularly in native samples, confirming their polysaccharidic nature [51-52]. Mineralized membranes reveal the presence of calcium and phosphorus, particularly when compared with native membranes, confirming the traditional chemical composition of apatite, beyond its characteristic layout already visualized in SEM and cryo-SEM images. These two elements were also present in higher percentage on NaOH modified samples of both types of BC, when compared with native sets (although not statistically significant), especially calcium. This supports the proposed mineralization mechanism and indicates that the surface of the NaOH modified samples already possesses the necessary apatite nucleating sites. In addition, the limited 1 day soaking time in NaCl-free SBF could be sufficient to start PO_4^{3-} nucleation, once phosphorus was also present in these samples.

Calcium orthophosphates are the principal mineral components present in several nature and living organisms, with associated structural diversity. In this sense, the Ca/P ratio is one of the distinctive features of these compounds, which in the produced mineralized BC membranes is lower than the stoichiometric ratio of 1.67 characteristic of hydroxyapatite [71-72]. This suggest that the hydroxyapatite layer present in the BC membranes is calcium deficient (CDHA) [71-72]. Formation of such calcium orthophosphate was also described in similar processes that involve BC modification followed by immersion in SBF 1.5x solutions [78-79]. Pure CDHA is not found in biological systems, where it is usually substituted with different ions present in surrounding body fluids, including Na^+, K^+, Mg^{2+}, Sr^{2+} instead of Ca^{2+}, F^-, Cl^-, CO_3^{2-} instead of OH^- and HPO_4^{2-} instead of PO_4^{3-} [66]. In fact, the EDS profile indicates an emergence of magnesium and chloride ions on BC

mineralized membranes, especially magnesium in gxBC membranes and chloride in cBC membranes.

On the other hand, CDHA is considered an interesting apatite phase, particularly if compared with HA, as the connection between a biomaterial and surrounding bone tissue is generally done through a CDHA layer [99][101-102]. This is closely related with the bioceramic solubility and an inverse relationship between Ca/P ratio and solubility exists: more soluble and acidic phases present a lower Ca/P ratio [71-72]. As CDHA has a lower Ca/P ratio than HA, it promotes faster connection with bone as it is readily dissolved, promoting Ca^{2+} and PO_4^{3-} release and consequent increase of local pH, creating an ideal environment for alkaline phosphatase action, osteoblasts proliferation and bone matrix synthesis [96].

The peaks characteristic of BC were identified on XRD diffractograms in the produced samples, from native BC to NaOH modified and mineralized BC membranes [59][78-79] [96][89]. In fact, chemical modification of BC did not introduce changes on BC XRD profile, for both types of BC, despite the presence of particles on the surface of this set. It is well known that Iα phase of cellulose I predominates in BC and the alkaline treatment enhances the conversion of cellulose I to cellulose II, but also of cellulose Iα phase to Iβ [56-57]. Thus 14.6° peak could be assigned to (100) Iα plane or (110)/(010) Iβ plane, 16.5° peak to (010) Iα plane or (011) Iβ plane and 22.6° peak to (110) Iα plane or (002) Iβ plane but also to (002) plane of cellulose II [100].

On the other hand, BC characteristic peaks on cBC membranes visualized on XRD diffractograms were generally more intense and broader than their gxBC homologues. According to Hong and co-workers, high peak intensity indicates that material is thicker and the greater width denotes lower crystallinity levels [73]. The producer strain and culture conditions are some of the parameters that influence these features [50-52][60].

In this context, both modification and mineralization did not alter the inherent characteristics of cellulose. On the other hand, the higher crystalline nature of gxBC was revealed.

Mineralized membranes present a novel set of peaks, located at 25.8°, 28.3°, 31.9°, 39.5°, 46.6°, 49.4° and 53.2°, characteristic of CDHA and demonstrating the successful biomimetic apatite deposition on BC substrates [59][78-79][96]. The emergence of these peaks, with equal intensity and width on both BC types, was accompanied by a decrease in characteristic BC peaks intensity, particularly evident in gxBC samples, due to CDHA deposition on the membranes [96].

Therefore, the chosen protocol to modify BC in order to stimulate the deposition of HA was effective, particularly on in-house produced BC membranes (gxBC), with complete mineralization,

from surface to the core of these samples. The same protocol applied on cBC membranes was effective on stimulating the deposition of a ceramic layer, limited to the surface of the samples. If a deeper mineralization is intended, this protocol has to be adjusted for this particular set of membranes.

These results evidence the differences among the diverse origin bacterial celluloses, which have to be considered when one is studying these materials for different applications. The differences between both types of bacterial celluloses are now evident and, as expected, reflected on their final properties.

In general, BC is characterized by its highly hygroscopic and hydrophilic nature and, therefore, when placed in water it is able to swell and retain large quantities of this solvent [45][51][55][62]. Moreover, the absence of chemical and enzymatic mechanisms on biological environment able to cleave the β-1,4-glycosidic linkages, makes this material non-biodegradable (unless it is combined with cellulases) [63].

Results herein presented reveal the higher water uptake ability, associated with higher weight loss, of gxBC membranes, in particular to NaOH modified samples. Water uptake capability or swelling is closely related with materials' network structure [90]. According to Chen and co-workers, during swelling breakage of inter-molecular bonds in partially amorphous regions of BC occurs, which remain available for establishing new connections with water molecules [101]. In addition, alkali treatment with NaOH also causes several structural changes, among which also the breakage of inter- and intra-molecular interactions and increase in materials' porosity [90][94]. In this sense, gxBC increased water uptake and weight loss may be related with these membranes' characteristic structural network. Although increased porosity does not appear evident in microscopic analysis, following chemical modification of these membranes, they demonstrate an open network, maintained after chemical treatment and mineralization. On the other hand, a dense and homogeneous layer was identified on mineralized cBC membranes' surface, as well as compact outer layer. Dense interfaces difficult solvent and solutes diffusion through materials, while this is facilitated in porous structures [102].

Materials' mechanical behaviour under biological environment is critical to achieve a successful performance during the expected period of implantation [58]. Polymers like BC and ceramics like apatite display an antagonistic behaviour: the former generally exhibits lower elastic modulus and strength while the later presents higher values for each of these parameters.

Combination of materials, through composite solutions, is an usual approach to cope with the limitations of each material and take advantage of its strong points [82][103].

As stated by Leitão and co-workers, membrane's density, dimensions, treatment and preparation methods influence materials' mechanical properties and the existence of hydrogen bonds within BC are considered fundamental for materials stiffness and lack of elasticity [46][54-56][104]. In fact, besides affecting water uptake and degradation, both drying state and the strong alkali treatment influences this interactions [90][94][101][105].

Within this context, these factors and effects are well displayed in the herein presented results, particularly for the young modulus, fracture strength and strain-at-failure.

As alkali treatment introduced a decrease the three parameters on both types of BC, the growth of a ceramic layer on these membranes resulted on increased values.

These results, along with previous observations, point out to a degradation of the membranes introduced by the strong alkali treatment. The alkaline treatment used to modify BC induces the break of strong intra- and inter-molecular bonds and the establishment of new interactions in order to form a mineral layer. Even if it was expected that the presence of an apatite layer would improve materials' properties, it did not completely mitigate the effect of the chemical modification protocol, especially in gxBC membranes, the type of BC where modification treatment induces the highest increase both on water uptake and degradation. However, the complete mineralization of gxBC membranes, from core to surface, was effective in re-establish the tensile modulus of these membranes, especially if compared with cBC membranes. Nevertheless, the overall mechanical performance of gxBC membranes was affected by the chemical treatment and, even though it was effective in allowing the complete mineralization of these membranes, it is demonstrating effects on the final physical properties of these membranes.

On the other hand, cBC membranes, as verified on swelling and degradation results, appear less affected, probably due to the lower degree of modification of these membranes and lower mineralization, restricted to their surface. In fact, the presence of apatite on mineralized cBC membranes was sufficient to bring strain at failure close to its initial values and to their improve fracture strength.

Furthermore, mechanical performance, as young modulus and fracture strength, decreases when membranes are studied in wet conditions. Swelling, and consequent materials' moisture is intimately associated with mechanical losses. In order to establish connections with water molecules, the break of intermolecular bonds in partially amorphous regions of BC occurs.

Therefore, more intramolecular hydrogen bonds are present on dry materials than in wet BC membranes [101][105]. On the other hand, as membranes, when both gxBC and cBC are placed in contact with an aqueous solution they become less brittle.

Recently, a study was published reporting the mechanical properties of three commercial membranes used in GBR applications, namely Biomend Extende®, TBR® and Bio-Gide® (Table 12) [106].

Table 12. Mechanical parameters of Biomend Extende®, TBR® and Bio-Gide® commercial membranes, used in GBR [106]

		E (MPa)	ε_r (%)
Wet tested	Biomend Extende®	20	25
	TBR®	10	26
Dried tested	Bio-Gide®	105	31.5

Comparing the obtained results with the ones reported for commercial products, it is possible to conclude that there is room for improvement of the mechanical performance of the herein produced membranes.

In vitro evaluation of materials' cytoxicity is essential when these are intended to consumer-oriented applications, in areas such as the pharmaceutical and biomedical ones [45][52][60][64][108][117-118]. This evaluation is imperative for the selection of materials as cytocompatibility may give indications of good *in vivo* biocompatibility [109]. BC membranes herein produced will be proposed for GBR applications. In this context, a GBR membrane will be at the interface between soft and hard tissues. For this reason, *in vitro* studies were performed with both fibroblasts and osteoblasts.

Both metabolic activity and live/dead results denote that all sets of BC membranes produced present long-term cytocompatibility with both 3T3 fibroblasts and MC 3T3-E1 osteoblasts. Nevertheless, 3T3 and MC 3T3-E1 cells react differently to the presence of the produced membranes, which are more rapidly colonized by 3T3 cells. According to Saska and co-workers, osteoblast-like cells are more sensitive than fibroblasts, which can explain their slower cell spreading [106]. Notwithstanding, there are several culture-related factors that could influence cells behaviour *in vitro*, namely type of culture medium and additives, cell seeding density and culture system [110].

Both fibroblasts and osteoblasts appear to prefer gxBC membranes, particularly the apatite non-covered and covered structures, respectively, with high visibility levels. Despite culture related

factors, cells behavior of cells could also be molded by several factors related to materials, including swelling and surface roughness [41][111]. In fact, gxBC samples are associated to the highest water uptake values as well as to higher roughness, as supported by microscopic analysis. In addition, the osteoblastic cells preference for apatite covered surfaces is also denoted by Rodríguez-González [112].

Promotion of fibroblasts and osteoblasts interaction with the herein produced structures is of the outmost importance, particularly if aiming integration with soft connective tissue with reduction of fibrous encapsulation and bacterial infection, but also control materials' performance during mineralization and bone ingrowth to the membrane [109].

F. Conclusions and perspectives

Development of bacterial cellulose membranes coated with hydroxyapatite for bone regeneration

Conclusions and perspectives

As the main component of skeletal system, innovative solutions to overcome bone related pathologies were always a goal of medical and scientific community.

The herein presented work was focused on the development of mineralized BC membranes envisaged for guided bone regeneration. Membranes herein produced are intended both to prevent soft tissue invasion but also to act as a promoter of bone regeneration. BC is an exopolysaccharide produced by bacteria, which is emerging as a promising material due to its transparency, *in situ* moldability, non-toxicity, inert behaviour, biocompatibility and high mechanical properties. To enhance BC properties and obtain suitable materials for the herein envisaged applications, coatings based on calcium phosphates are studied due to their known bioactivity, biocompatibility and osteoconductivity. In fact, this approach is intended to increase the potential use of BC for bone-related applications.

In this study, several variables were considered, both material-related (pre-modification treatment, viz. drying conditions, and material's origin) and methodological (modification conditions, viz. contact time with alkali and SBF solutions). These parameters are considered for their suspected impact over the final properties of the produced membranes.

Drying conditions influence BC microstructure, being freeze-drying considered the most appropriate procedure to ensure a porous and fibrillated network.

The effectiveness of the employed mineralization protocol, based on chemical BC modification followed by biomimetic apatite deposition, was confirmed, where influence of BC origin, drying pre-treatment and NaOH and SBF contact time was evaluated.

BC is fundamentally non-bioactive and the herein presented mineralization methodology was effective in stimulating the formation of ceramic layer on BC membranes. Furthermore, BC origin, the employed drying procedure and NaOH and SBF contact time are key variables, which influence the formation of an apatite covering.

Both diverse origins herein used BC membranes present different properties, including overall structure and morphology, thickness and crystallinity, water uptake and degradation, mechanical performance and cellular interaction. Consequently, obtained NaOH modified and mineralized structures also present diverse properties.

Chemical modification with strong alkali treatment followed by limited immersion in NaCl-free SBF and normal SBF solution was effective in promoting the formation of an apatite layer, namely calcium deficient hydroxyapatite. In fact, complete mineralization, from core to surface, was

observed in gxBC membranes. However, this is associated with some degree of material degradation with considerable impact on its final properties. Nevertheless, all of the analysed membranes demonstrated good cytocompatibility, both towards fibroblast-like and osteoblast-like cells.

Comparing the two types of BC studied, cBC and gxBC, it becomes evident that an equilibrium must be found between the mineralization effectiveness and the required material's properties for a given application. This equilibrium must be met always keeping in mind the influence of the different variables that play a role in this process.

Due to attractive characteristics over existing bone regeneration strategies, GBR is being developed with promising results in preliminary human studies [113]. According to Dimitriou and co-workers, the ideal solution should be carefully designed and therefore these complete characterization and proposed improvements are recommended before *in vivo* and possible pre-clinic and clinic validation studies, where efficacy and safety but also cost-effectiveness should be counted in [113].

To complete the characterization and understanding the mechanisms of action of the herein proposed materials, different tests could be performed.

These include DMA analysis, to ascertain materials mechanical performance; micro-computed tomography (μ-CT), to ascertain about materials porosity, pore size, distribution and interconnectivity; atomic force microscopy (AFM), to characterize the surface of the produced membranes, including the size and morphological distribution of BC fibers and hydroxyapatite; fourier transform infrared spectroscopy (FTIR), to determine materials' characteristic functional groups and study of BC chemical modifications introduced by the chemical modification and mineralization protocol; contact angle measurements, which enable the assessment of materials' hydrophobicity or hydrophilicity; and mass transfer performance, through diffusion and permeability assays, using relevant solutes and quantify their transport through the produced BC matrices.

To compete with commercial solutions, there is room for improvement of the mechanical performance of the proposed membranes. Taking into consideration the requirements for GBR membranes, particularly proper mechanical properties to ensued space making ability for isolation and stabilization of the defect and its filler, the improvement of fracture strength and control over elongation capability and elastic modulus (strain at failure and young modulus) is important.

Supplementary *in vitro* biological characterization is also crucial for a better understanding of cellular-materials interactions and responses, namely cell adhesion assessment, proliferation assays through dsDNA quantification, alkaline phosphatase and mineralization assays.

In conclusion, the produced BC membranes demonstrate interesting characteristics to be proposed for GBR, combining occlusive properties, desired interaction with target cells and promotion of bone formation within a unique material.

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