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CELL - MATRIX INTERACTIONS:

CELL CULTURING IN COLLAGEN - BASED SCAFFOLDS

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Abstract

The primary aim of tissue engineering is to develop bio-functional matrices that mimic native tissues *in vitro* and help to stimulate healing under normal or chronic situations. Collagen based scaffolds are widely used as temporary or permanent coverings to help dermal wound healing. Under natural conditions, wound healing is affected by many factors, including different cell types, growth factors and several components of the extracellular matrix. Due to the complexity of the cell-to-matrix interaction, many cell-based mechanisms are not yet properly known. The aim of this work was to study the ultrastructure of equine collagen type I scaffolds that are used in tissue regeneration. Furthermore, to simulate the healing process *in vitro* the mechanisms by which a stabilized cell line of the connective tissue interacts with such supports were also carefully studied.

Scanning (SEM) and transmission electron microscopy (TEM) techniques were used to assess the structural features that this collagen scaffold should have to support cell migration and interaction with the host tissue. This study has clearly demonstrated that fibroblasts NIH3T3 can actually migrate through the collagen matrix by embracing collagen fibers with long filopodia and fold them back onto the cell surface to form large intracellular vacuoles.

Gelatin Zymography and Western Blot techniques were also used to verify what role gelatinase B or MMP-9 play in the elaboration of the collagen matrix. Although the activation of this matrix-metalloproteinase is not conditioned by the presence of the collagen scaffold, the extracellular release of MMP-9 and its diffusion onto the collagen matrix was found to be highly correlated with shedding of the microvesicles from the cell membrane. This finding suggests that the MMP-9 activation and the following degradation could be triggered by the release of microvesicles. Their interaction with the prosthetic collagen is probably a precondition for fibroblasts to laying new extracellular matrix during wound healing.

Several comparative studies were carried out to verify the extent by which native equine collagen is structurally modified if combined with other elements (active substances or nanoparticles). Data have shown that, even under these conditions, collagen maintain enough structural stability to sustain cellular migration and proliferation. This finding opens the way to develop innovative dermal substitutes by making collagen bio-functionalized with probes specifically developed to address it toward certain target tissues.

On the whole, the morpho-functional analysis carried out in this study demonstrates that equine type I collagen-based matrices provide conditions favorable for mimicking wound healing *in vitro*. In spite of the complexity of the process *in vivo*, and the variety of factors actually involved in wound healing, it is conceivable that the type of cell-to-matrix interactions – as due to migration, adhesion and proliferation – envisaged in this study may represent the first step in a cascade of reactions leading to tissue remodeling. Given this possibility, the experimental conditions worked out in this thesis for the establishment of a 3D culture in a collagen matrix may constitute a first pivotal attempt for testing additional cell parameters under conditions that could not be adequately controlled *in vivo*.

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Aim of work

The present work is part of a larger project aimed at studying a collagen-based scaffold produced by the Euroresearch Company. The scaffold is used as a medical device in the process of wound healing. The purpose of the project was:

1. to characterize the overall morphology and the ultrastructure of the native equine collagen used to fabricate this scaffold;
2. to establish how selected cell lines interact *in vitro* with the collagen matrix of this scaffold;
3. to study the quality of the regenerative process through the analysis of cell behavior in the prosthetic collagen; and finally
4. to verify how the collagen matrix is modified - both in ultrastructure and cell behavior - when combined with other substances.

To accomplish these goals chapter II investigates the ultrastructural features of the native equine collagen type I scaffold, the BIOPAD® Collagen Scaffold (BCS). The main objective was to establish which structural and biochemical properties should this collagen scaffold have to meet the wound healing requirements *in vivo*. It was my intent to use BCS *in vitro* and verify how this device simulates the overall function of the extracellular matrix under these culturing conditions. The underlying idea of this project was to find out which structural features of the collagen scaffold should allow it to improve the regenerative process of the injured area under normal and chronic situations.

Chapter III addresses the question of how this collagen matrix interacts *in vitro* with fibroblasts. The expectation was to evaluate the mechanisms by which these cells interact with the collagen matrix and, above all, how they contribute to the degradation and remodeling of the collagen fine structure. These questions were experimentally approached by using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). In the course of these studies it was observed that fibroblasts take specific contact with the collagen fibers through the extension of numerous filopodia. In addition, they also proved capable of anticipating their migratory behavior toward the collagen fibers by releasing a number of microvesicles into the surrounding medium.

Chapter IV aims at further investigating the role played by microvesicles in the extracellular processing of the collagen matrix. The primary objective was to verify whether shedding of the microvesicles from the fibroblast plasma membrane could be a precondition for the collagen matrix to be first degraded and be subsequently incorporated intracellularly. The question was approached experimentally by using both enzyme zymography and gold immunocytochemistry with specific anti-MMP9 antibodies. It was found that microvesicles act as vehicles spreading their pro-metalloproteinases cargo through the collagen matrix and conveying it to nearby collagen fibers. Evidence suggests that activation of the metalloproteinase cargo may be triggered by its release from the microvesicles.

As stated in the literature cited in this thesis, wound healing is a rather complex process resulting from the highly elaborate interplay between different cell types, various growth factors and several components of the extracellular matrix. This is why so much research is dedicated to the development of new biomaterials to improve wound healing. Based on these considerations, chapter V of this thesis reports an extensive study aimed at the structural characterization of the collagen matrix following addition of various substances, including:

1. Sodium hyaluronate as an additional constituent of the extracellular matrix (BCS & NaYal),
2. Silver nanoparticles for their anti-inflammatory properties (BCS & Ag) and
3. Biosecure[®] as an antimicrobial agent (BCS & Biosecure[®]).

The aim of this study was to evaluate how these new BIOPAD[®] scaffolds could improve the quality of the collagen matrix and make it more suitable for cell interaction.

As a final objective, this thesis aims at verifying the possibility to load some lipid nanoparticles (the Lipidots™) onto the collagen-Biopad based scaffold and set up the conditions for their stable binding to the collagen matrix. Lipidots™ (LNPs) are lipid nano-emulsion particles composed of an outer lipid shell stabilized by PEGylated surfactants. Because of these properties, LNPs can be employed *in vivo* for the vectorization and delivery of drugs under varying conditions. Chapter VI reports an extensive study that deals with the preparation of the Biopad Collagen Scaffold (BCS) combined with Lipidots™ (BCS/LNPs). The aim was to characterize chemical/physical properties of this new product and explore the potential responses that it may induce in various cell types under varying *in vitro* and *in vivo* conditions.

CHAPTER I

1. INTRODUCTION

1.1. The cell biology of tissue engineering

1.1.1. Structure and function of the extracellular matrix (ECM)

Much of the volume occupied by multicellular organisms is comprised of an extracellular space. This is largely filled with an intricate network of macromolecules that, altogether, constitute the extracellular matrix (ECM). Here cells proliferate, migrate and die. The extracellular matrix includes a variety of proteins and polysaccharides that are secreted and assembled into an organized meshwork in close association with the cells that have produced them. Cells synthesize, assemble and degrade each component of the ECM in response to specific stimuli. At the same time, the ECM is endowed with the capacity to guide and control the functional activities of all cells that contains. This constant interaction between cells and ECM is the essential feature of every living tissue and its capacity to develop and regenerate.

Macromolecules of the extracellular matrix may differ in the extent are assembled, processed and adapted to the functional requirements of the tissue. For instance, the ECM in vertebrates is known to act as a relatively inert scaffold, with the primary role of stabilizing the tissue physical structure (Fig. 1a). However, it is also clear that the matrix plays an active and complex role in the regulation the cell behavior in accomplishing several metabolic functions, including development and differentiation (Alberts *et al.*, 2002).

The two major classes of macromolecules that make up the extracellular matrix are proteoglycans and fibrous proteins. This latter type of proteins includes two additional groups: a first group comprised of such proteins as collagen and elastin whose role is primarily structural; and a second one which includes such molecules as fibronectin and laminin that have an adhesive function. While proteoglycans form a highly hydrated gelatinous substance with the main scope of resisting to the compressive forces exerted on the matrix, the collagen fibers provide the

complementary function of a tensile strength against traction and bending. Adhesion proteins contribute to the matrix organization by providing stable interfaces between cells and fibrous proteins (Fig. 1b). On the whole, however, every component of the tissue has to be so flexible and dynamic to allow the ECM to be remodeled during differentiation and development (Beningo *et al.*, 2004).

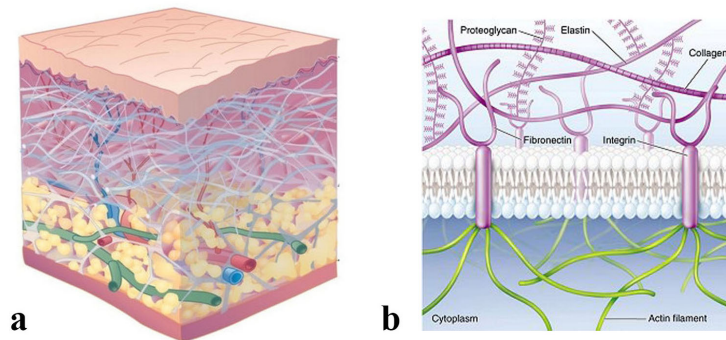


Figure 1. (a) Schematic drawing of ECM. (b) Schematic view of interface between cells and fibrous proteins of ECM.

1.1.2. Cell adhesion

In many connective tissues, cells are immobilized in the extracellular matrix. Cell adhesion plays a key role in regulating how cells are assembled and spatially arranged in these tissues. Cells and ECM interact by Focal Adhesions (FA) and Focal Contacts (FC). The FA are specialized elongated structures that extend for a few square microns along the cell border. On the cytoplasmic side, FA are associated with bundles of actin microfilaments (stress fibers), a cytoskeleton component that provides an internal scaffolding to the cell. FA are docked to the sites where ECM is bounded to the actin cytoskeleton through a complex network of “anchor membrane proteins” (Alberts *et al.*, 2002). These proteins play the key role of activating such specific signaling pathways as protein-tyrosine kinase, serine-threonine kinases, GTPases, phosphatases and other enzymes, in response to the association with actin, tensin, vinculin, paxillin, α -actinin, talin and parvina (Zamir and Geiger, 2001). The “anchor proteins” interact with the

cytoplasmic portion of transmembrane proteins by acting as a bridge between the intracellular environment and the ECM.

Trans-membrane proteins act as receptors integrating intracellular and extracellular signals. Among these, integrins form a family of heterodimeric transmembrane glycoproteins composed by two α and β subunits (Hynes, 1992). There are at least eight β and 16 α subunits of integrins resulting from alternative splicing that, in combination, may give rise to 24 different heterodimers (Van der Fuler and Sonnenberg, 2001).

Integrins control cell behavior by interacting with several specific ECM components. Among these, fibronectin (FN) has perhaps the most relevant role in ECM. It can in fact exist in two main forms: one soluble, called plasma FN (pFN), that is present in the blood and does not react with any adhesion receptors, and a second, multimeric insoluble form, called cellular FN (cFN), which is instead highly adhesive. The cFN is synthesized in a fibrillar form by fibroblasts, epithelial cells and other cell types. Both fibronectin forms are derived from a common precursor of approximately 450kDa comprised of two identical 225kDa subunits linked by a disulfide bridge. FN is involved in a wide variety of cellular functions, such as cell adhesion, cytoskeletal organization, migration, differentiation, oncogenic transformation, phagocytosis and haemostasis. To accomplish these roles, FN needs to be polymerized in a highly controlled manner (Hynes, 1990). Under this form, its adhesive function is carried out through the specific recognition of the repeating sequence of three amino acids, Arg-Gly-Asp (RGD), by the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (Ruoslahti, 1991).

FA have been extensively studied in the past to determine their protein composition and the mechanism by which they are gradually assembled during development. There are three classes of FA, each characterized by a different combination of integrins and "anchor proteins" (Zamir and Geiger, 2001). The "classics" FA, which is normally located along the cell periphery, is made of integrin $\alpha_v\beta_3$, vinculin and paxillin. The Fibrillar Adhesions, located towards the center of the cell, contain integrin $\alpha_5\beta_1$, tensin and few or no phosphor-tyrosines and are associated with fibronectin fibrils (Zamir and Geiger, 1999). Along the cell periphery, especially in

association with lamellipodia of migrating cells, there are also some small adhesions referred to as Focal Complexes (FX) (Nobes and Hall, 1995).

A number of recent studies have clearly shown that FA may change in composition depending on whether fibroblasts are cultured *in vitro* in a two-dimensional system or in a three-dimensional system as it occurs naturally *in vivo* (Cukierman *et al.*, 2001). FA thus represent a key model system to study how ECM and cytoskeleton are structurally linked and how signals from the ECM may affect cell interaction and vice versa (BurrIDGE and Chrzanowska-Wodnicka, 1996).

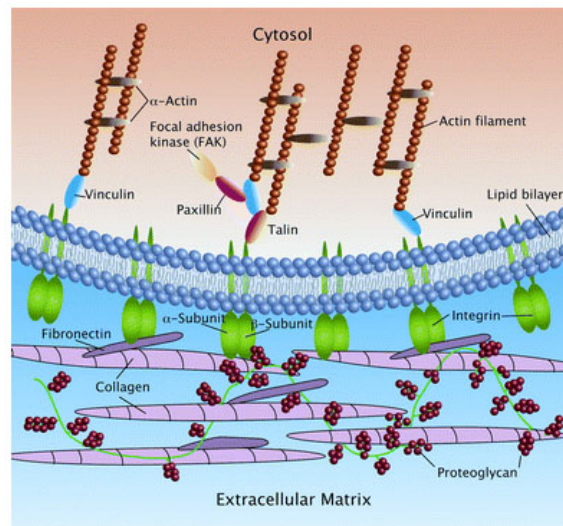


Figure 2. Schematic drawing of focal adhesions (FA) showing the interactions of Integrin molecules with other proteins on both sides of the lipid bilayer (unpublished picture).

1.1.3. Cell migration

Cells and the extracellular matrix interact not only to ensure a proper adhesion, but also to guide cell migration. In many physiological and pathological processes cell migration plays a central role by determining the overall morphology of organs and tissues. Furthermore, cell migration has a fundamental role in many inflammation and regeneration processes. For instance, leukocytes migrate to infected areas in response to chemotactic gradients; endothelial cells migrate to form new blood vessels during angiogenesis (Gobin and West, 2002); fibroblasts and epithelial cells are directed to damaged skin areas for sustaining wound healing (Friedl and Brocker EB, 2000). Even in metastases, tumor cells may leave the original tumor mass and migrate into the surrounding environment to disseminate new cell clones (Friedl and Wolf, 2003).

The extent by which cells are allowed to migrate through the extracellular matrix depends in large measure on how tightly they adhere to the substrate. If adhesion is enough weak, cells cannot adopt a flatten profile and are consequently impeded to migrate for lack of a proper traction on the substrate. If, however, cells are too strongly linked to the substrate, they may certainly change profiles, but locomotion might not yet take place in the absence of a proper flexibility of FA. Cells can actually migrate along a substrate only if interactions are sustained by an intermediate type of force, such as that guaranteed by the synchronous occurrence of many weak, but numerous, binding sites of the matrix proteins (Di Milla *et al.*, 1993).

The occurrence of a cytoskeleton anchoring the cell membrane from inside makes it impossible for the extracellular matrix to tear it apart, while, at the same time, it allows the cell periphery to extend and expand in the form of lamellipodia or filopodia for sustaining cell migration. Knowledge of these mechanisms shows how difficult is to simulate *in vitro* the full range of signals that characterize cell interaction under natural conditions. Cell migration can in fact be considered the result of a periodic alternance between forward and rearward traction forces due to lamellipodia extension along the leading edge and motion of the remaining cell body along the tail.

The mechanical forces associated with cell locomotion induce actin filaments to form a complex network both in the cytoplasm and around the nuclear membrane

(Maheshwari and Lauffenburger, 1998). This in turn changes the overall geometry of the cytoskeleton in such a way as to cause the cell shape to become flat in response to the mechanical traction of the focal contacts. The forward cell movement is thus due to the simultaneous occurrence of two different types of forces: (1) a tensile force, which is generated by lamellipodia protrusions; and (2) a contraction force caused by the following cytoplasmic spasm (Raucher and Sheetz, 2000). These forces are not evenly distributed. They are asymmetrically placed between the leading and the rear edges of the cell, and the difference between the two is such as to create a net force capable of propelling the cell forward. It affects cell migration in response to a gradient of soluble biochemical signals - such as growth factors – that are associated with the ECM (Maheshwari and Lauffenburger, 1998).

The mechanisms involved in cell migration are of chemotactic or non-chemotactic type. Chemotactic mechanisms are directional and guided by specific soluble biochemical signals, e.g. chemotaxis of endothelial cells is activated by the presence of oxygen gradients (Lauffenburger and Horwitz, 1996). The non-chemotactic mechanisms are strongly influenced by the structure and mechanical properties of the substrate material, and by the presence of specific adhesion sites. This latter type of adhesion depends on the presence of specific receptors on the cell membrane and on the chemical properties of the adhering proteins. The extracellular matrix can affect the cytoskeleton organization in relation to the number and distribution of ligands associated with it, and this, in turn, may affect both the speed and the direction of cell migration. However, cells may also remodel the overall geometry of the surrounding ECM by virtue of their capacity to change morphology through the reversible assembly of actin filaments.

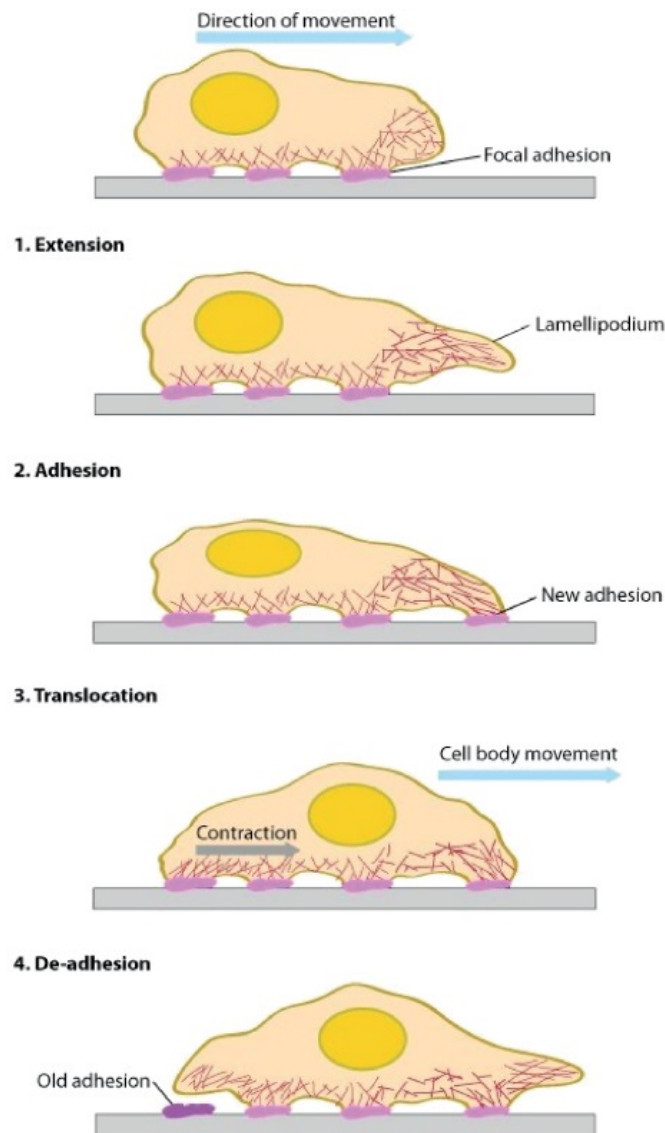


Figure 3. Schematic representation of the different steps of cell migration on 2D substrates. 1. Polymerization of actin filaments at the leading edge is translated into protrusive force. 2. Membrane protrusion facilitates the binding of transmembrane cell surface receptors to the substratum components. New adhesions are rapidly linked to the network of actin filaments. 3. The combined activity of retrograde actin movement and contractile forces produced by stress fibers generates tension to pull the cell body forward. 4. The forces produced by the contractile network combined with actin filament and FA disassembly help to retract the trailing cell edge. Image of the Mechanobiology Institute, National University of Singapore.

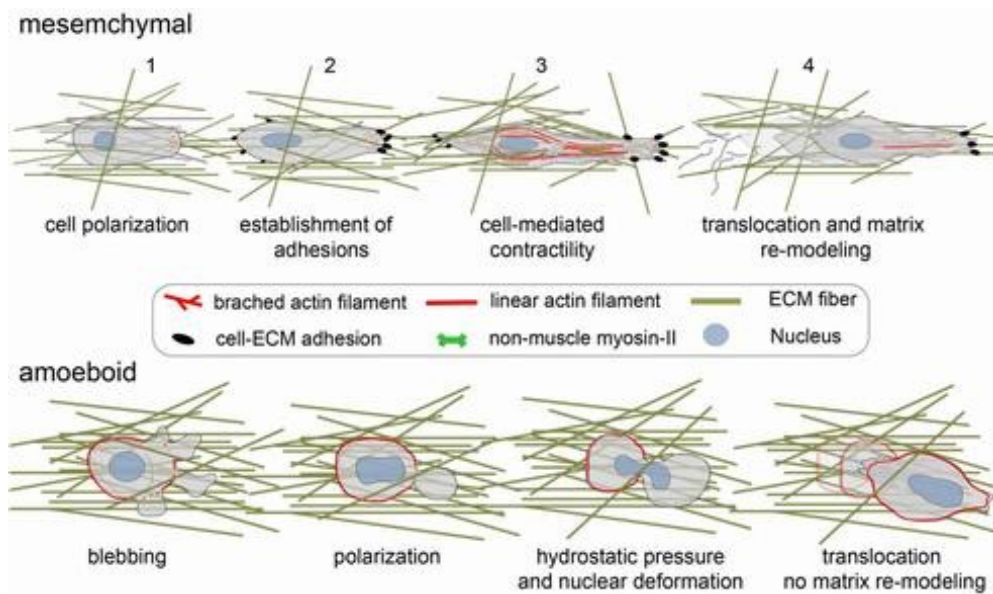


Figure 4. Modes of interstitial migration. In 3D networks, mesenchymal migration proceeds through 4 steps: 1. Cells polarize by producing a pseudopodial leading edge. 2. The pseudopodia contacts the ECM fibers and cell-ECM adhesions are established. 3. Cell contractility tests the adhesion strength and the matrix deformability. 4. Adhesions at the rear are lost and the cell advances leaving behind a path of re-modeled fibers. Activated immune cells migrate in an amoeboid mode by the formation of polarized blebs due to local disassembly of the cell actin cortex. Hydrostatic pressure generated by actomyosin contractility squeezes the cell mass through small interstitial gaps. The nucleus is deformed to adapt to the pore, and once it penetrates the pore the cell can move forward (from Ladoux and Nicolas, 2012).

1.1.4. Cell communication: the membrane vesicles

Cells and the extracellular environment entertain a continuous exchange of information. As well known, cell communication plays a key role in multicellular organisms by allowing tissues to be properly coordinated during development and differentiation (Gilbert, 2000). Cells may communicate by either ligand-receptor interaction, surface recognition of specific cell adhesion molecules or transfer of cytoplasmic components through junctional coupling (Goodenough and Paul, 2009). In recent years, it has been revealed that cells may also communicate through the release of extracellular vesicles and affect the metabolic fate of nearby cells in different ways. Although originally assumed to arise as artifacts in tissues processed for microscopic observations, and therefore regarded as cellular debris with no apparent function (Chargaff and West, 1946; De Broe *et al.*, 1977), microvesicles are now recognized as specific structures released by cells under a variety of physiological and pathological conditions (Vittorelli, 2003; Ratajczak J *et al.*, 2006; Cocucci *et al.*, 2009; Pap *et al.*, 2009; Turturici *et al.*, 2014).

Many different cell types have been shown to release microvesicles (e.g. epithelial, fibroblast, hematopoietic, immune, tumor and stem cells). These vesicles may originate as either exosomes upon fusion of the endosomal compartment with the plasma membrane or be directly released from the cell membrane in the form of shedding vesicles (Heijnen *et al.*, 1999; Ratajczak *et al.*, 2006; Cocucci *et al.*, 2009; Dignat and Boulanger, 2011; Théry, 2011). When released, both types of vesicles, may either be dispersed in the extracellular space next to the site of release and quickly broken down, or reach other tissues at distant sites through such biological fluids as plasma, urine, milk, cerebrospinal fluid and others. Cells can also release some apoptotic bodies in the form vesicular structures larger than exosomes and shedding microvesicles (György *et al.*, 2011). These bodies are formed only during the latest stages of apoptosis and are characterized for containing some nuclear material, several cell organelles and a variety of membrane/cytosolic contents (Elmore, 2007). These different types of vesicles are sketched in the following Figure 5.

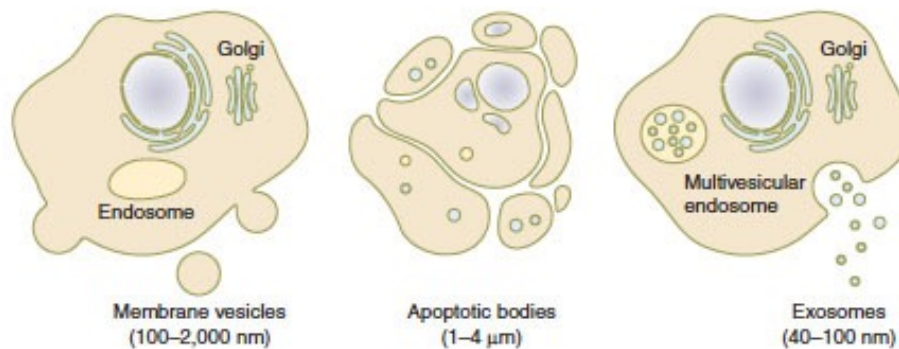


Figure 5: Origins of various classes of extracellular vesicles. The membrane vesicles are formed by the outward blebbing of the plasma membrane and the subsequent release of the vesicle into the extracellular space. Apoptotic bodies are formed during the late stage of apoptosis. Exosomes are formed from the multivesicular endosomal cell compartment (from Turturici *et al.*, 2014).

Since microvesicles may deliver different molecules as enzymes, mRNA, microRNA or RNA interference, the significance of their potential role in cell communication and the mechanisms by which they may act on specific target cells is largely dependent on the nature of their cargo and on that of the receptor(s) eventually exposed on their limiting membrane (Cocucci *et al.*, 2007; Deregibus *et al.*, 2007; Mathivanan *et al.*, 2010).

Transfer of cargo molecules is a selective process, for microvesicles differ from their parental cells by a number of functional properties and roles. Microvesicles are in fact involved in several pathologies, including inflammation (Distler *et al.*, 2005; Köppler *et al.*, 2006), cardiovascular and renal diseases (Collino *et al.*, 2010) and cancer metastasis (Muralidharan-Chari *et al.*, 2010), besides having a beneficial role in modulating a variety of cell responses under physiological conditions (Ginestra *et al.*, 1998, 1999; Sidhu *et al.*, 2004; Kim *et al.*, 2005; Scanu *et al.*, 2008; Collino *et al.*, 2010). Many stimuli have been shown to increase vesicle shedding, e.g. hypoxia, oxidative stress, exposure to shear stress, increased cytosolic calcium ions and

degradation of the cytoskeleton (Beaudoin *et al.*, 1991; Février and Raposo, 2004; Horstman *et al.*, 2004; Hugel *et al.*, 2005; Ratajczak *et al.*, 2006).

Microvesicles may be rapidly broken to release their cargo into the nearby extracellular space. However, interaction with their target cells may also occur by: (1) membrane fusion and transfer of their cargo into the cytoplasm; (2) endocytosis and release of their cargo into endosomal vesicles; and (3) signaling through receptor binding (Mathivanan *et al.*, 2010). These mechanisms are summarized in Figure 6.

Microvesicles shedding produce a number of effects in many cell processes (Cocucci *et al.*, 2007; Deregibus *et al.*, 2007). Tarin (1967, 1969) was able to show for the first time that release of microvesicles and ECM degradation are causally related phenomena in tumorigenesis. Now it is widely recognized that proteolysis of the extracellular matrix during cell migration in tumor growth is due to the release of the protease content of the shedding vesicles. As it has been shown by Taraboletti *et al.* (2002), Candela *et al.* (2010) and Lozito and Tuan (2012) these vesicles contain proteases, including MMP-9 and MMP-2 as well their zymogens and the urokinase-type plasminogen activators (uPA) (Angelucci *et al.*, 2000). The role played by these vesicles in several pathological conditions has been explained as due to a bidirectional exchange of genetic information between stem cells and injured cells (Camussi *et al.*, 2011). The mRNAs and miRNAs released by injured cells may reprogram the phenotype of stem target cells to induce them to acquire specific features of the tissue (Ratajczak *et al.*, 2006). In contrast, vesicles released from stem cells may induce the de-differentiation of those cells that survive injury, resulting in cell cycle re-entry and tissue self-repair by specific mRNAs and miRNAs.

Microvesicles and exosomes have many characteristics that make them ideal drug-delivery vehicles: they can contain both proteins and genetic material, are well-tolerated in the body, as demonstrated by their presence in all biological fluids and proved capable of crossing the plasma membrane to release their contents to specific target cells (Alvarez-Erviti *et al.*, 2011; El Andaloussi *et al.*, 2012; Lee *et al.*, 2012). For a better understanding of the microvesicle properties further studies would be required to clarify how: (i) bioactive molecules are assembled within the vesicles; (ii) what type

of signal(s) trigger their release; (iii) which type of receptors confer them their target-specificity, and finally (iv) what kind of diagnostic potential they might play in several diseases.

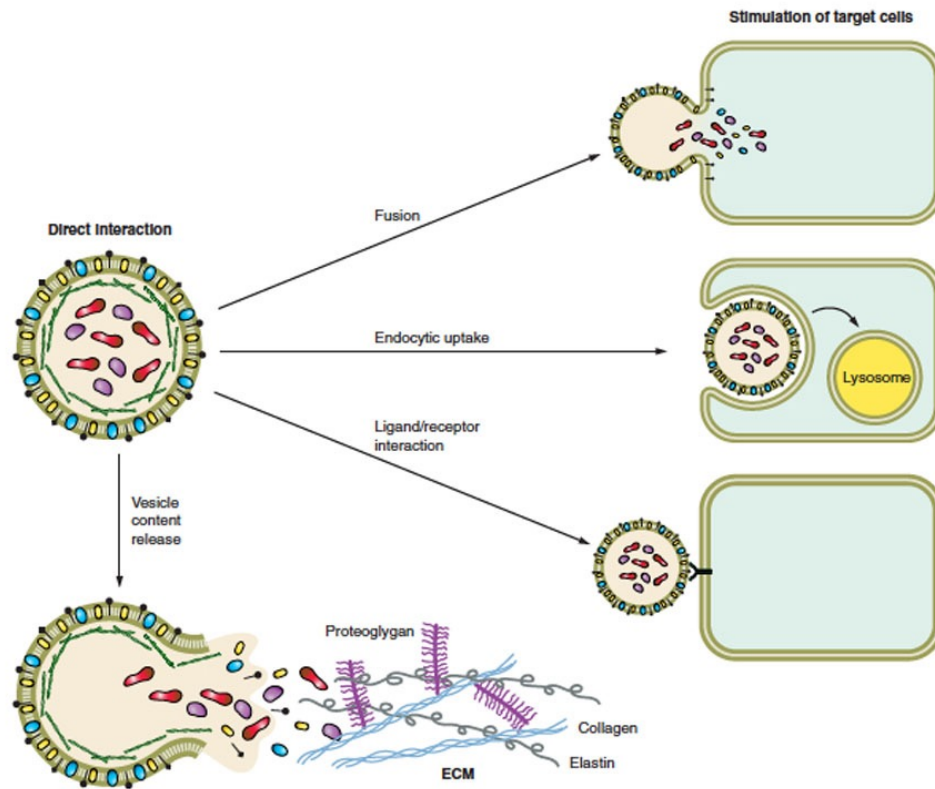


Figure 6. Mechanisms whereby the membrane vesicles achieve their biological effects. The microvesicles may activate cell signaling by physical ligand/receptor interactions or by fusing with their target cells and transferring their contents. They may also be endocytosed by the target cells or may release their contents into the extracellular space. ECM, extracellular matrix (from Turturici *et al*, 2014).

1.2. The process of wound healing

The term wound refers to the disruption of the normal anatomical structure that may occur under certain accidental or pathological conditions. Therefore, the term healing is referred to the complex and dynamic processes that take place in the affected tissue in the attempt to restore its anatomical integrity (Lazarus *et al.*, 1994).

1.2.1. Normal and chronic wound

Under normal conditions, tissue repair entails the re-establishment of an equilibrium between scar formation and scar remodeling. However, multiple factors may impair wound healing by affecting one or more phases of the process. These include local and systemic factors whose influences are not mutually exclusive. The overall outcome of the healing process may be due to single factors playing specific roles or to many working synergistically for a predetermined end (Guo and DiPietro, 2010).

Normal wound healing includes a cascade of four orderly events: hemostasis, inflammation, proliferation and remodeling (Figure 6) (Clark, 1996; Martin, 1997; Werner and Grose, 2008). *Hemostasis*: repair is initiated immediately after injury by the release of various growth factors, cytokines, and low-molecular weight compounds from the serum of the injured blood vessels and from the degranulated platelets. Disruption of blood vessels leads also to the formation of blood clots comprised of cross-linked fibrin and such extracellular matrix proteins as fibronectin, vitronectin, and thrombospondin (Clark, 1993,1996; Martin, 1997). The fibrin clot serves as a provisional matrix and sets the stage for the subsequent healing events (Clark, 2001). Besides providing a barrier to invading microorganisms, the blood clot can also serve as a matrix for migrating cells and as a reservoir of several growth factors required. *Inflammation*: Within a few hours after injury, inflammatory cells invade the wounded tissue. Neutrophils arrive within a few minutes, followed by monocytes and lymphocytes. They produce a wide variety of proteinases and reactive oxygen species as a defense against contaminating microorganisms and for removing cell debris. In addition to these defense functions, inflammatory cells are also important sources of

growth factors and cytokines that stimulate the proliferative phase of wound repair. *Proliferation and Remodeling*: this phase begins with the migration and proliferation of keratinocytes along the wound edges and is followed by proliferation of dermal fibroblasts in the wound neighborhood. Subsequently, these cells migrate into the provisional matrix where they deposit large amounts of newly synthesized extracellular matrix. Furthermore, wound fibroblasts differentiate into myofibroblasts by acquiring the contractile phenotype required for the wound contraction. Massive angiogenesis leads to the formation of new blood vessels, while nerve sprouting occurs along the wound edges. The resulting wound connective tissue is known as granulation tissue due to the granulated appearance of numerous newly formed capillaries. Finally, the occurrence of a new collagen synthesis and collagen catabolism causes the granulation tissue to mature into a scar (Clark, 1996; Martin, 1997). In a normal tissue, collagen forms highly organized molecular arrays. In contrast, the collagen fibers formed in a scar tissue are much smaller and have no organized appearance. A scar tissue is always weaker than a healthy tissue for a wound could never restore the tensile strength typical of a normal tissue. The maximum tensile strength attainable by a wound is approximately 80% of that of a normal skin (Diegelmann and Evans, 2004). Finally, collagen remodeling is followed by collagen degradation. Specific collagenase present in fibroblasts, neutrophils and macrophages process the triple molecular structure of the collagen and break it down into characteristic three-quarter and one-quarter pieces. These collagen fragments undergo further denaturation and digestion by other proteases (Pilcheret *et al.*, 1999; Parks, 1999). Numerous studies have demonstrated a beneficial effect of many growth factors on each of these healing phases. For instance, platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs) and granulocyte-macrophage colony stimulating factor (GM-CSF) have been shown to act on the healing process both in many animal models and also in patients with different types of wound healing disorders (Abraham and Klagsbrun, 1996).

Under pathological conditions the tissue response to injury is at variance from the normal repairing response. On one hand, there might be too much deposition of connective tissue resulting in an altered structure and eventually in a loss of function

(Van Zuijlen *et al.*, 2002). Fibrosis, strictures, adhesions and contractures are examples of excessive healing. Keloids and hypertrophic scars in the skin are examples of fibrosis (Rahban and Garner, 2003). Contraction is part of the normal process of healing but if excessive, it becomes pathological (Nedelec *et al.*, 2000). Chronic non-healing ulcers are the opposite of fibrosis; they are due to insufficient deposition of the connective tissue matrix, thus causing the tissue to become so weakened to fall apart. Ulcers are in a state of chronic inflammation due to abundant neutrophil infiltration along with reactive oxygen species and destructive enzymes. Under these conditions, healing could begin only if inflammation is first brought under control (Diegelmann and Evans, 2004).

A proper balance between degradation and synthesis of collagen is thus essential for a new granulation tissue to be formed on the wounded region and to be later transformed into a mature scar (Nedelec *et al.*, 2000; Madsen *et al.*, 2007; Rohani *et al.*, 2014). During this transition a number of specific growth factors are known to stimulate both synthesis and extracellular release of several matrix metalloproteinases (Werner and Grose, 2003; Armstrong and Jude, 2002; Grotendorst *et al.*, 2004). The resulting generation of collagen fragments in the extracellular milieu is followed by uptake into nearby cells and then by intracellular degradation (Everts *et al.*, 1996; Abraham *et al.*, 2007).

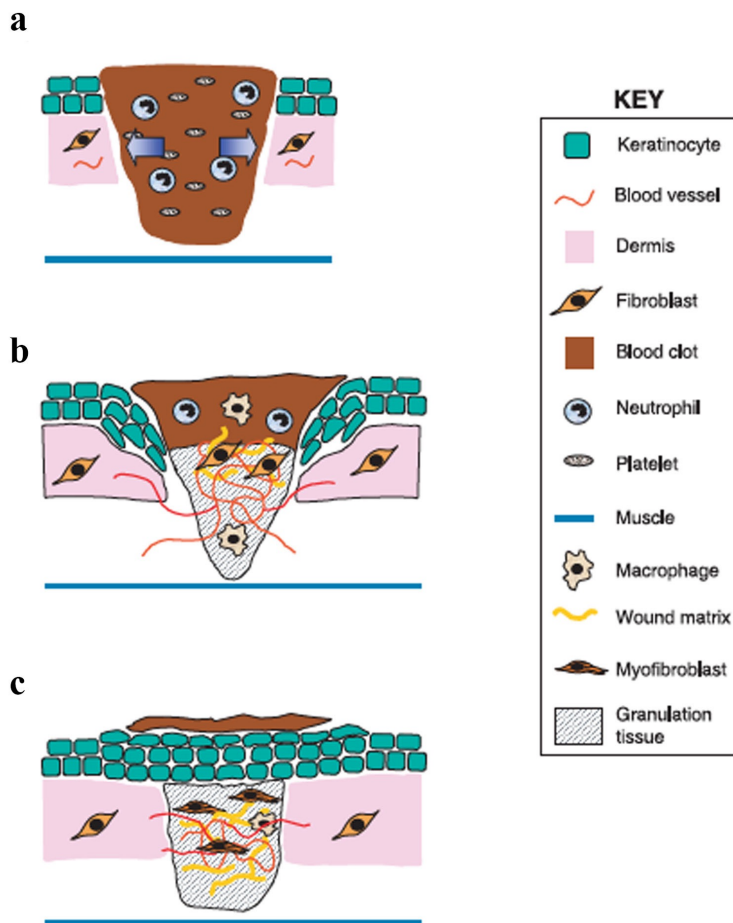


Figure 7. Schematic representation of different stages of wound repair. (a): 12–24 h after injury the wounded area is filled with a blood clot. Neutrophils have invaded the clot. (b): at days 3–7 after injury, the majority of neutrophils have undergone apoptosis. Instead, macrophages are abundant in the wound tissue at this stage of repair. Endothelial cells migrate into the clot; they proliferate and form new blood vessels. Fibroblasts migrate into the wound tissue, where they proliferate and deposit extracellular matrix. The new tissue is called granulation tissue. Keratinocytes proliferate at the wound edge and migrate down the injured dermis and above the provisional matrix. (c): 1–2 wk after injury the wound is completely filled with granulation tissue. Fibroblasts have transformed into myofibroblasts, leading to wound contraction and collagen deposition. The wound is completely covered with a neoepidermis (from Werner and Grose, 2008).

1.2.2. Role of metalloproteinases in normal and chronic wound

Matrix metalloproteinases (MMPs) are a class of collagen-degrading enzymes known to play a key role in many physiological and pathological tissue remodeling processes, including embryogenesis and cancer, besides wound repair (Nelson *et al.*, 2000; Chakraborti *et al.*, 2003; Vu and Werb, 2000).

For wounds to heal the ECM needs to be laid down and then progressively remodeled to reach maturity. MMPs are the enzymes primarily involved in this degradative process. They comprise a family of some 24 distinct but structurally related enzymes that, when acting together, can degrade almost all ECM components (Birkedal-Hansen, 1995; Shapiro, 1998). They can be divided into four subgroups based on their substrate specificity: collagenases, stromelysins, type IV collagenases and membrane type-MMPs (MT-MMPs).

MMPs activity can be controlled at various levels: transcription, activation of the zymogen form, and inhibition of the active enzyme (Stamenkovic, 2003). In resting-state adult tissues, MMPs may be expressed at low levels or not be expressed at all, but their expression can be rapidly stimulated by numerous cytokines and growth factors as well as by specific cell interactions (Nelson, 2000; Vu and Werb, 2000; Sternlicht and Werb, 2001). The MMPs are secreted in latent forms which are then cleaved to become biologically active. Their activities are also tightly regulated by inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which bind the active MMP enzyme with high affinity. Four TIMPs (TIMP 1-4) have been identified; each binding and inhibiting MMP activities to a varying extent (Mannello and Gazzanelli, 2001). MMPs are produced by many of the cell types involved in wound healing, including fibroblasts, keratinocytes and inflammatory cells. Their expression is modulated in response to signals from cytokines, growth factors, cell-matrix interactions and altered cell-cell contacts (Stamenkovic, 2003).

Owing to their wide range activity, MMPs were initially considered as equivalent to proteolytic “bulldozers” with the main function of destroying the ECM scaffold. However, it became soon evident that these proteolytic enzymes not only control ECM homeostasis and remodeling, but also affect many physiological processes during

development (Vu and Werb, 2000; Mannello *et al.*, 2005). By virtue of their proteolytical activity, MMPs can regulate cell migration and generate ECM fragments with new functions. For example, cleavage of laminin-5 and collagen type IV makes accessible some of the cryptic sites that promote migration and cell motility (Egeblad and Werb, 2002). Evidence suggests that MMPs also have some "shedase" activity, i.e. the ability to release bioactive molecules from the cell surface that may act as growth factor precursors or adhesion receptors (Mannello *et al.*, 2005). The number of potential substrates on which MMP may act is so high for the biological processes in which they are actually involved remain to be fully explored (McCawley and Matrisian, 2001).

MMP expression in normal wound healing is complex, MMP concentration and activities changing according to the phase of healing (Werner and Grose, 2003; Armstrong and Jude, 2002; Grotendorst *et al.*, 2004). During the inflammatory phase, an increase in MMP-9 expression is correlated with neutrophils and macrophages infiltrating the wound to phagocytose bacteria (Mannello *et al.*, 2005). MMP-9 activity decreases in the subsequent proliferative phase, when fibroblasts begin their migration, while expression of other MMPs, particularly MMP-2 and MMP-1 begin to increase (Armstrong and Jude, 2002). Besides being key players in wound repair and in ECM remodelling, MMPs have other important functions, including regulation of cell growth and differentiation. They can alter cell motility, affect cell to cell interactions and release growth factors and cytokines to induce cell proliferation and growth (Löffek *et al.*, 2011).

MMPs have been shown to be abnormally expressed in diabetic wounds to such an extent as to be taken as reliable markers and mediators of many impaired wound healing processes in diabetes (McLennan *et al.*, 2008). In chronic diabetic foot ulcers an increased proteolytic environment contributes to the failure of the wound to heal due to a raise in MMPs activity and a decrease in TIMP-2 concentration. New treatment strategies for healing chronic diabetic foot ulcers could therefore be directed towards reducing MMPs activity and increasing the local levels of TIMPs (Lobmann *et al.*, 2002, 2006; McLennan *et al.*, 2008).

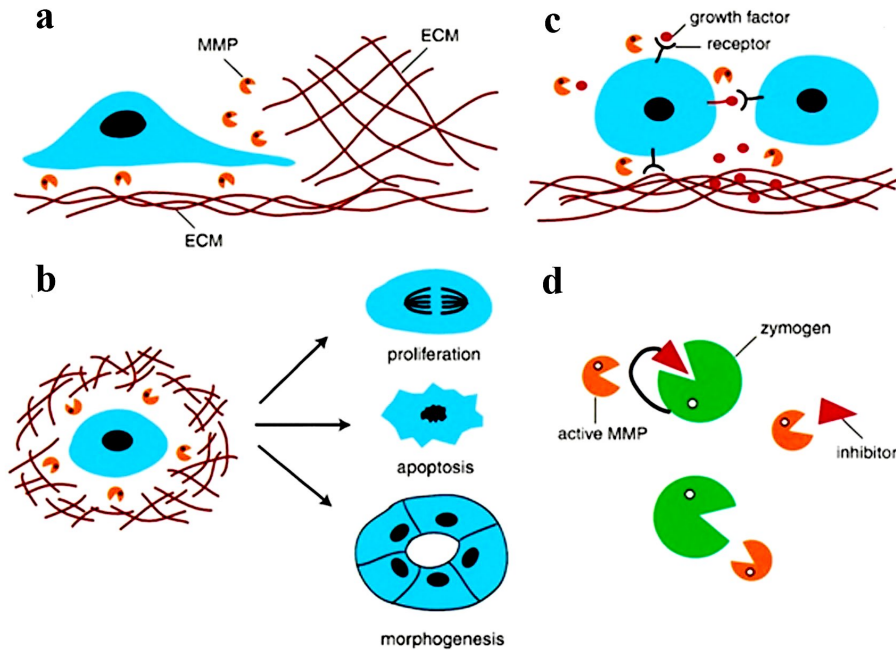


Figure 8. Modes of action of the matrix metalloproteinases. (a), MMPs may affect cell migration by changing the cells from an adhesive to non adhesive phenotype and by degrading the ECM. (b), MMPs may alter ECM microenvironment leading to cell proliferation, apoptosis, or morphogenesis. (c), MMPs may modulate the activity of biologically active molecules such as growth factors or growth factor receptors by cleaving them or releasing them from the ECM. (d), MMPs may alter the balance of protease activity by cleaving the enzymes or their inhibitors (from Ansari *et al.*, 2013).

1.3. Skin substitute for wound healing

1.3.1. Tissue engineering

Tissue engineering is an interdisciplinary field of regenerative medicine. It makes use of principles from biology and engineering to develop skin substitutes that may temporarily replace functions partially lost by damaged tissue(s). The adoption of skin substitutes is expected to restore the structural and functional properties of the wounded tissues at conditions preceding injury (Langer, 1997; Clark *et al.*, 2007). There are three general strategies to develop tissue-engineered products: (1) make use of cells to be transplanted in the injured tissue; (2) develop some synthetic polymers or other biomaterials in the presence of specific additives; or (3) use of cells embedded within a three-dimensional matrix, i.e., skin substitutes comprised of keratinocytes and fibroblasts encapsulated in a biological or synthetic bio-absorbable polymer (Figure 9) (Bannasc *et al.*, 2003; Jimenez and Jimenez, 2004; Kopp *et al.*, 2004).

Some of the methods developed for *in vivo* or *in situ* tissue engineering make use of scaffolds tailored for maintaining an adequate internal “space” inside the regenerating tissue so that the original features may be restored by cell growth (Hollister *et al.*, 2002). A much “simpler” approach to tissue regeneration is realized through the use of bioactive scaffolds that stimulate angiogenesis with the patient’s own cells (Hing, 2004). Many biological scaffolds simulating new deposition of the skin extracellular matrix (ECM) are also available for wound healing (Balasumabramani *et al.*, 2001; Ehrenreich and Ruszczak, 2006; Boateng *et al.*, 2007). As compared to *in vitro* tissue engineering, this latter approach has the advantage that scaffolds can be applied on the wound in the absence of any cell manipulation.

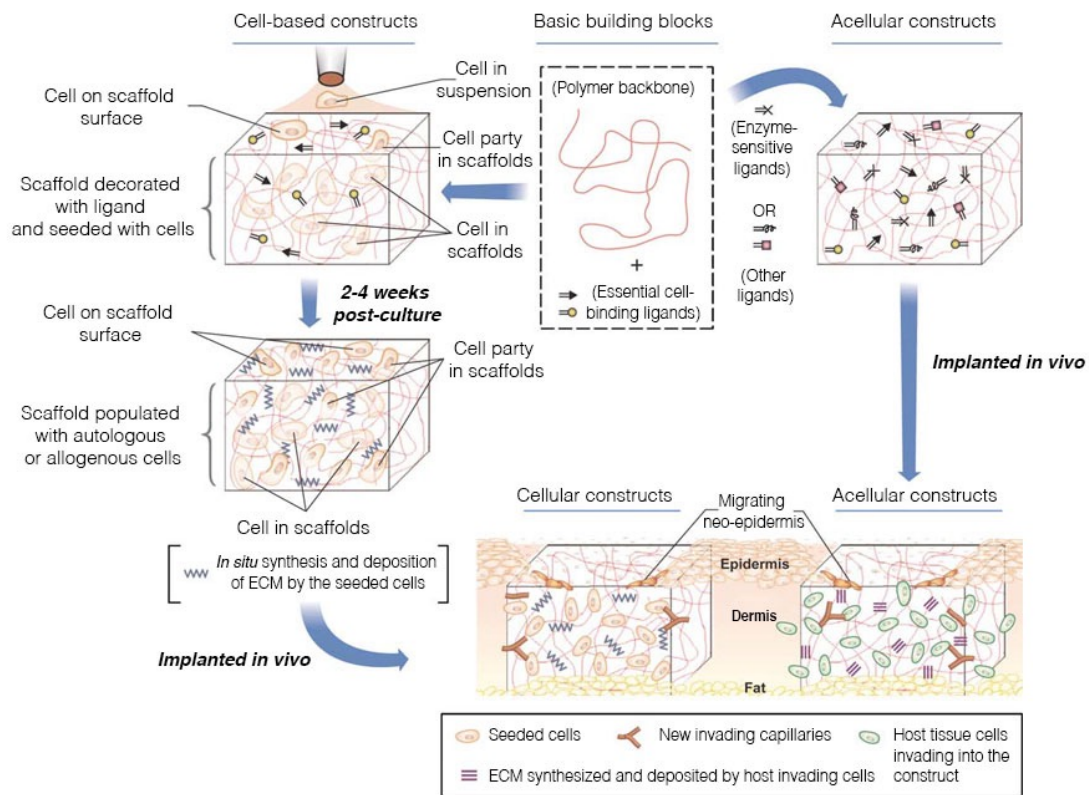


Figure 9. The basic building blocks of a tissue-engineered construct are a biopolymer, one or more biomimetics, and perhaps cells. The biopolymer must be biocompatible and biodegradable. Biomimetics are selected to add function to the biopolymer. The bioactive function may be cell-binding activity, growth factor activity, or growth factor-binding activity, or enzymatic activity or enzyme-binding activity. Cells added exogenously to the engineered biopolymer may be used to induce a functioning tissue-substitute for transplantation (left schemata). These cells may be stem cells or genetically engineered cells. When an acellular biopolymer is implanted, enough information must be available within the engineered construct to support endogenous tissue cell ingrowth and appropriate differentiation for tissue formation (right schemata). (from Clark *et al.*, 2007)

1.3.2. 3D scaffolds for tissue engineering

Tissue engineering has traditionally made use of 3D scaffolds as temporary surrogates for the extracellular matrix (ECM) to host cells from natural tissues. In that respect, the role of scaffolds was limited to that of ‘cell-friendly’ vehicles that could support cell viability and growth, and efficiently deliver cells to target sites (e.g. wound bed, bone repair site etc.). However, the field is currently experiencing a steady transition from cell scaffolds intended as largely inert and permanent supports to that of biodegradable scaffolds capable of conferring certain bio-functionalities to them (Wang *et al.*, 2013).

The criteria for an ideal skin substitute could be defined by the capacity to express the same functional and structural characteristics that closely match those of a skin autograft (Phillips, 1998; Aldini *et al.*, 2008). Based on these criteria, a 3D-scaffold should then provide: i) a mechanical support for the regenerating tissue; ii) a dynamic interaction with the surrounding tissues; iii) an efficient transport for nutrients and metabolites; iv) an adequate biocompatibility to minimize toxicity both at tissue and systemic levels; and finally v) the techniques employed for its preparation should be easily accessible (Balasubramani *et al.*, 2001). The choice for the *right* biomaterial is a key issue in the production of 3D-scaffolds for tissue engineering. Different materials have been used so far and the choice is largely correlated with the type of tissue to be regenerated (whether soft or hard connective tissues) and with the functions it accomplishes in the organism. Although a large variety of materials can be employed to produce three-dimensional scaffolds, biocompatibility is the prime feature to take into account to avoid unwanted secondary reactions (Balasubramani *et al.*, 2001; Cornwell *et al.*, 2009).

To date the use of such biomaterials has given numerous positive results, particularly for the regeneration of relatively simple tissues, such as epithelia, bone and cartilage (Ungaro *et al.*, 2006). Regeneration of complex tissues (e.g., liver tissue, smooth muscle, highly vascularized tissues) is made more difficult by the present impossibility to control the actual concentration of signaling molecules as well as cell differentiation and proliferation during tissue repairing (Murphy and Evans, 2012). For

highly cellularized tissues, cell differentiation is highly dependent upon the capacity to control cell–cell interactions through cell signaling, cell adhesion, nutrient perfusion and O₂ consumption. For connective and contractile tissues, the emphasis is in fact on the mechanical and substrate properties that 3D scaffold must have to define the cell topographical and directional cues. By comparison, 3D scaffolds for simpler tissues should have such basic properties as suitable pore sizes for cell to perfuse and an adequate stability to sustain cell migration during repairing time.

The bulk fabric for such scaffolds can be made of: (1) Synthetic polyesters such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), their copolymers (PLGA, PLLA, etc.), and poly (caprolactone) (PCL) (Gunatillake and Adhikari, 2003); (2) Natural polymers such as alginates, chitosan, collagen, GAGs and elastin, gelatin and fibrin (Malafaya *et al.*, 2007).

The distinction between these two categories of products has proved crucial in relation to the question of the scaffold persistence and degradation on the wound itself. It is inescapable that whatever the scaffold composition, its persistence on the wound has to be only temporary, for it must disappear with healing. Furthermore, its disappearance must be temporally related with the formation of a new tissue by the resident cells. Given the diversity of the physico-chemical conditions provided by any given injured site, this level of control is almost unattainable for conventional biodegradable polymers. This is because they may be hydrolyzed and/or dissolved independently of any cell activity by the host. On the other hand, scaffolds constructed from native protein aggregates are commonly susceptible to metabolic degradation by the usual array of extracellular matrix proteases. Biomimetic (native protein-based) scaffolds represent in fact a natural constituent of the cell-matrix and hence are equally subject to its biological turnover and remodeling. Biomimetic scaffolds will thus tend to be removed by the host cell activity as new matrix is gradually produced. This is not the case for conventional polymers, which may either disappear too rapidly or too slowly. In the first case they would not provide a mechanical support strong enough for cells to migrate, while, in the second, they might actually inhibit or counteract cell-based renewal (Brown and Phillips, 2007). Scaffold removal and replacement cycle are known

to be central issues for sustaining growth and repair natural under natural conditions (Fini *et al.*, 1992; Moses *et al.*, 1996; Page-McCaw *et al.*, 2007). In connective tissues, scaffold proteins are known to be sequentially removed and then replaced by more permanent substrates through well coordinated cell-dependent process. Although it is neither feasible nor desirable to try to mimic the entire sequence, cells could not fabricate any new native structures if they are actively blocked from completing the entire sequence of events. Such a blocking is likely to occur when the synthetic polymer fail to disappear from the wound bed and so its persistence create a sort of mechanical hindrance to the surrounding cell environment (Brown and Phillips, 2007).

A fundamental question for the scaffolding design is how much or how little similarity with the target tissue is needed for it to qualify as biomimetic. The success of tissue engineering therefore rests on the assumption that adequate function can be achieved via compositions and structures that are much simpler than the native tissues, and which are realizable via manipulation of cells and biomaterials *in vitro* (Freed *et al.*, 2006).

The ideal scaffolds should provide a framework and initial support for cells to attach, proliferate and differentiate and form an extracellular matrix (ECM) (Agrawal and Ray, 2001). It should be noted that scaffold surface topography and chemistry (wettability, softness and stiffness, roughness), microstructure (porosity, pore size, pore shape, interconnectivity, specific surface area) (O'Brien *et al.*, 2005) and mechanical properties (Engler *et al.*, 2006; Peyton and Putnam, 2005) have been shown to significantly influence such cell behaviors as adhesion, growth and differentiation, and to affect the bioactivity of scaffolds used in *in vivo* regeneration applications of such tissues as cartilage, skin and peripheral nerves.

For tissue engineering, it is important to understand how cell behavior is physiologically related to the mechanical properties of 3D extracellular scaffolds and this knowledge is obviously highly relevant for the design of bioactive scaffolds. Controlling cell behavior by modulating the local extracellular environment is also a critical step in the development of the next generation of bioactive scaffolds. Therefore, given the complex nature of many biological tissues and the cell ability to maintain it

through elaborate turnovers, the scaffold has to be sufficiently robust to support local cell/tissue interactions, but also enough flexible to be susceptible of cell-mediated remodeling.

1.3.3. *In vitro* cell-matrix interaction (the 3D cell-culture)

The spatial dimensions of the scaffold constitute one of the fundamental features accounting for biomimesis. It is selfevident that the use of a scaffold, as opposed to that of a flat culture surface, constitutes the essence of a three dimensional growth. Under these conditions, cells are not expected to grow in an additional vertical axis, but rather to expand in all three dimensions to generate a completely new topological setting, highly comparable to that of an *in vivo* tissue (Lawrence and Madihally, 2008). This topological information can be either homogeneously distributed or polarized, unlike the one in two dimensional cultures that provide only polarized cues. As such, it may not be used to simulate native conditions for fibroblasts or other stromal cells.

It has already been reported that the *in vivo* 3D extracellular environment strongly influences cell shape and differentiation (Di Milla *et al.*, 1993; Behonick, and Werb, 2003). While it is highly recognized that the extracellular matrix may exert strong influences on these processes by surface chemistry, it is also known that such physical cues as topographic distribution, mesoscale mechanical forces and 3D spatial organization may have great influence on cell orientation, locomotion and direction (Brown and Phillips, 2007; Grinnell and Petroll, 2010; Chang and Wang, 2011).

Considering the role that cell-ECM interaction may play in the organization of a scaffold matrix, it is essential to determine meaning and ambiguities behind the concepts of 2D and 3D cell culture. Many *in vitro* experiments have shown that cells have different responses with respect to colonization, proliferation and differentiation on 3D scaffolds than on traditional 2D-tissue cultures. Under 2D conditions, *in vitro* cultured cells are spread and attach to a flat rigid glass or plastic surface (Figure 10). Cells in 3D adopt more *in vivo* like morphologies by responding more specifically to mechanical signals through intercellular signaling and cellular migration (Nisbet *et al.*, 2009; Grinnell *et al.*, 2003). A 3D environment also influences the surface diffusion of

adhesion proteins, growth factors and consequently of all enzyme activities that are essential for an optimal cell viability (Dutta and Dutta, 2009). All of these cell-to-cell interactions have of course a bearing on the cell capacities to attach and spread on the intercellular scaffold itself. A 3D environment is more complex than it may appear at first sight. Cell adhesion to a 3D scaffold changes from point to point of the cell contour. In fact cells similarly attached on a substrate, may differ in locomotion, interaction of neighboring cells and lateral inhibition or convergent cell extension in the scaffold. Tissues differing in overall architectures may require different microenvironments to undergo repair and regeneration. This basic principle can be easily demonstrated by using scaffold matrices differing in pore sizes (Loh and Choong, 2013).

Scaffold matrices should have the following basic properties: (1) be biocompatible, bioresorbable and biodegradable during tissue regeneration process, (2) be porous to such an extent to allow cells and nutrients, oxygen and wastes to diffuse unimpaired through the matrix (3) express such surface properties as wettability, stiffness and compliance to support cell attachment, proliferation and differentiation, and finally (4) provide sufficient mechanical strength to withstand stresses at the site of implantation (Lawrence and Madhally, 2008).

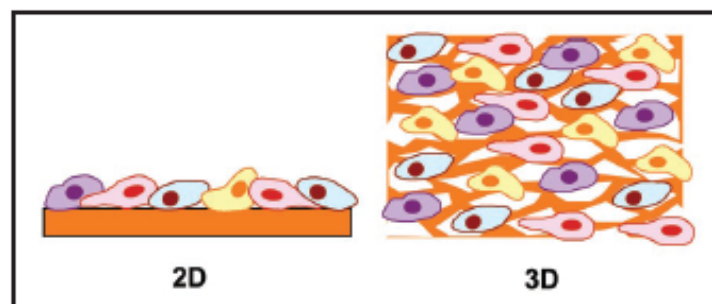


Figure 10. 2D and 3D cell culture (from Lawrence and Madhally, 2008).

1.3.4. Effects of scaffold physical structure on cell behavior

The influence exerted by the spatial architecture of skin substitutes on cell behavior has been extensively explored. Studies have shown that such physical properties as stiffness, hydrophilicity, porosity, pore size and void volume affect cell morphology, attachment and function (Lawrence and Madhally, 2008). Particularly significant in this respect is the scaffold topography that may directly affect cell alignment, orientation, spreading and cell attachment (Ranucci and Moghe, 2001; Zeltinger *et al.*, 2001; Stevens and George, 2005). Other features as pore size affect cell binding, migration, depth of cellular in-growth, cell morphology and phenotypic expression (O'Brien *et al.*, 2005). An appropriate pore size allow cells to spread into the matrix through specific cell to cell interactions. Cell ingrowth is supported an “optimum” pore size range. Outside this range, cells fail to spread and are thus impeded to form interconnected networks. Many cell types, including endothelial cells, may not colonize certain matrix scaffolds if pores exceed 300 μm in mean size due to the difficulty spreading over long distances (Zeltinger *et al.*, 2001; Salem *et al.*, 2002). It has been determined that 100–150 μm pores are within the best range for supporting cell ingrowth for the majority of connective cell types, except than for osteoblasts and osteocytes (Yannas *et al.*, 1989). Furthermore, high porosity and pore interconnectivity have been conventionally perceived as critical properties in ensuring spatially uniform cell distribution, cell survival, proliferation and migration (Leong *et al.*, 2003). Matrices with high porosity values provide healthier healing environments, especially in case of chronic wounds, due to their capacity to absorb more efficiently blood and lymphatic fluids from the wounded area (Lawrence and Madhally, 2008; Wang *et al.*, 2013).

Tissue topography is a complex concept for it accounts for both migration and movement. Cell migration is the statistical availability of sufficient scaffold binding sites for cells to exert some traction forces, whereas movement depends on the actual existence of adjacent gaps in the scaffold which cells can accede (i.e. *space to move*). Indeed, a similar principle has also been evoked to account for the influence of the

adhesion site density on cell migration (Ahmed *et al.*, 2000; Grinnell and Petrol, 2010). It has been recently demonstrated that cells move through the pores of a 3D scaffold matrix by aligning along the collagen fibers. For example, fibroblasts can be guided by the microchannels (30-40 μ m diameter) that fibers of soluble phosphate-based glass (PGFs) may form in dense collagen scaffolds (Nazhat *et al.*, 2007; Pena *et al.*, 2010).

The role of substrate mechanics in conditioning cell behavior has been extensively investigated and is now well established chapter of tissue engineering (Karamichos *et al.*, 2006; Dado and Levenberg, 2009). Cell interaction with the ECM is sustained by two different, but concurrent, signaling mechanisms. (1) Ligation-induced signaling, i.e. ECM ligand-receptor mediated signaling (e.g. RGD-Integrin binding) which depends on the ECM surface chemistry, and (2) traction-induced signaling which depends on the mechanical stimuli transmitted through the ECM. Discher *et al.* (2005) have discussed how the substrate mechanics may modulate the cell phenotype and the proliferation activity in a manner similar to biochemical signals. These studies have been performed on a variety of substrates, more commonly on collagen-coated silicone or polyacrylamide gels (Wang and Pelham, 1998). Ghosh *et al.*, (2007) found that adult human dermal fibroblasts can modify their mechanical response in relation to the substrate stiffness. In particular, they observed that these cells can support higher cytoskeletal moduli and a more stretched actin cytoskeleton, if allowed to grow on stiffest hydrogels comprised of hyaluronan and fibronectin, proliferated normally.

In order to fully understand cell proliferation in the context of specific tissues, it is important to create a three-dimensional environment that mimics as closely as possible the cell-matrix interaction *in vivo*. This has necessitated the development of physiologically relevant, tissue-engineered constructs that could be controlled in relation to the aimed cell responses (Carlson and Longaker, 2004; Freed *et al.*, 2006; Dado and Levenberg, 2009).

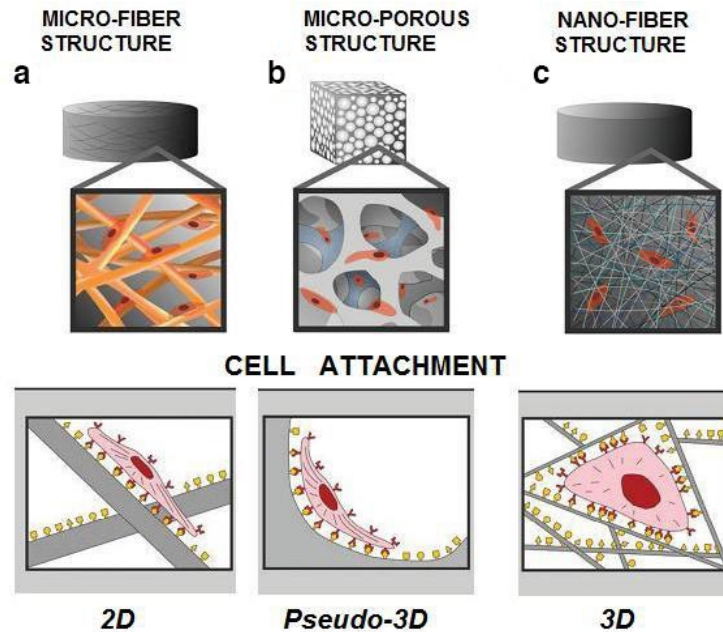


Figure 11. Schematic view of how scaffold architecture can affects cell binding and spreading. (a) and (b) Cells binding to scaffolds with microscale architectures flatten and spread as if cultured on flat surfaces. (c) Scaffolds with nanoscale architectures have larger surface areas to adsorb proteins, presenting many more binding sites to cell membrane receptors. The adsorbed proteins may also change conformation, exposing additional cryptic binding sites (from Stevens and George, 2005).

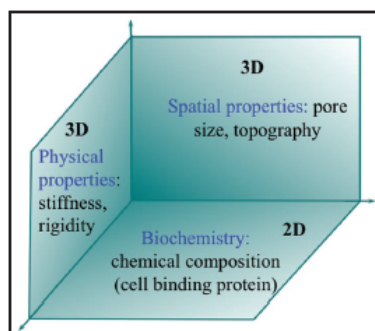


Figure 12. Factor influencing cel colonization in 3D (from Lawrence and Madihally, 2008).

1.3.5. Collagen-based matrices

As a major constituent of the extracellular matrix, the collagen plays a key role in wound healing by acting as an anchoring support for migrating cells (Friess, 1998; Friedl and Bröcker, 2000). Because of its relative abundance in bodily proteins, low immunogenicity and high stability, the collagen is *par excellence* the best biomaterial employed as a prosthetic medical device to treat dermal injury (Murphy and Evans, 2012; Chattopadhyay and Rainers, 2014). However, to satisfy these requirements, the collagen scaffold should be mechanically integral, structurally uniform and have a matrix porosity suitable for cells to migrate and be retained up to the completion of tissue remodeling (Laurence and Madihally, 2008).

Prosthetic collagen matrices may differ in type and concentration as well as in the chemical nature of the substances added to modulate matrix absorption and/or stability (Lin and Liu, 2007; Brett, 2008; Miron-Mendoza *et al.*, 2010). Therefore, a wide variety of collagen-based dressing differing in structural and biochemical properties have been developed with the intent to simulate the overall function of the extracellular matrix and improve the regenerative process of the injured area in normal or chronic situations (Ghibaud *et al.*, 2009; Karr *et al.*, 2011). Amongst the physiological variables that characterize the collagen matrix, its overall three-dimensional architecture is most relevant since it conditions such cell behaviors as proliferation, migration and ultimately signaling and differential gene expression (Astashkina *et al.*, 2012; Iyer *et al.*, 2012; Lu *et al.*, 2012).

An important structural feature of 3D matrices is porosity. During collagen extraction, this parameter can be affected by several factors, including the type of solvent, the enzyme activity and the pH. In addition, time and freezing temperature during lyophilization do also affect the appearance of the final product (Murphy and O'Brien, 2010). Earlier studies have clearly shown that for an optimal pore size range the collagen matrix should be freeze-dried at a temperature comprised between -20°C to -196°C . In general, however, the lower the temperature, the smaller the pore size (Doillon *et al.*, 1986; Karageorgiou and Kaplan, 2005). Collagen dressings are meant to create a wound environment favorable for wound healing. Wound closure is in fact

attained by cells migrating from the wound margins that ultimately give rise to a new granulation tissue (Schultz *et al.*, 2003).

Certain collagen-based dressings are known to enhance fibroblast infiltration in the wound bed, due perhaps to their hydrophilic properties. They may also stimulate collagen deposition by attracting fibroblasts and promote their migration via fibronectin binding. They may help preserve leukocytes, macrophages, fibroblasts, and epithelial cells within the wound and maintain constant the chemical and thermostatic microenvironment of the wound (Doillon and Silver, 1986; Palmieri, 1992). Of the various effects that collagen dressings may induce the inhibition or deactivation of an excessive titre of MMPs in the wound should also be mentioned (Brett, 2008). As well known, in fact, a MMP excess may contribute to install a wound chronicity.

Application of an exogenous collagen may ultimately enhance the wound tensile strength and promoting re-epithelialization, although it is still unclear how these cell processes would also affect scarring on the long run (Doillon and Silver, 1986).

1.3.6. Bioactive molecules for regenerative medicine

One of the main purpose of tissue engineering is to design biomaterials that promote tissue regeneration by favoring cell invasion of the scaffold matrix. Many medical devices incorporate new chemicals with therapeutic values to overcome some of the disadvantages associated with the topical application of pharmaceuticals. Drugs may affect the wound healing process either directly or indirectly as cleansing agents removing necrotic tissues, antimicrobials preventing infections or growth factors aiding tissue regeneration (Boateing *et al.*, 2007).

A bioactive 3D-scaffold can be obtained in several ways. The main strategy is the incorporation of a bioactive molecule in the polymer matrix by simple diffusion or immobilization through electrostatic interactions or covalent bonds (Biondi *et al.*, 2008). However, the molecule bioactivity and the structural organization of the matrix can be both affected during production of the scaffold. There are a number of different collagen dressings available today, which employ a variety of carriers and cross-linking agents such as gels, pastes, polymers, oxidized regenerated cellulose (ORC) and

ethylene diamine tetra-acetic acid (EDTA). The amount of collagen present in these products vary in concentration and type (i.e. bovine, porcine, equine, or avian sources) and in relation to the degree of purity. Ingredients as alginates and cellulose derivatives can enhance absorbency, flexibility and comfort, and help maintain a moist wound healing environment and control pathogens within the wound (Murphy and Evans, 2012).

Several factors need to be considered to ensure a successful wound healing. In case of chronic wounds such factors as drug therapy and the patient health records must all be reviewed and properly addressed before a particular wound dressing is applied.

Advanced wound dressing products do indeed optimize healing conditions for the wound environment. With the advent of tissue engineering, skin substitutes are being created that not only provide novel effective temporary coverage of wounds, but are also changing the paradigm of wound management. By supporting the wound with growth factors and biologic substances, they can help augment or modulate the wound healing process itself.

CHAPTER II

2. The BIOPAD® Collagen Scaffold (BCS)

This section aims at investigating the ultrastructural features of the native equine collagen type I scaffold, the BIOPAD® Collagen Scaffold (BCS). This type of scaffold is a primary wound dressing already used as a medical device to control minor bleedings and any kind of ulcer or skin lesions. It is a sponge-shaped device constituted exclusively by lyophilized type I native equine collagen which transforms into a soft gel when placed in contact with the wound bed. At the present this type of heterologous material is the ideal first-aid means to cure wound healing problems.

2.1. Ultrastructural characterization

To characterize the overall morphology of this type of collagen matrix, the three-dimensional architecture and the density of its internal texture were analyzed by both light and electron microscopy. A second objective of this study was also to verify whether the series of treatments leading to the finished product could preserve the structural organization of the native collagen.

When observed at the stereomicroscope, the BCS dressing appeared to apparently offer two different external surfaces. The upper surface, hereafter referred to as “air surface” – because in contact with the atmosphere during the manufacturing process – is characterized by regular large pores, perhaps due the polypropylene membrane employed for freeze-drying (Fig. 13A). By contrast, the lower surface, hereafter referred to as “pan surface” - due to its contact with a plastic tray - is rather homogeneous, showing in the main a prevailing fibrillar structure (Fig. 13B). When observed by scanning electron microscopy (SEM), the upper surface of the collagen dressing appeared collapsed in the form of a compact layer, with several regular depressions over the entire area (Figs. 13C and 13E). The opposite surface had instead a laminar organization with regular small pores bordered by thin fibrils (Figs. 13D and 13F).

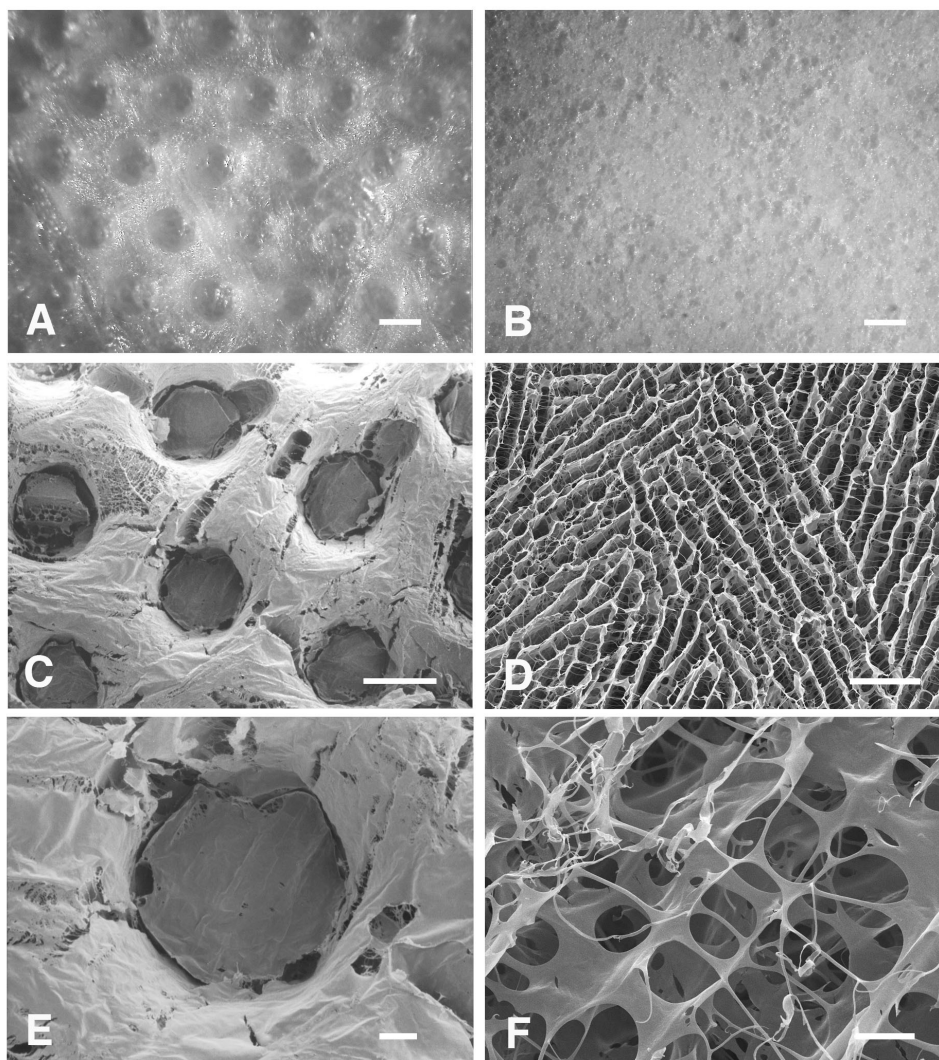


Figure 13. Morphological characterization of the BCS surfaces. (A) and (B), LM images of the upper and the lower surface respectively, Bars 500 μm . (C) and (D), SEM images of the two surfaces, as before. (C), Bar 500 μm ; (D), Bar 100 μm . (E), high magnification of one depression on the upper surface, Bar 100 μm . (F), laminar organization of the lower surface showing the regular small pores, Bar 10 μm .

In cross sections, the internal architecture of the collagen scaffold appeared characterized by numerous vertically oriented laminae interconnected by thin scattered fibrils (Figs. 14A and 14B). The overall architecture of the collagen scaffold gave the impression of a trabecular structure with a regular porosity delimited by bundles of collagen fibers (Fig. 14C). To define the pores sizes of this trabecular structure, a number of SEM images were elaborated with the ImageJ program and the results are shown in Table 1.

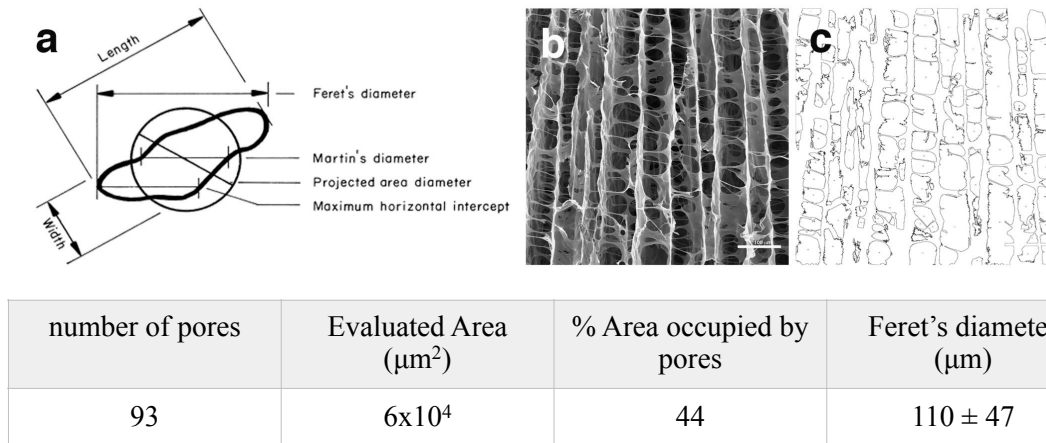


Table 1. Results of pore size analysis. (a), schematic drawing of Feret's diameter used to measure the sizes of pores. (b), SEM images used for analysis, Bar 100 μm . (c), drawing of pores obtained by imageJ program.

When observed at the transmission electron microscope (TEM) on ultra-thin sections and by negative staining, the structural arrangement of the collagen scaffold could be examined in more details (Figs. 14D and 14E). Under these conditions, the trabecular laminae, although rather compact, appeared as rather well-organized collagen fibers characterized by the regular banding periodicity of the native structure (Fig. 14E).

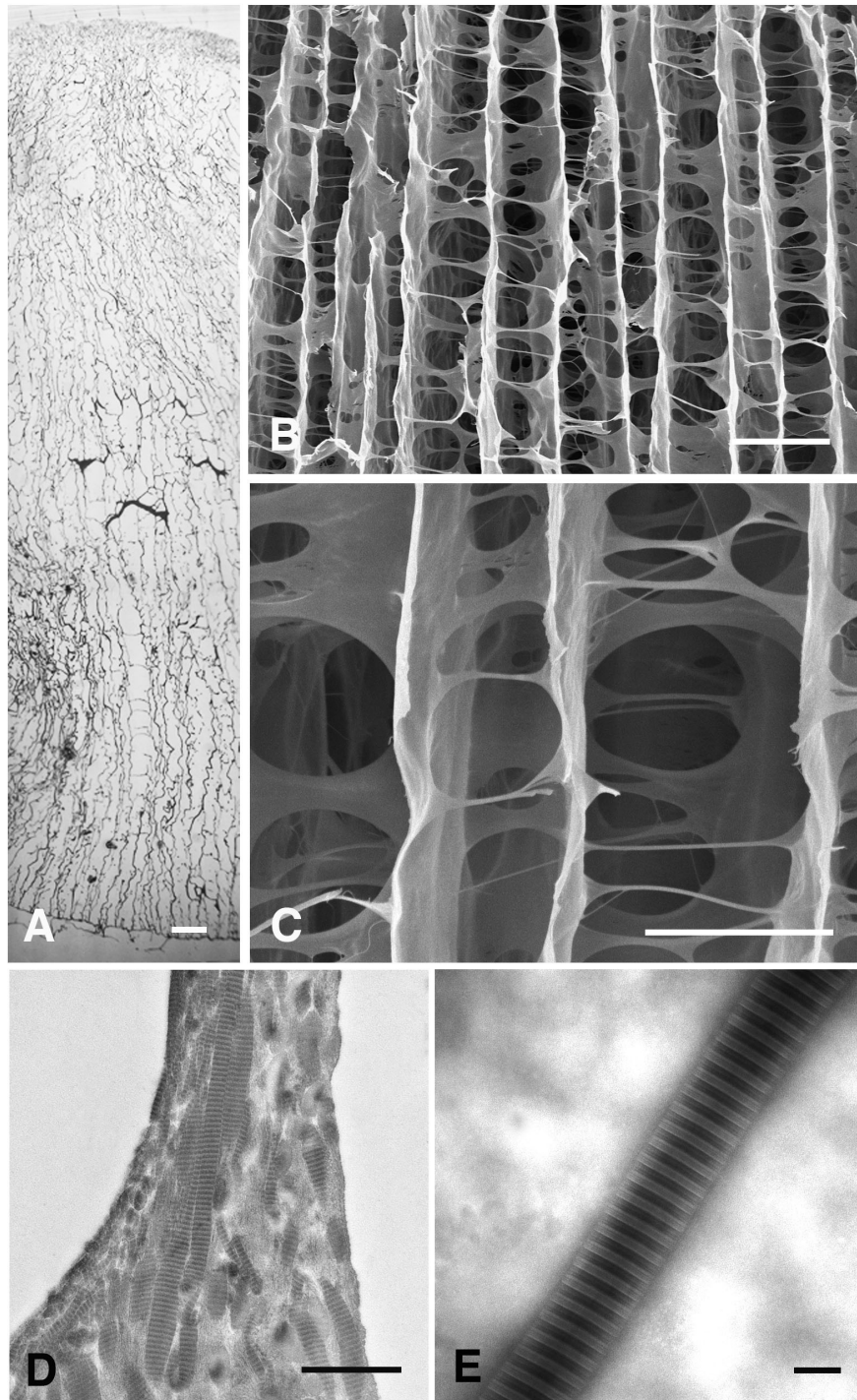


Figure 14. Internal architecture of BCS. (A), LM image of longitudinal semithin section of collagen matrix, Bar 100 μm . (B) and (C), SEM images of collagen laminae interconnected by thin fibrils. (B), Bar 100 μm ; (C), Bar 50 μm . (D), TEM micrograph of BCS ultrastructure showing the collagen fibers with the regular banding periodicity of the native structure, Bar 1 μm ; (E), negative staining of a collagen fiber, Bar 100 μm .

2.2. Functional characterization

To assess the overall stability of the collagen scaffold, 0.5 x 0.5 mm blocks were cultured *in vitro* for periods of time ranging from a minimum of 4h for up to 14 days, either in the presence or absence of cells. Figure 15 shows how the collagen scaffold changes during this culturing period. From a compact structure with highly intertwined collagen fibers (Figs. 15A to C), the scaffold is gradually transformed into a rather loose assembly of dispersed collagen fibers (Figs. 15D to F). These changes occur regardless of whether cells are present or not. However, they are somehow accelerated, and collagen fibers become even more dispersed, if the scaffold is cultured in the presence of cells (compare Figs. 15E and 15F).

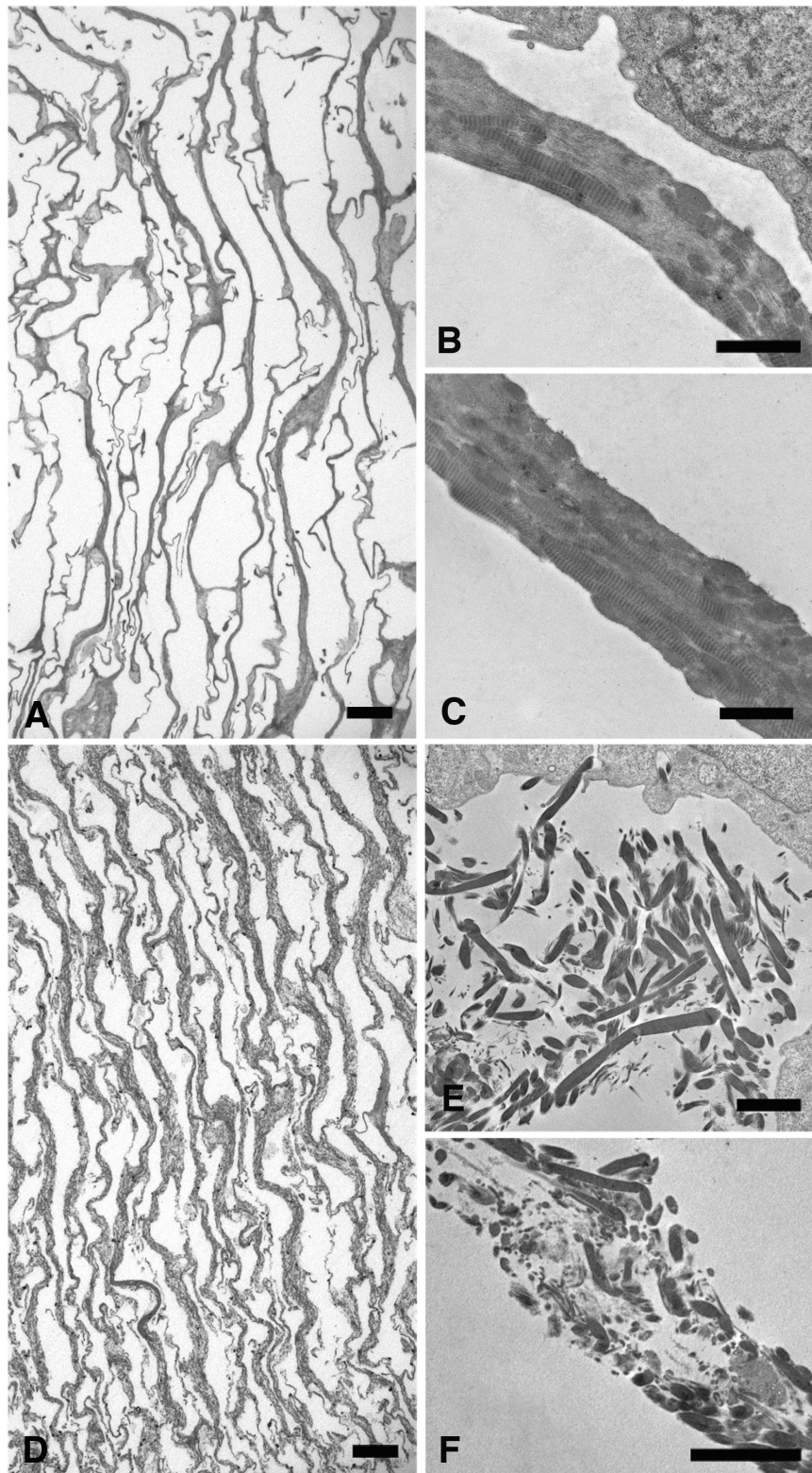


Figure 15. LM and TEM images showing laminas of the collagen scaffold following 4 hours (A and B) and 14 days (C and D) of *in vitro* culture in the presence of cells (A, B, D and E) or without cells (C and F); (A) and (D), Bars 50 μm; (B) and (C), Bars 1 μm; (E), Bar 2,5 μm. (F), Bar 3 μm.

2.3. Discussion

This study was carried out to verify the Biopad Collagen Scaffold capacity to withstand the harsh conditions of the wound healing, as far as they could be simulated *in vitro*, i.e.: (1) how long it could persist on the wounded bed, and above all (2) how much it is structurally altered during the culturing period being tested. The knowledge gained through this type of studies should help, not only to understand the mechanisms underlying the wound healing process, but also to gain new information as to how collagen-based matrices behave *in vivo* and eventually how they could be chemically enriched to improve this behavior.

A variety of collagen scaffolds are available today to help wound healing and skin regeneration. To accomplish these goals, scaffolds ought to satisfy several requirements. First, the pore size has to be maintained within a restricted size range for cells to adhere to the collagen matrix and migrate inside the scaffold. Second, the scaffold itself should be sufficiently stable to allow new granulation tissue to mature while the injured tissue is replaced by new collagen deposition. Third, the collagen matrix should not persist indefinitely, but be degradable within the time periods compatible with the healing process.

A careful series of observations and measurements allowed us to establish that the collagen scaffold used in this study offers a rather uniform matrix structure for cells to migrate through, with pore sizes falling within the expected range of about $100 \pm 150 \mu\text{m}$ (Lawrence and Madihally, 2008). This value is in line with earlier observations that explored the effects of upper and lower limits of this range (O'Brien *et al.*, 2005) and tested cell migration in scaffolds of different collagen compositions (Karr *et al.*, 2011). Recent reviews stressed the importance of pore sizes ranging between 50 and 200 μm as a factor providing favorable conditions for cell infiltration, adhesion, and proliferation. All these features are known to play key roles in wound healing by allowing fibroblasts to initiate and sustain synthesis and secretion of a new extracellular matrix at the damaged site (Yannas *et al.*, 2010). At the same time, these conditions should facilitate the release of any biofactor that could have been loaded on the matrix itself to help wound healing (Loh and Choong, 2013). On the whole, the capacity to preserve the

native collagen structure should allow the scaffold to maintain its stiffness for longer periods on the wound bed, and this, in turn, should provide better conditions for fibroblasts to infiltrate and proliferate within the matrix itself so as to accelerate the formation of new granulation tissues. Besides these features, the BCS scaffold is also known to constitute a barrier against infective agents, and this capacity to seal off the wounded area from any exogenous influence has important practical consequences for wound management on the long run (Vaes, 1980; Brett, 2008).

CHAPTER III

3. The BCS simulates wound healing

This section addresses the question as to how collagen based matrices interact with *in vitro* cultured fibroblasts for periods of time up to two weeks. The question is experimentally approached by using transmission electron microscopy (TEM) and scanning microscopy (SEM). The intent is to examine (1) the overall collagen infrastructure of these matrices, (2) to determine how they are structurally modified under various conditions and finally (3) whether interaction with the matrix entails also degradation of the collagen fibrils. In answering these questions it is also shown that fibroblasts prove capable of anticipating their migratory behavior through the release of a number of microvesicles into the surrounding medium.

3.1. *In vitro* cultured 3T3 fibroblasts

NIH3T3 fibroblasts were cultured on collagen based scaffold matrices as specified in Materials and Methods, and their distribution assessed at regular time intervals by LM and SEM. Figure 16 shows that at 4 h after seeding, numerous fibroblasts were retained on the upper scaffold surface, and some cells had already migrated into deeper regions of the collagen matrix (Fig. 16A). When entering the matrix, fibroblasts became highly intertwined with the collagen fibers, having most of their cell surface in close contact with the collagen fibers and occasionally forming specific adhesion plaques (Figs. 16B and 16C). In the presence of the collagen scaffold, fibroblasts apparently assumed different shapes, ranging from very roundish cells (Fig. 16D) to highly polarized ones (Fig. 16E). As culturing time was prolonged to 14 days, fibroblasts became even more entangled with the collagen fibers, eventually forming thick bundles of weaved filaments (Fig. 16F).

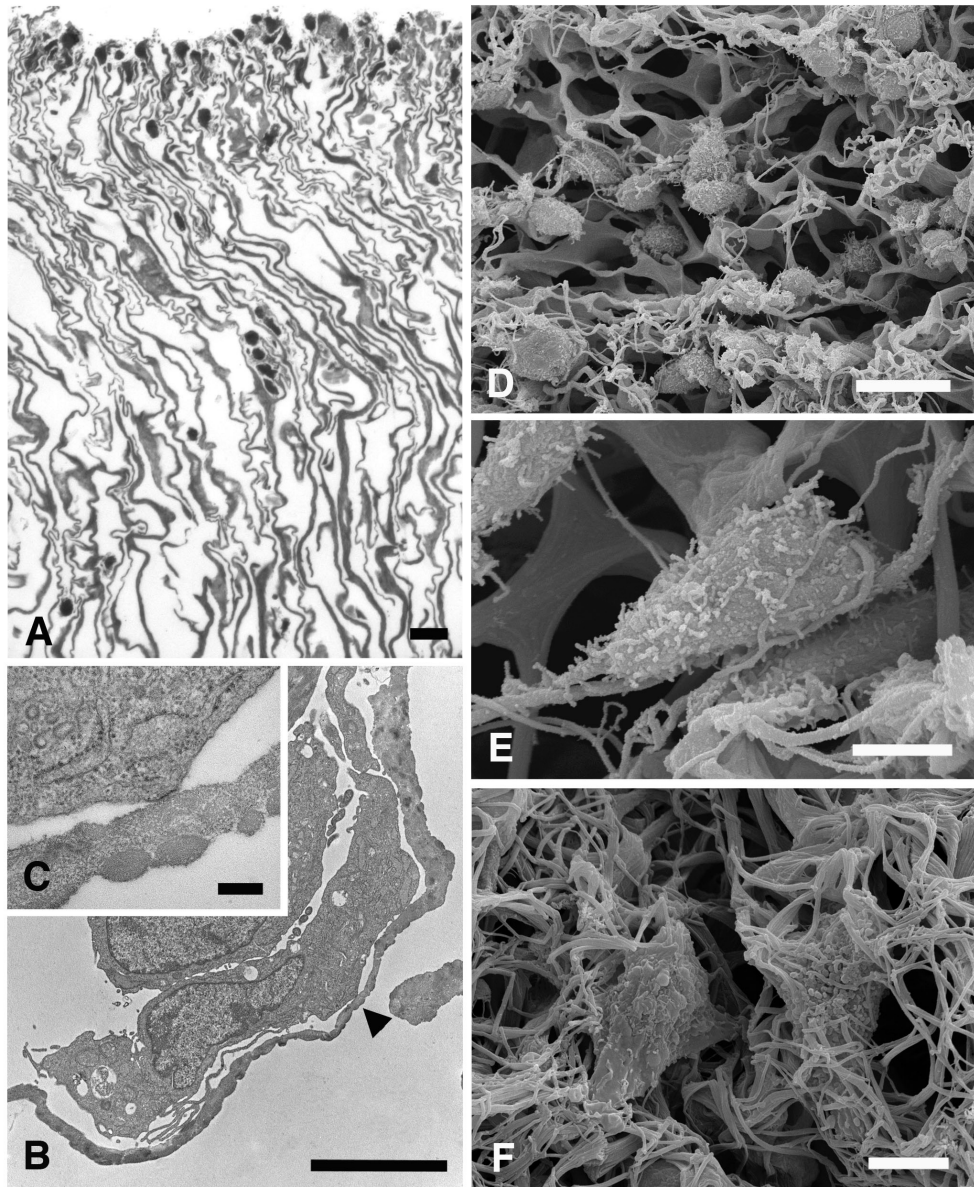


Figure 16. (A), LM image showing numerous fibroblasts on the upper surface of the BCS. Note that some cells have already migrated inward, Bar 20 μm . (B), TEM micrograph showing fibroblasts closely adhering to a collagen fiber (see arrowhead), Bar 5 μm . (C), enlargement of the adhesion point, Bar 200 nm. (D), SEM micrograph showing a number of fibroblasts on the top surface of the collagen scaffold, Bar 20 μm . (E), SEM micrograph showing a fibroblast amidst collagen fibers 4h after seeding, Bar 5 μm . (F), SEM micrograph showing fibroblasts on the collagen scaffold 7 days after seeding, Bar 5 μm .

As a control of the cell-to-collagen interaction experiments, fibroblasts were also cultured *in vitro* and allowed to grow for up to 14 days. Under these conditions, fibroblasts grew and migrated as flat elongated cells, remaining firmly attached onto the substrate (Figs. 17A and 17B).

Although variable both in shape and extension in relation to such conditions as cell crowding and incubation time, the number of filopodia present on the fibroblast cell surface appeared to increase steadily during the entire culture period (Figs. 17C and 17D). Numerous microvesicles were also present along the cell contours of many fibroblasts, even though they could not be discerned in regions far from the cell surface. This indicated that they had to remain in the cell proximity due to the poor additional anchoring site.

Fibroblasts could also release microvesicles *in vitro* in much the same way as in the presence of collagen scaffolds, i.e., as buds from the filopodial tip, the only difference being their number and position relative to the cell body (Figs. 17E and 17F). Fewer microvesicles were in fact released *in vitro* as compared to that recorded in the presence of the collagen scaffold.

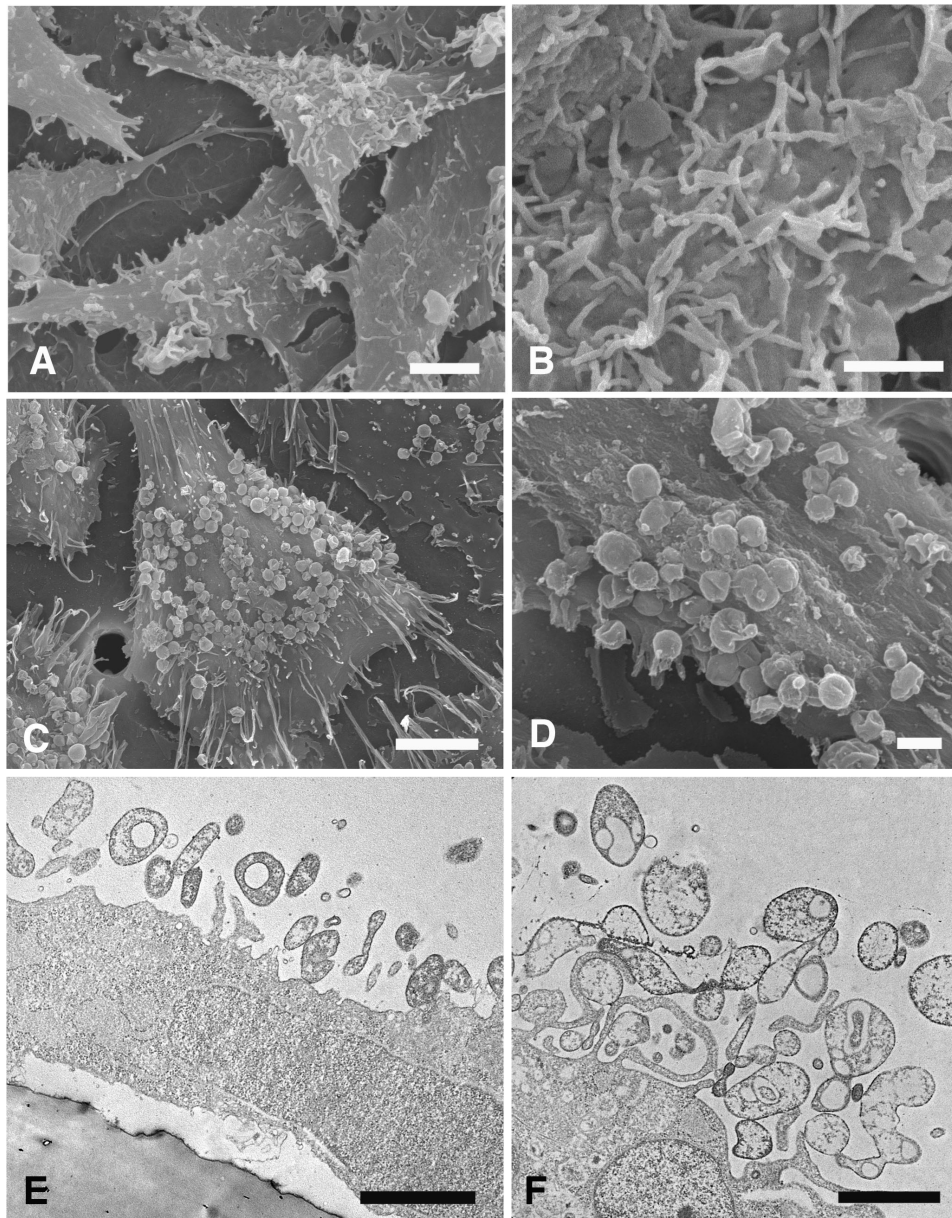


Figure 17. (A), SEM micrograph of 1 day *in vitro* cultured fibroblasts, Bar 5 μm . (B), SEM micrograph showing a number of filopodia on the fibroblast cell surface, Bar 2 μm . (C), SEM micrograph of 7 day *in vitro* cultured fibroblasts showing a number of cells highly enriched with filopodia and microvesicles, Bar 5 μm . (D), enlargement of the previous figure showing the extent of microvesicle budding from the cell surface, Bar 1 μm . (E), TEM micrograph of an *in vitro* cultured fibroblast showing a number of filopodia and microvesicles protruding from the cell surface, Bar 2 μm . (F), TEM micrograph showing several microvesicles amidst some tortuous filopodia, Bar 2 μm .

3.2. Cell kinetics

A rough estimate of the number of fibroblasts migrating in collagen scaffolds as a function of the culturing time indicated that a progressively higher number of fibroblasts could be found in matrix regions farther away from the seeded side of the scaffold (data not shown). However, taking the MTT test as a reliable tool to measure cell viability and cell proliferation, fibroblast/collagen interact in a manner more complex than simple cell counting could suggest. Figure 18 indicates that cells increased their MTT activity quite rapidly within a day of culturing and then stayed constant for the rest of the incubation period. This is probably caused by fibroblasts starting to proliferate soon after entering the collagen matrix, and declining upon becoming involved in collagen breakdown and absorption.

By comparison, cells cultured *in vitro* with no collagen scaffold grew according to a symmetrical kinetics inclusive of slow rise followed by an equally slow decline. This is probably due to the fact that confluence *in vitro* is attainable only by 3 days of cell culture. Afterwards cells may stop growing and presumably start to be removed by apoptosis.

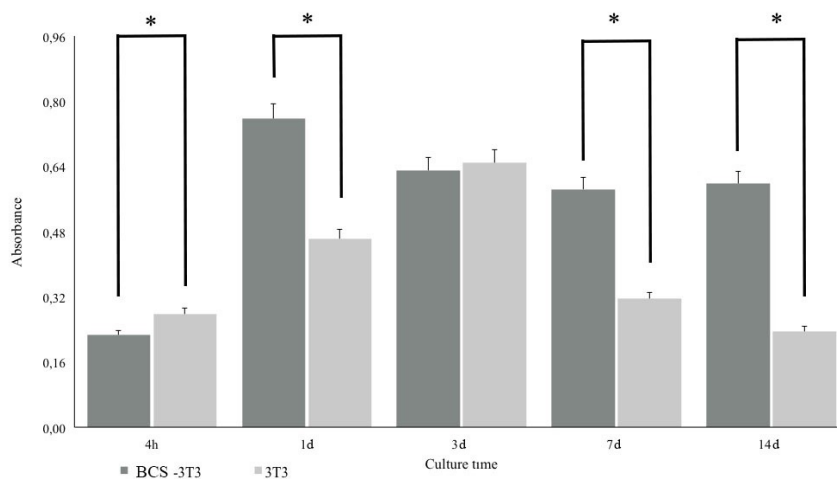


Figure 18. Histogram showing cell viability of 3T3 fibroblasts cultured for over a period of 14 days (grey columns) or co-cultured for the same length of time in the presence of a BCS (black columns). Asterisks (*) for $P < 0.05$.

3.3. Cell/collagen matrix interaction: incorporation

Following 1 day of culture, fibroblasts were already anchored onto the collagen laminae, but on average they maintained a low cell surface roughness with very few filopodia extending outwardly (Figs. 19A and 19B). However, with the continuation of the co-culture period for up to 14 days, the fibroblast plasma membrane weaved intimately with the collagen matrix by extending an increasing number of filopodia (Fig. 19C). Figure 19D shows how some of these filopodia can actually embrace entire collagen fibers. A number of small vesicles appeared to be shed from the fibroblast cell surface in the proximity of the embraced fibril (Fig. 19E). Additional disaggregation of the collagen scaffold could be observed when fibers started to be gradually frayed into thinner fibrils (Fig. 19F). As these changes occurred, fibrils were seen gradually reduced in mean diameter, deprived of their staggered banding patterns and eventually taken up into large vacuoles by fibroblasts (Fig. 19G). Fibroblast filopodia are likely to play an active role in this engulfment process by first forming hairpin-like protrusions and then folding them back onto the cell surface to trap collagen fibers in a sort of vacuolar enclosure (Fig. 19D).

An extensive ultrastructural analysis indicated that filopodia are dynamic structures, highly variables both in mean diameter and overall length (Fig. 20A). Besides taking direct contact with the collagen fibers (Fig. 20B), filopodia are also involved in releasing a number of microvesicles onto the collagen matrix. In all likelihood, these microvesicles emerge from the fibroblast surface through an active process of budding from the filopodial tips. Instances of actual continuities of filopodia with emerging microvesicles have been recorded several times (Fig. 20C). As microvesicles are shed from the fibroblast cell surface they recruit part of the cortical cytoplasm inside, including a number of smaller vesicles (Fig. 20D). From this cell site they may migrate at a certain distance from the cell surface and disperse into the collagen matrix (Fig. 20E). With the continuation of the co-culture for up to 14 days, microvesicles increase in number, appearing also at a far distance from the fibroblast cell surface and highly intermingled with frayed collagen fibrils (Fig. 20F).

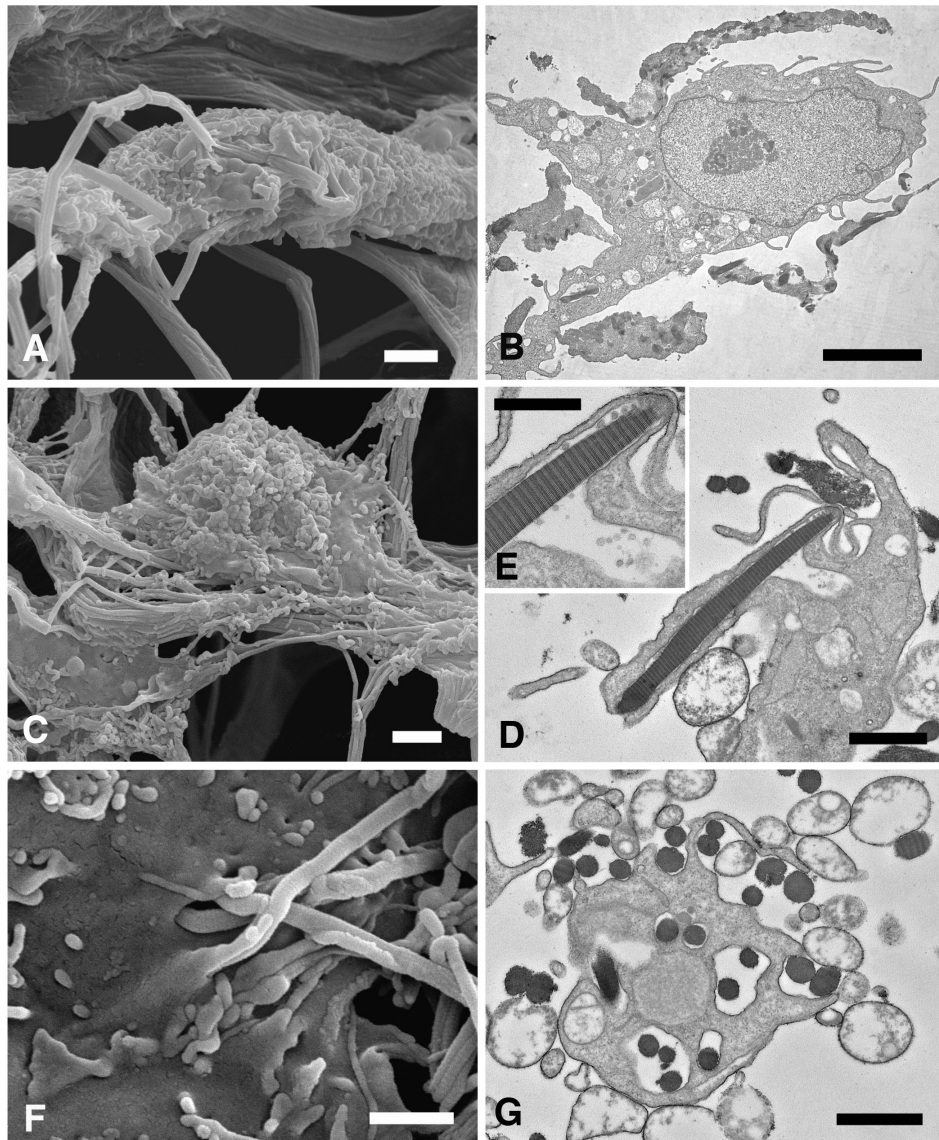


Figure 19. 3T3 fibroblasts cultured on BCS for up to 7 days. SEM (A) and TEM (B) micrographs showing fibroblast anchored onto collagen fibers after 1 day of culture. (A), Bar 2 μm . (B), Bar 5 μm . SEM (C) and TEM (D) micrographs showing a fibroblast highly intertwined with a collagen fiber after 4 days of culture. (C) and (D), Bars 2 μm . (E), enlargement of Fig. 17D showing a number of microvesicles, Bar 500 nm. SEM (F) and TEM (G) micrographs showing an enlargement of a fibroblast surface with several collagen fibers entering the cell interior, as seen in cross sections. (F) and (G), Bars 1 μm .

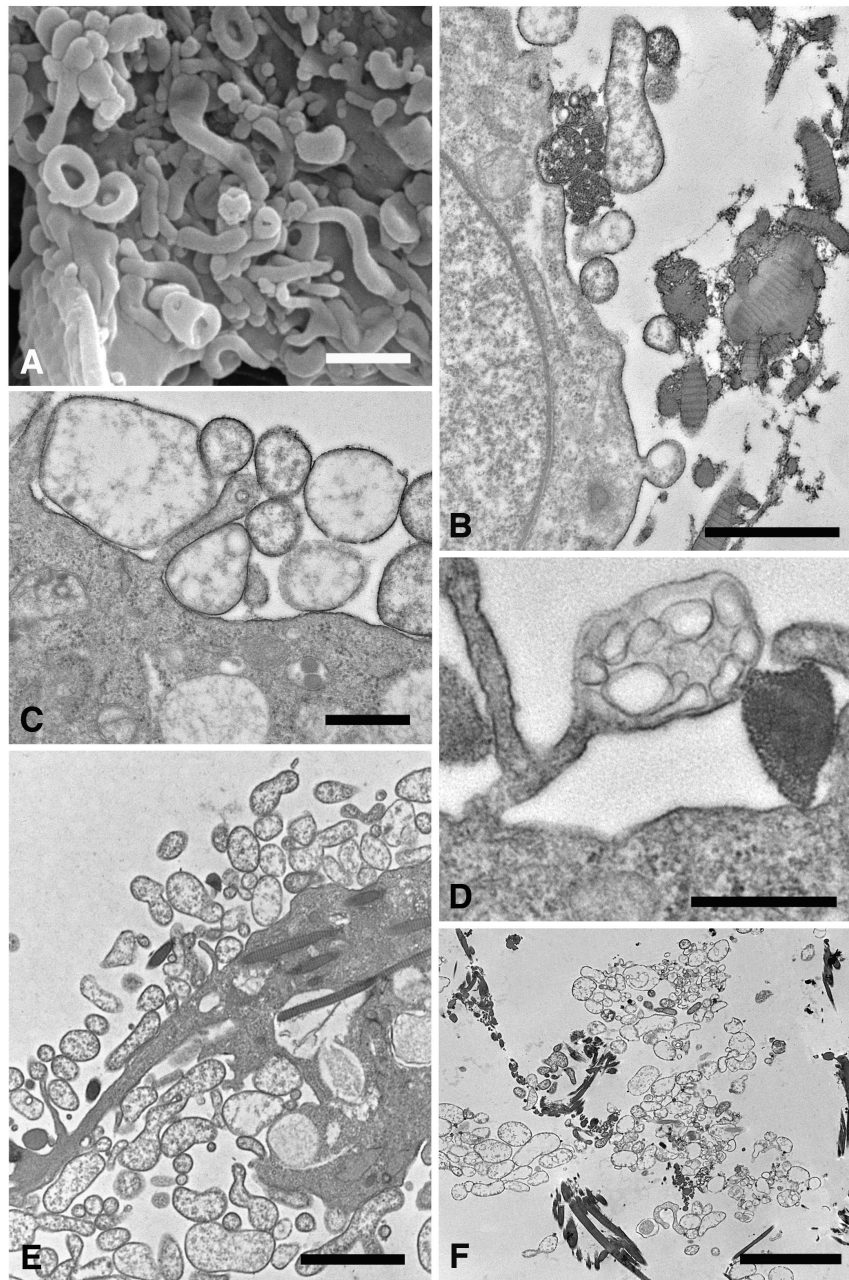


Figure 20. (A), SEM micrograph showing the rough surface of a fibroblast, Bar 1 μm . (B), TEM micrograph showing a fibroblast surface with numerous filopodia and microvesicles closely adjoined to collagen fibers, Bar 1 μm . (C), TEM micrograph of fibroblast showing numerous filopodia and microvesicles protruding from the cell surface, Bar 500 nm. (D), TEM micrograph showing a microvesicle budding from a filopodium tip, Bar 500 nm. (E), TEM micrograph of a 7 day fibroblast highly entangled with collagen fibers and microvesicles, Bar 2 μm . (F), TEM micrograph of collagen fibers and microvesicles in regions of the collagen matrix far way from the fibroblast cell body, Bar 5 μm .

With the continuation of cell culture, both fibroblasts and the collagen matrix undergo additional changes. In particular, the fibroblast cytoplasm becomes gradually filled with vacuoles containing material of varying consistency and density. Occasionally, some of the material in these vacuoles becomes continuous with collagen fibrils (Fig. 21A). These vacuoles are likely to be formed by phagocytosis of collagen fibers through embracement and folding of the filopodia followed by invagination of the enclosed plasma membrane (Fig. 21B). However, these collagen vacuoles are complex organelles containing not only exogenous material, but also material conveyed by other vesicles. These latter are likely to be Golgi-associated vesicles delivering their load of hydrolytic enzymes to the collagen containing vacuoles for additional processing (Figs. 21B and 21C).

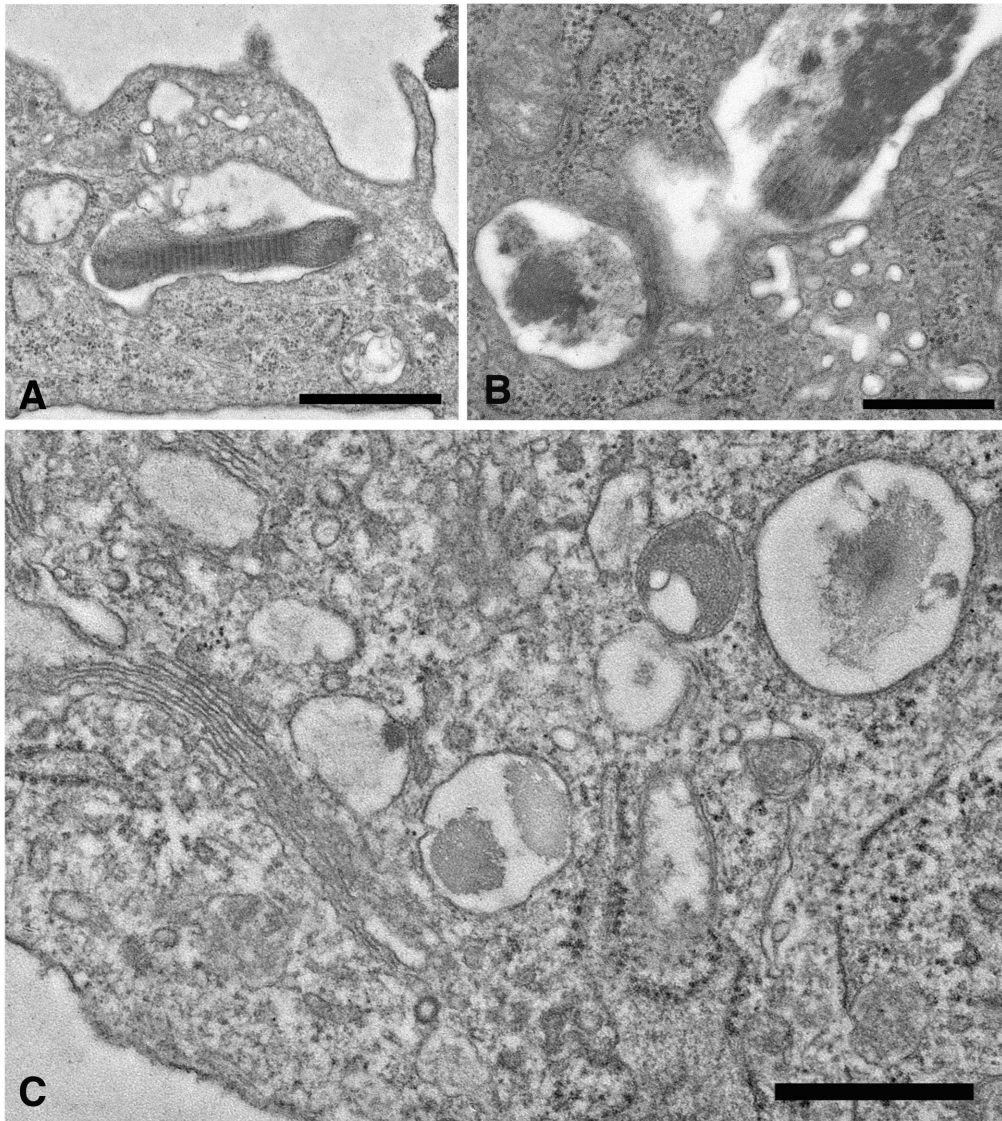


Figure 21. (A), TEM micrograph showing a large vacuole in the cortical cytoplasm of a fibroblast containing a collagen fiber, Bar 1 μm . (B), TEM micrograph showing some collagen-containing vacuoles merging with smaller vesicles deriving from the Golgi apparatus, Bar 500 nm. (C), TEM micrograph showing several vesicles with some electron dense material therein, close to a Golgi apparatus, Bar 500 nm

3.4. Discussion

Wound healing is a rather complex phenomenon requiring the contribution of several cell types and the activation of a variety of growth factors and cytokines (Werner and Grose, 2003), primarily for sustaining extensive syntheses and remodeling of the dermal tissues. Fibroblasts appear to play a major role in the process by providing collagen for the extracellular matrix to be regenerated (Lamme *et al.*, 1998). In this study, I have followed the fate of fibroblasts as they migrate and proliferate in a collagen scaffold for up to 14 days of *in vitro* culture. The observed effects included adhesion to the collagen matrix, changes in the overall fibroblast morphology, disaggregation and fraying of the collagen fibers and eventually phagocytosis and intracellular digestion of the engulfed collagen fragments. That fibroblasts may actually migrate inside the collagen scaffold is clearly indicated both by cell counting and by their recurrent interaction with collagen fibers by means of focal adhesion plaques (see Fig. 16C). Through these type of cell junctions fibroblasts appear to be highly involved in complex mechanical interactions with the collagen matrix, presumably as a precondition for their attachment onto fibrils located far distant from the seeded side. Small adhesion plaques are in fact formed close to the leading edge of the migrating fibroblasts where contractile forces act to propel migration (Benigno *et al.*, 2001). The extent by which cells migrate into the collagen scaffold is an important parameter to determine how quickly collagen matrices are processed *in vivo* and how long it takes for a new extracellular matrix to be fully deposited. Understanding of these parameters should in principle help to design better collagen substrates for wound healing (Abraham *et al.*, 2004).

Micrographs shown in this study are strongly suggestive of the occurrence of collagen remodeling as sustained by both extracellular and intracellular degradation. Clear indications of collagenolysis stem from the observation that collagen fragmentation increases as culture time is prolonged from 1 to 14 days. During this time period, collagen fragments accumulate in the culture medium and gradually fray into bundles of woven thin fibrils. Numerous instances of intracellular collagen degradation have also been recorded as more and more material, ranging from banded collagen

fragments to bundles of highly disorganized filaments, accumulate in the fibroblast vacuoles. These observations are in line with the notion that intracellular collagen degradation in fibroblasts requires the presence of specific collagen receptors and that collagen may accumulate in the culture medium if co-cultured with receptor-deficient fibroblasts incapable of undergoing endocytosis (Madsen *et al.*, 2007). This condition may actually simulate what have been previously described *in vivo* as fibrotic lesions, where fibroblasts with marked deficiencies in phagocytic activity affect the process of collagen degradation and eventually lead to an excessive deposition of extracellular matrix in the impaired organ (McCulloch and Knowles, 1993). Additional evidence that collagen remodeling entails intracellular degradation, comes from the observation that the extent of collagen degradation can be changed *in vitro* by modulating the pH-dependent activity of lysosomal enzymes in the presence of acidotropic agents (Everts *et al.*, 1995). However, for collagen to be taken up by fibroblasts it has first to be partially degraded by specific collagenases. Collagen phagocytosis and degradation are in fact inhibited whenever the expression of these enzymes is blocked by treatment with specific RNA interference (Lee *et al.*, 2006). Taken together, this evidence makes it highly probable that collagen remodeling *in vivo* requires both the action of secreted collagenases in the extracellular milieu and the activity of some hydrolytic enzymes in the intracellular space (Madsen *et al.*, 2013).

As clearly documented in this study, filopodia are dynamic structures involved in a number of different cell functions. They are endowed with the capacity to explore the external milieu through transient adherence to the substrate and spreading along the collagen laminae. This behavior is typical of cells capable of altering their actin cytoskeleton in response to variations in substrate roughness (Dalby *et al.*, 2004; Albuschies and Vogel, 2013). However, filopodia have also the additional function of acting as cellular tentacles to engulf some partially degraded collagen fragments and retreat them into vacuole-like enclosures. By doing so they are likely to predispose the fibroblast plasma membrane for phagocytosis. Indeed, it has been demonstrated that the appearance of new filopodia in myoblasts coincides with the activation of metalloproteinase precursors involved in collagen degradation (Knoblauch *et al.*, 2007).

Interestingly, this type of correlation is not restricted only to connective tissues, since metalloproteinases are also controlling the distribution of postsynaptic receptors in dendritic spines through a similar mechanism (Michaluk *et al.*, 2011; Szepesi *et al.*, 2013).

However, besides providing a substrate attachment for the migrating fibroblasts and protruding tentacles for engulfing and degrading collagen fibers, filopodia are also involved in the release of microvesicles. This study has clearly documented the occurrence of extensive budding and fission of microvesicles from the filopodia tips and the inclusion of numerous smaller vesicles inside them. The role played by this shedding process remains to be proved experimentally, although microvesicles are likely to serve as vehicles for the horizontal transfer of enzymes required for collagen degradation. Microvesicles are in fact known to be a universal type of organelle that cells release as carriers of factors involved in many metabolic and developmental functions (Taverna *et al.*, 2003). Shedding from filopodial protrusions has recently been demonstrated to occur in endothelial cells where microvesicles appear to regulate vessel sprouting during human brain vascularization (Virgintino *et al.*, 2012).

In conclusion, the study of fibroblast interaction in collagen scaffolds has allowed to show that such basic functions as cell migration and adhesion, along with shedding of microvesicles are preconditions for the degradation of the prosthetic collagen and presumably for the deposition of a new extracellular matrix in wound healing.

CHAPTER IV

4. Role of microvesicles in wound healing

This section aims to deepen the previous observations on fibroblasts microvesicles release into culture medium, when cultured *in vitro* in presence of collagen. The primary objective of this study was thus to verify whether the release of microvesicles can be preconditions for the degradation of the prosthetic collagen and presumably for the deposition of a new extracellular matrix in wound healing.

4.1. Microvesicles shedding from *in vitro* cultured fibroblasts

Fibroblasts co-cultured under 3D conditions for up to 7 days i.e., in the presence of a prosthetic collagen scaffold, can be shown to migrate rapidly inside the matrix and take close contact with the collagen fibers. Figures 22A-C depict an initial condition of this *in vitro* culture. At this stage, roundish fibroblasts appear firmly attached to a collagen matrix, still characterized by a prevailing laminar structure. By the end of this culture period (4 hours), cells have become highly intermingled with the collagen fibers, the matrix itself has lost its laminar structure and acquired a rather fibrillar appearance (Fig. 22D). The collagen fibers themselves are no longer aligned in continuous laminar sheets, but are rather scattered in the form of a fragmented network with no recognizable orientational order (Fig. 22E).

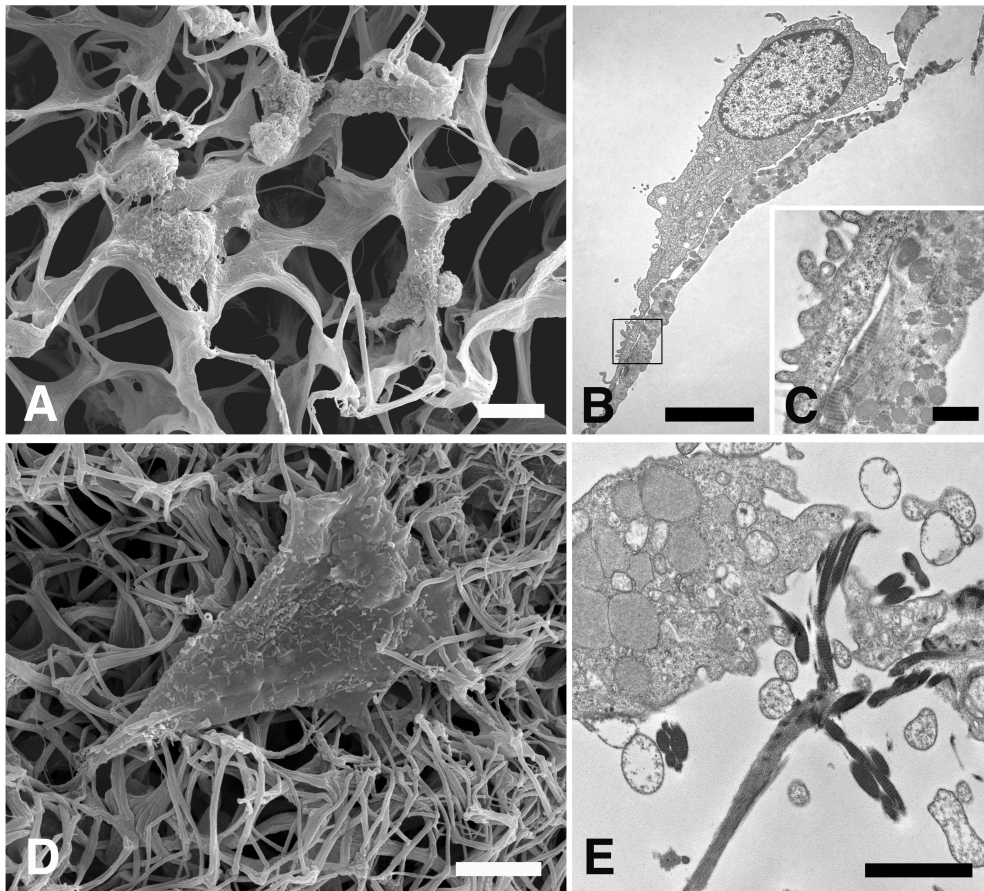


Figure 22. SEM and TEM micrographs showing the extent by which the collagen matrix is structurally modified in the presence of migrating fibroblasts. Figures (A), (B) and (C) show the collagen matrix at day 1 following cell seeding. Notice the laminar structural appearance of the collagen matrix. Figures (D) and (E) depict fibroblasts after 7 days of in vitro culture. Notice that the collagen matrix has in the mean time acquired a prevailing fibrillar organization. (A), Bar 10 μm ; (B), Bar 5 μm ; (C), Bar 500 nm; (D), Bar 5 μm and (E), Bar 2 μm .

4.2. Microvesicles shedding in collagen matrix

To account for the laminar-to-fibrillar transformation of the prosthetic collagen I attempted to verify whether, under this culturing conditions: (1) migrating fibroblasts may release metalloproteinases into the surrounding medium and (2) the released enzyme activities may actually be responsible for collagen processing. Of the numerous metalloproteinases identified so far, I chose to study the MMP-9 because it is known to be constitutively expressed in many cell types, including fibroblasts, besides being endowed with the capacity of remodeling the extracellular matrix and degrading a broad variety of collagen types (Fanjul-Fernández *et al.*, 2010). Figure 23 shows that, when tested by gelatin zymography in polyacrylamide gels, cell lysates from fibroblasts co-cultured in a 3D collagen matrix reveal the presence of two major protein fractions. These are identifiable as bona fide pro-MMP-9 and active MMP-9 on the basis of their respective molecular weight of 92 and 82 kDa and by their common reactivity to an anti-metalloproteinase antibody, as detected by western blot analysis (see Fig. 23, WB line). Since these MMP-9 fractions are antigenically related in a precursor-product relationship, only the lower molecular weight fraction could be expected to be enzymatically active by zymography. However, the activation of the pro-MMP-9 is thought to result from the enzyme artificial exposure to the denaturing conditions adopted for gel electrophoresis (Frankowski *et al.*, 2012).

Results showed that the relative concentration of the active form is gradually increased from 1 to 7 days of *in vitro* culture while the pro-MMP-9 is instead reduced. This correlation is interpreted as indicating that co-culture with the collagen matrix is a condition necessary, though not sufficient, for the MMP-9 to be both released and activated extracellularly. By comparison, fibroblasts cultured under 2D conditions i.e., in the absence of any collagen interference, appear to have a much lower MMP-9 activity and a higher enzyme turn-over, both MMP fractions having already disappeared after 3 days of *in vitro* culture (see right panel on Fig. 23). When gelatin zymography was used to examine extracts from 3D culture media, both MMP fractions appeared to be expressed with titre values even higher than in their respective cell lysates (Fig. 24).

By comparison, the MMP expression in 2D culture media had much lower titres and it was instead restricted to the sole 82 kDa active form.

Extracts from all culture media were routinely checked by negative staining (data not shown), regardless of their origin from either 2D or 3D *in vitro* cultures. This suggests that microvesicles might have a role to play in the extracellular release of MMP-9 and probably in their diffusion within the collagen matrix.

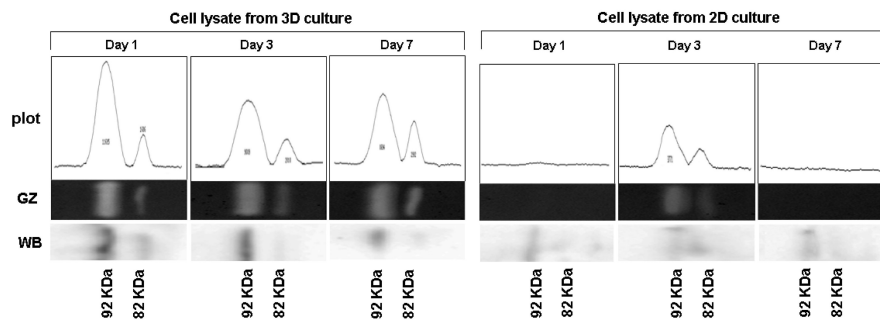


Figure 23. Immune detection of pro-MMP-9 and relative enzyme activity in cell lysates following *in vitro* culture in the presence of a collagen matrix (3D system) or in the culture medium alone (2D system) for 1, 3 or 7 days. (GZ), Gelatin Zymography; (WB), Western Blot with anti-MMP-9 antibody. Zymographs were scanned with densitometer equipped with ImageJ program.

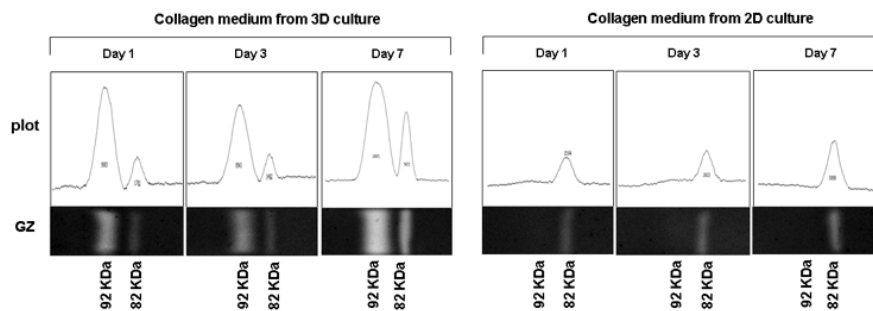


Figure 24. Immune detection of MMP-9 and enzyme activity in the culture medium extracted from the collagen matrix (3D system) or following *in vitro* cell culturing (2D system) for 1, 3 or 7 days. (GZ), Gelatin Zymography; (WB), Western Blot with anti-MMP-9 antibody. Zymographs were scanned with densitometer equipped with ImageJ program.

4.3. Role of metalloproteinases

To verify this possibility, cells from 2D and 3D *in vitro* cultures were processed for ultrastructural immunolocalization of MMP-9 with gold-tagged specific antibodies. Data showed that, under these experimental conditions, the microvesicles associated with the cell periphery of many fibroblasts appear heavily labelled with gold particles (Fig. 25A). As microvesicles are shed from the cell surface, they appear to become loaded with numerous smaller vesicles ranging in size from 100 to 300 nm (Fig. 25B), thus giving rise to a sort of extracellular multivesicular body. Interestingly, gold labelling with specific anti-MMP antibodies appears to be associated with some of these internal vesicles as well as with similar vesicular structures present in the nearby cortical cytoplasm (Fig. 25C). Instances of gold labelling have also been encountered in many microvesicles shed from the cell surface and enclosing a number of smaller vesicles inside (Fig. 25D). Given this multiple vesicular structure, it is quite intuitive that the enclosed MMP-9 cargo might gain access to the extracellular milieu by allowing the inner vesicular membrane to first bind to and then fuse with the enclosing microvesicular membrane, as suggested by the images depicted in Figure 25E.

Vesicular budding occurs also in 2D cultures, for fibroblasts proved capable of synthesizing and releasing MMP-9 even in the absence of the collagen matrix. However, the extent by which microvesicles are released into the surrounding milieu under these latter conditions is much less pronounced than in 3D cultures (data not shown). A detailed analysis of 3D cell cultures from 1 to 7 days has clearly shown that microvesicles can accede to regions of the collagen matrix far away from their cell of origin. It is not clear whether microvesicles actually move away from their budding site or simply remain anchored to the collagen fibrils while cells migrate along the collagen fibers. Whatever the mechanism(s) underlying the physical separation of vesicles from their cells of origin, microvesicles can either occur in isolation or in association with numerous frayed collagen fibrils (Figs. 26A and 26B). In both circumstances, microvesicles are still loaded with a MMP-9 cargo (Fig. 26C and 26D). However, when observed in close contact with the collagen, gold label appears spread on the frayed fibrils (Fig. 26D), indicating that the MMP-9 cargo has been released from the

microvesicles and dispersed in the surrounding extracellular environment. That the amorphous or fuzzy material contacting either microvesicles or cells should actually be identified as collagen, in spite of the difficulty to recognize it in the absence of a typical banded pattern, it is clearly demonstrated by its reactivity to the anti-collagen antibody (see Fig. 26E).

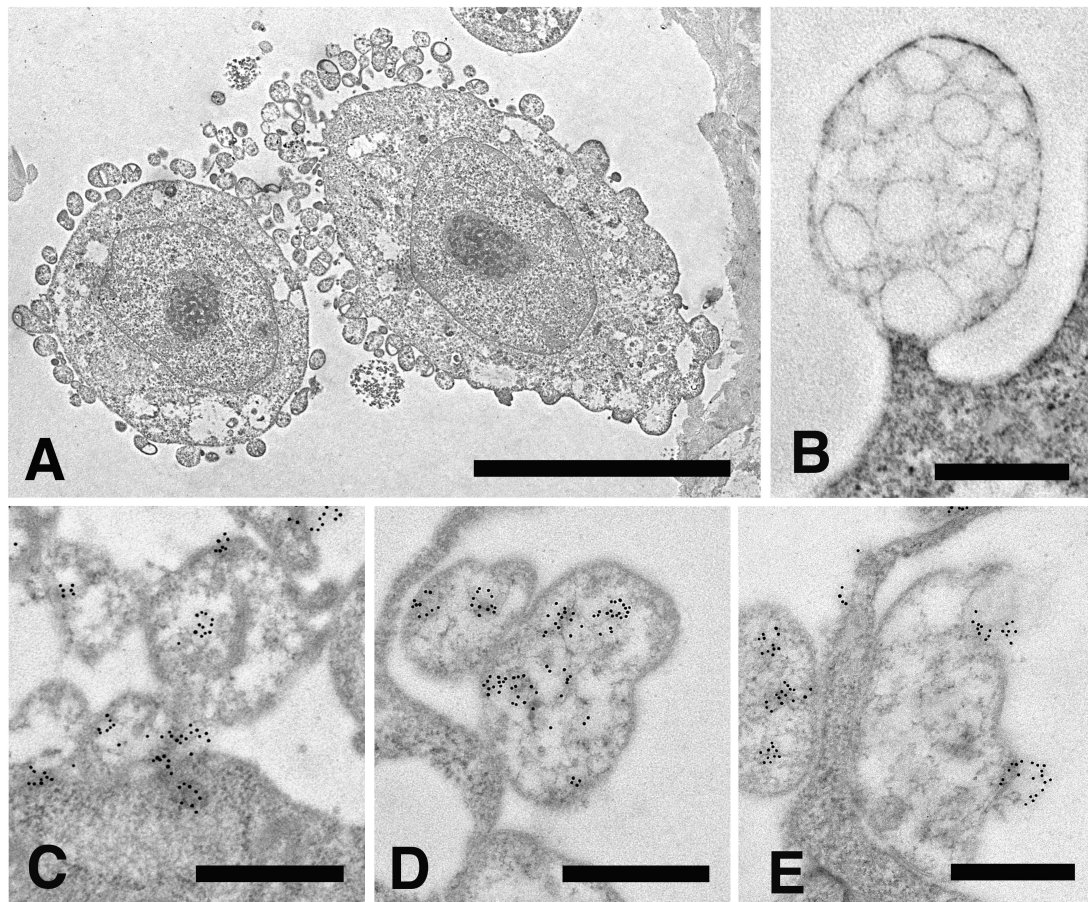


Figure 25. Immunolocalization of MMP-9 by post-embedding gold-staining with anti-MMP-9 antibody. (A), low magnification micrograph showing two fibroblasts from a 3D culture with multiple microvesicular budding along the cell periphery. Many of the microvesicles are labelled with gold particles, Bar 10 μ m. (B), high resolution TEM micrograph showing a microvesicle still continuous with the cell membrane. Note how many minor vesicles are included in the budding microvesicle, Bar 500 nm. (C-E) immunolocalization micrographs depicting an hypothetical sequence of an MMP-9 cargo being loading into a budding microvesicle and eventually being released into the surrounding milieu, Bars 500 nm.

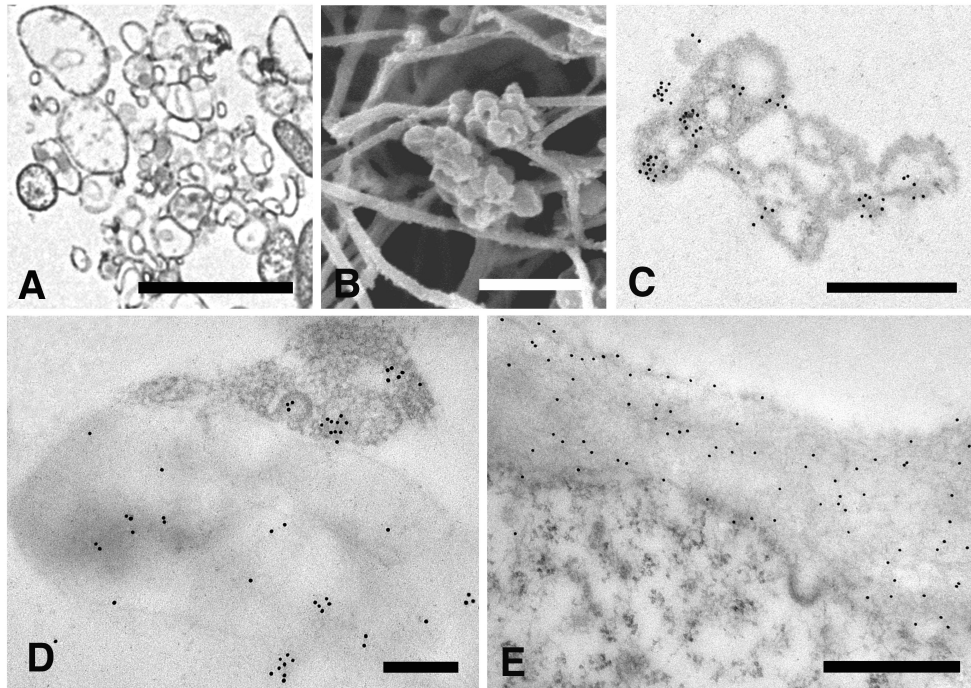


Figure 26. (A), High resolution TEM micrograph showing isolated microvesicles, Bar 1 μm ; (B), SEM micrograph showing a cluster of microvesicles associated with collagen fibres, Bar 2,5 μm ; (C), Immunogold localization of MMP-9 in association with a cluster of isolated microvesicles. Note that gold label occurs inside the microvesicles but also along the enclosing membrane, Bar 4 μm ; (D), Immunogold localization of MMP-9 in a microvesicle associate with a bundle of frayed collagen fibrils; the arrow points to the gold label being extruded from the vesicle. Gold labeling is also present on the collagen itself, Bar 200 nm; (E), immunogold localization of anti-collagen antibody on a bundle of frayed collagen fibrils, Bar 500 nm.

The ultimate result of the MMP-9 release from microvesicles is the spreading of the enzyme all over the collagen matrix. As clearly indicated by the sequence of images depicted in Figures 27A-C, MMP-9 antibody staining becomes progressively more pronounced on the collagen matrix as culture times are gradually prolonged from 1 to 7 days. This observation can also be verified at the ultrastructural level where collagen fibers exposed to the anti-MMP-9 antibody appear clearly labeled with gold particles.

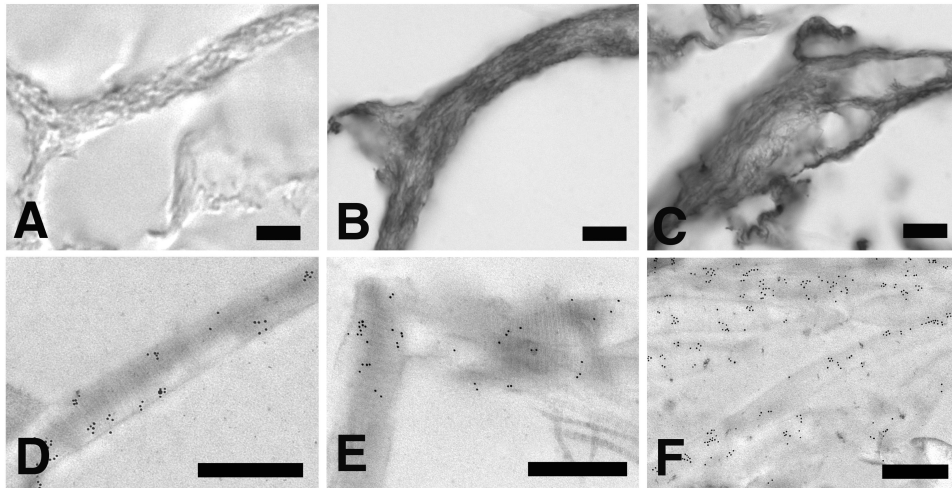


Figure 27. Immunolocalization of MMP-9 on collagen matrix following 1, 3 or 7 days of 3D *in vitro* culture in the presence of fibroblasts. (A-C), thick sections of formaldehyde-fixed and paraffin-embedded collagen matrix exposed to MMP-9 antibody and stained with DAB and nickel enhancement as described in material and methods, Bars 10 μ m. (D-F), thin sections of formaldehyde-fixed and glycol-methacrylate embedded collagen matrix exposed to MMP-9 antibody and treated with a secondary gold labelled antibody. Note how gold particles remain associated with the collagen even when the banding pattern is apparently lost, Bars 500 nm.

4.4. Discussion

The main objective of this study was to find out what causal relationship holds between collagen degradation, microvesicle release and metalloproteinase activities. When co-cultured with fibroblasts *in vitro*, the collagen matrix is gradually transformed from a prevailing laminar to an exclusive fibrillar type of architecture, indicating that collagen fibers are gradually frayed, disassembled or even fragmented. Given their temporal coincidence over the period of *in vitro* culturing tested in this study, both microvesicle release and metalloproteinase activity could be potential candidates for causing this collagen transformation. Experiments using gelatin zymography on cell lysates from fibroblasts demonstrated that the concentration of the active MMP-9 form is gradually increased as the relative precursor pro-MMP-9 is reduced. During the same time period, the extent by which microvesicles are released from the fibroblast plasma membrane is also significantly enhanced, as clearly documented by the numerous

micrographs depicting fibroblast cell peripheries or regions far more distant of the gel matrix.

However, coincidences can be meaningful only if causally connected. Microvesicle shedding and MMP activation could in fact be unrelated events and act independently on collagen degradation. In this case there would have to be different mechanism(s) affecting collagen transformation, without necessarily being mutually dependent on each other. One of the best result obtained in this thesis is the demonstration that shedding of microvesicles from the fibroblast filopodia and MMP secretion into the surrounding medium are indeed causally related events. The demonstration has come from the remarkable immunogold finding that microvesicles act as actual carriers for MMP-9. A large body of morphological and ultrastructural evidence from this thesis shows in fact that microvesicles become loaded with numerous smaller vesicles emerging from the nearby cortical cytoplasm as they are shed from the cell surface. Furthermore, numerous gold particles linked to MMP antibody label exactly these inner vesicles, suggesting that the MMP-9 cargo gains access to the extracellular milieu by means of an exocytic mechanism controlling fusion between the inner vesicles and the enclosing microvesicular membrane. To the best of my knowledge, this is the first ultrastructural and immunocytochemical demonstration that a metalloproteinase is secreted from a fibroblast cell type through a mechanism of membrane shedding.

In spite of its novelty, however, the finding that metalloproteinase and microvesicle shedding are causally related events is consonant with numerous earlier studies on microvesicles in various cell types and with their role on the persistence and stability of the extracellular matrix under normal and pathological conditions.

As well known, cell communication plays a key role in multicellular organisms. For cells to differentiate and remain sufficiently stable upon completing development, they need to be properly coordinated and their tissue integration strictly controlled. Cells achieve these ends by communicating through either ligand-receptor interaction, surface recognition of specific cell adhesion molecules or transfer of cytoplasmic components through junctional coupling (Sluijter *et al.*, 2014). In recent years, it has become apparent that cells may also communicate through the release of extracellular vesicles.

In spite of having been interpreted for many years as artefacts produced during tissue preparations, microvesicle shedding is now widely recognized as a general phenomenon occurring in every cell type (Lee *et al.*, 1993; 2011). Microvesicles may originate as exosomes from the endosomal compartment or be shed directly from the cell plasma membrane, especially in relation to filopodia extensions. In the first case, they are extruded from the cell surface following fusion of multivesicular bodies with the plasma membrane, while, in the second, they are simply detached from cell surface extensions by budding (Camussi *et al.*, 2010).

Extensive analyses by mass spectroscopy have revealed the variety of molecular contents associated with microvesicles and exosomes in different cell types. In general, the most abundant proteins present in these vesicles are related to cytoplasmic organelles or to the extracellular matrix and to cell adhesion molecules (Comelli *et al.*, 2014). Microvesicles and exosomes have also been implicated in the exchange of genetic information in the form of microRNA and RNAi from donor to acceptor cells (Gtiérrez-Vázquez *et al.*, 2013). Microvesicles released from breast carcinoma cells have been found to release specific metalloproteinase into the extracellular matrix (Dolo *et al.*, 1998). Under these circumstances, the load of associates metalloproteinase is likely to facilitate metastatic diffusion during cancer cell migration. Since several growth factors stimulate shedding of microvesicles loaded with pro-MMPs from the endothelial cells, it is currently assumed that the resulting proteolytic activity on the extracellular matrix constitutes a precondition for these cells to install a cascade of morphogenic events eventually leading to angiogenesis (Taraboletti *et al.*, 2002).

A large body of evidence has by now suggested that, during wound healing, the amount of collagen deposited on the damaged tissue is directly correlated with the level of MMP-9 expression, suggesting the extent of repairing is conditioned by the tissue capacity to remove any impaired collagen matrix (Agren *et al.*, 1998). The results obtained in this thesis are in line with this observation in indicating that MMP-9 expression on the collagen matrix is progressively more pronounced as it is gradually transformed from a laminar to a fibrillar type. The causal role played by microvesicles is once again demonstrated by the fact that this enhanced MMP-9 expression is

correlated with vesicles spreading in regions of the matrix far distant from their cells of origin.

Through the use of western blotting, two metalloproteinases fractions were detected in *in vitro* cultured fibroblasts. As well known, the higher molecular weight fraction is secreted as a stable, inactive zymogen, pro-MMP-9. Although a number of potential factors could activate pro-MMP-9 *in vitro* and *in vivo*, a recurrent observation – confirmed in this study – is that adhesive interactions with the extracellular matrix play an important role in MMP activation and hence in tissue remodelling (Abel and Vliagoftis, 2008). It is likely that activation of metalloproteinase may eventually result in the release of a number of collagen breakdown products that by themselves can exert chemotactic effects on cells invading the wound bed during formation of the granulation tissue (Brett, 2008).

In conclusion, the experimental evidence shown in this chapter could be summarized by saying that collagen breakdown and extracellular remodelling in wound healing are both causally dependent on the release of metalloproteinases and on microvesicle shedding. Given this relation, what remains to be verified is how in the first place microvesicles and pro-MMP9 come to be associated and eventually how they may be targeted to their substrate during cell migration.

CHAPTER V

5. The BCS and bioactive molecules.

Advances in the fabrication of biomaterials have led to the development of a new generation of engineered skin substitutes promoting both cell migration and tissue regeneration. As already shown, BCS has been proven optimal for sustaining cell interaction *in vitro* and for yielding high quality healing when applied *in vivo* (Lambert and Slaughter, 2011). The Euroresearch Company has developed new collagen based scaffolds to create even more favorable conditions for tissue growth and regeneration. The application of these scaffoldings has been shown to protect wounds from any external factors potentially hindering the healing process. The aim of this chapter is to verify if the BCS is structurally modified when combined with certain active substances, and in case it is, to what extent this modification affect or improve cell migration and/or adhesion when used for *in vitro* co-culturing.

Various combinations of collagen-based materials were studied. BCS was combined with 2,5% of sodium hyaluronate (BCS & NaYal), with 10 ppm of silver nanoparticles (BCS & Ag) and with 2% of Biosecure (BCS & Biosecure). Each of these combinations was then compared with the collagen scaffolding comprised of BCS and Collagen & Oxidate Regenerated Cellulose (Coll & ORC or Promogran®).

5.1. Ultrastructural characterization

Regardless of the substance they were combined with, Biopad Collagen based scaffolds were found to remain invariant as respect to the overall morphology of the upper external surface that maintained the same collapsed or sheet-like form in all cases. For this reason, only on the pan surface and the internal structure of the varying collagen combinations were compared in this study.

When examined by SEM, all Biopad Collagen based scaffolds appeared structurally similar to the controls, i.e. obtained under the same conditions with no substance added (Figs. 28A and 28B). The pan surface of these BCS (Figs. 28C to 28H) exhibited a uniform sponge morphology comprised of a network of thin collagen

laminae and fibers delimiting ample pores. By comparison Coll & ORC surface examined under similar conditions appeared made of irregularly displaced fibers and larger pores (Figs. 28I and 28J).

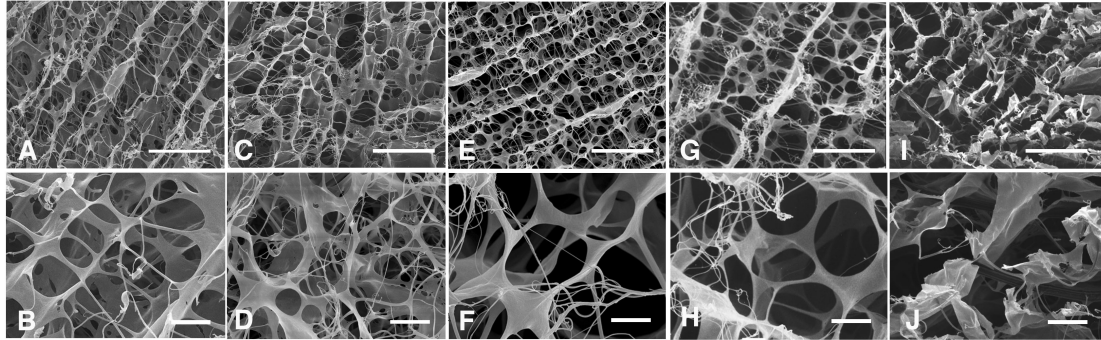


Figure 28. The pan surfaces of the different materials compared at SEM level. (A) and (B), the BCS; (C) and (D), the BCS & NaYal; (E) and (F), the BCS & Ag; (G) and (H), the BCS & Biosecure®; (I) and (J), the Coll & ORC. (A) and (C), Bars 50 μm ; (E), (G) and (J), Bars 100 μm ; (B), (D), (F) and (H), Bars 10 μm ; (I), Bar 500 μm .

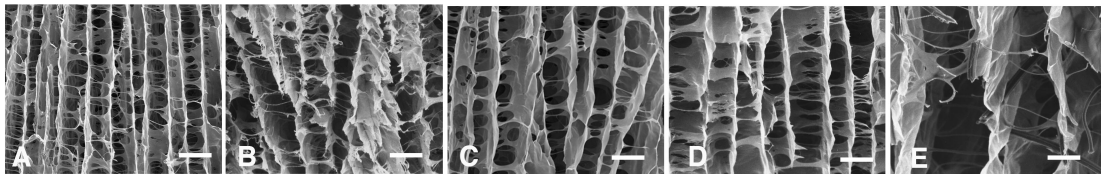


Figure 29. SEM images of the cross sections. (A), the BCS; (B), the BCS & NaYal; (C), the BCS & Ag; (D), the BCS & Biosecure®; (E), the Coll & ORC; Bars 100 μm .

When observed in cross sections, all Biopad Collagen based matrices exhibited the typical architecture of a BCS with a laminar trabecular pattern characterized by regular channels enclosed by bundles of collagen fibers (Figs. 29A to 29D). Coll & ORC had apparently the same overall architectural organization, although the collagen fibers did not maintain the same orientation for the entire thickness of the scaffold. As a result, this type of collagen scaffold had larger, though not uniform, pores.

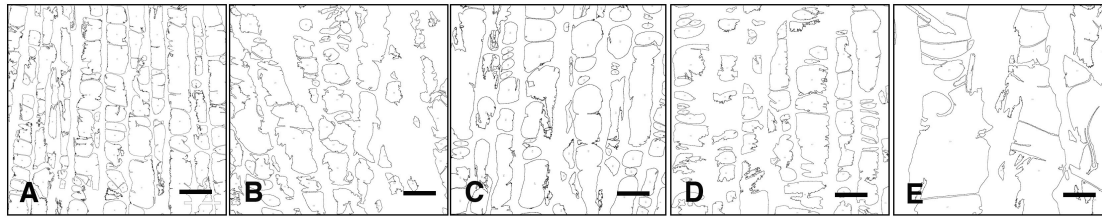


Figure 30. The SEM images of cross sections were used for pore size analysis and the pores drawing by using the imageJ program. Drawings of pores: (A), the BCS; (B), the BCS & NaYal; (C), the BCS & Ag; (D), the BCS & Biosecure®; (E), the Coll & ORC. Bars 100 μm .

	BCS	BCS & NaYal	BCS & Ag	BCS & Biosecure	Coll & ORC
Feret's diameter $\mu\text{m} \pm \text{sd}$	110 ± 47	78 ± 26	102 ± 41	79 ± 21	128 ± 92
n° of pore	93	73	72	28	28
% of area occupied by pore	44	41	56	42	55
Evaluated area μm^2	6×10^4	6×10^4	6×10^4	6×10^4	6×10^4

Table 2. Results of pore size analysis. The size of pores were measured extracting the Feret's diameter (see figure (a) in Table 1).

The scaffold pore sizes were measured by analyzing numerous SEM images of cross sections with the ImageJ program (Figure 30). The results are shown in Table 2. It appears that addition of NaYal and Biosecure® to BCS causes a slight reduction in the pore sizes of the collagen matrix. The Coll & ORC scaffold had pore sizes larger than all other samples examined in this study.

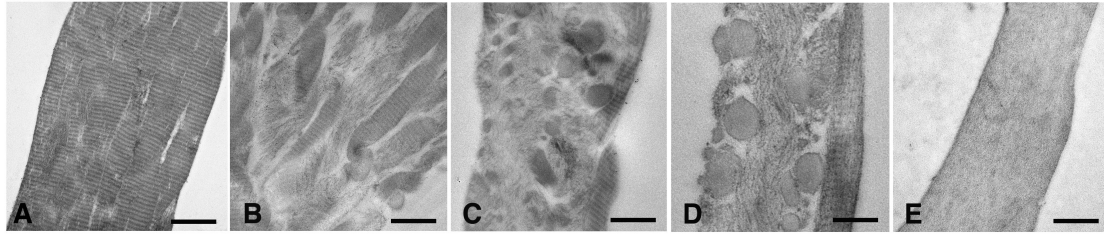


Figure 31. TEM micrographs of all BCS combinations. (A), the BCS; (B), the BCS & NaYal; (C), the BCS & Ag; (D), the BCS & Biosecure®; (E), the Coll & ORC. Bars 500 nm.

At the ultrastructural level, the collagen matrices of all collagen combinations were more loose than that of the BCS controls. In all cases, however, they were characterized by the presence of native collagen fibers (Figs. 31A to 31D). In the Coll & ORC sample, the laminae of the collagen scaffolding were still organized into fibrillar bundles, but no periodic banding could be envisioned on any of them (Fig. 31E). When the BCS & Ag sample was examined at the TEM, silver nanoparticles appeared associated with the collagen fibers either their margins (Fig. 32A), or as isolated cluster inside some of the dispersed fibrillar material (Fig. 32B).

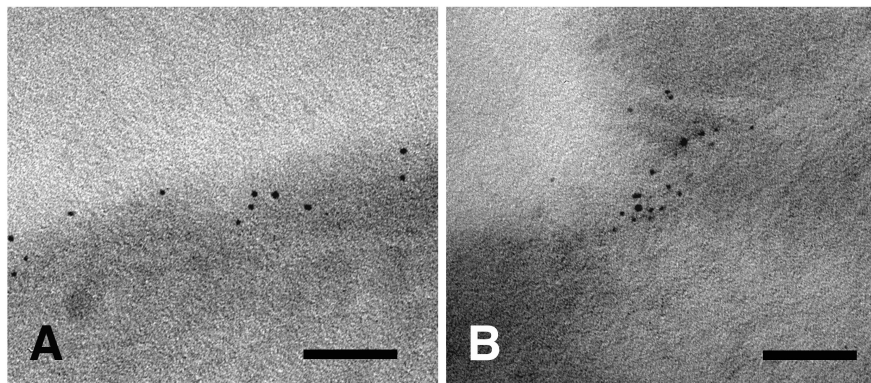


Figure 32. TEM images of BCS&Ag. (A), Ag nanoparticles along the collagen fiber; (B), cluster of Ag nanoparticles into a fibrillar portion of the material. Bars 100 nm.

5.2. Cell-matrix interaction

As shown above, addition of some external substance to the collagen scaffold changes the pore size of the matrix architecture. In this chapter, I ask the question whether such parameters as cell adhesion, migration and proliferation may be affected by this change and if so, whether this change may in turn represent an improvement over earlier formulations or simply an irrelevant variation. To verify how cell behavior is affected by the different pore sizes of these new collagen scaffoldings, aliquots of fibroblasts were cultured *in vitro* with the BCS, BCS&NaYal and Coll&ORC and maintained in under these conditions for time intervals ranging from 4 hours to 14 days.

A quantitative analysis of cell adhesion was carried out by directly counting the number of cells adhering to the seeded surfaces of every collagen scaffold. Data showed that addition of NaYal to BCS does not change significantly its capacity to retain cells within the matrix and allow them to migrate inward. By comparison the Coll&ORC sample appears to retain a significantly lower number of cells, thus suggesting that large pore sizes somehow hamper the cell capacity to enter the matrix (Figure 33).

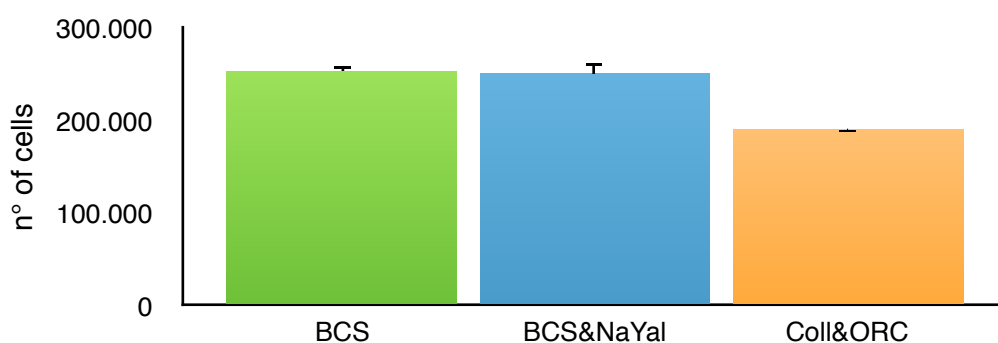


Figure 33. Results of cell adhesion.

Figure 34A is a SEM micrograph showing several cells adhering on the scaffold surface of the BCS&NaYal sample at 4 hours after seeding. Under these conditions, fibroblasts have already assumed a flattened shape, and are tightly anchored to some

collagen laminae (Fig. 34B). This result could be confirmed by light microscopy that allows to detect more cells on larger superficial collagen surface as well as inside the matrix itself (Figs. 34C and 34D).

More structural details could be revealed by analyzing the collagen surfaces at TEM level. In particular, it could be observed that along these sites cells had already assumed a spindle-like morphology, having been anchored with their lateral profiles to the vertical laminae (Fig. 34E). In this case, cells appeared rather thin, elongated and equipped of numerous filopodia creeping into the underlying collagen matrix. These changes in cell shape and adhesion can be taken as significant parameters to indicate that, by this time, fibroblasts have already begun migrating inside the collagen scaffold. Figure 34F shows a cell that has gained access into the collagen matrix and has already been anchored onto a collagen fiber. Some of collagen fibers are seen inside the cell, suggesting that fibroblasts may have already incorporated some of the material they have come in contact.

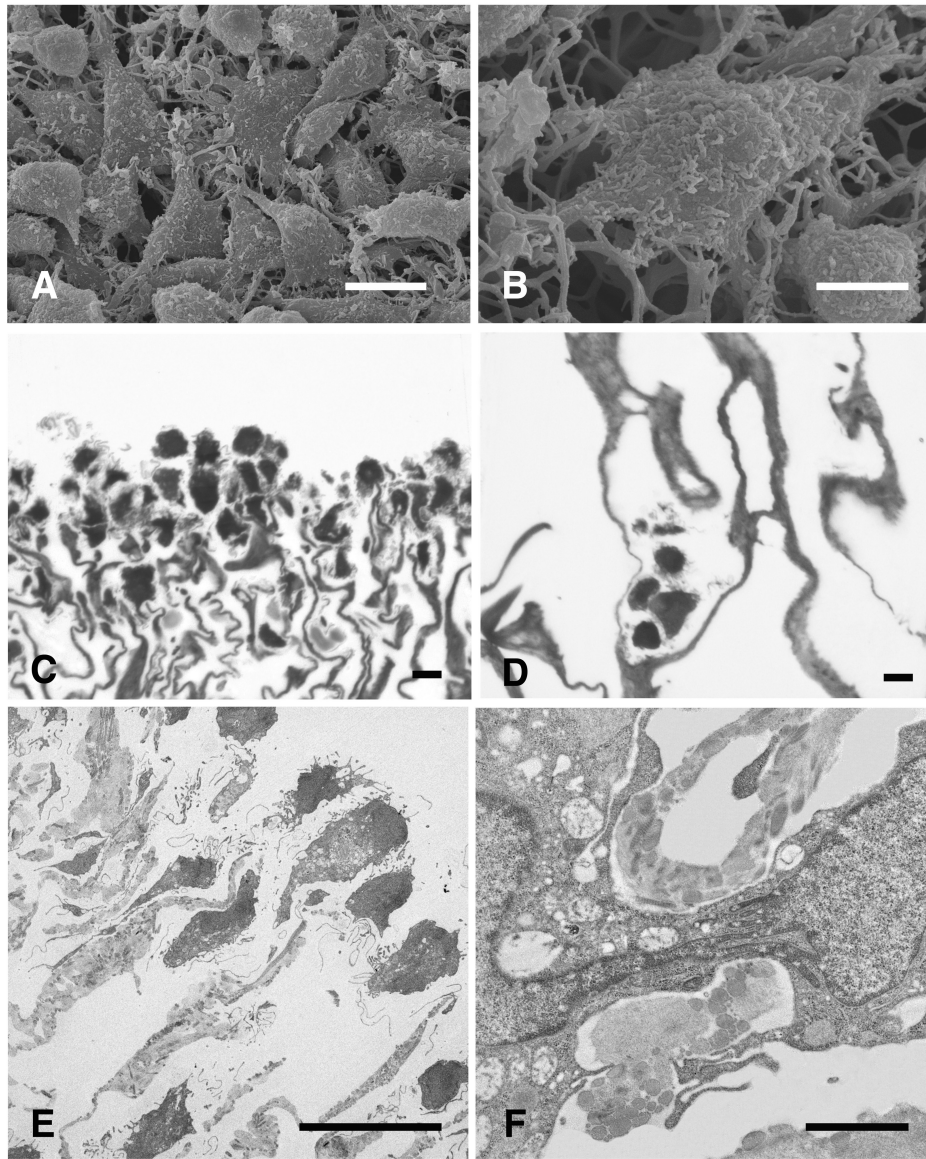


Figure 34. Cell-BCS & NaYal interaction at 4 hours from seeding. (A) and (B), SEM images of fibroblasts on the scaffold surface. At this time of co-culture the cells are numerous and strongly adherent to the material.(A), Bar 10 µm; (B), Bar 5 µm. (C) and (D), LM views showing the cells on the BCS & NaYal surface as well as inside the matrix, Bars 10 µm. (E), TEM micrograph showing thin cells elongated and equipped of filopodia that are creeping into the collagen matrix, Bar 20 µm. (F), NIH3T3 fibroblast anchored onto a collagen fiber, Bar 2 µm.

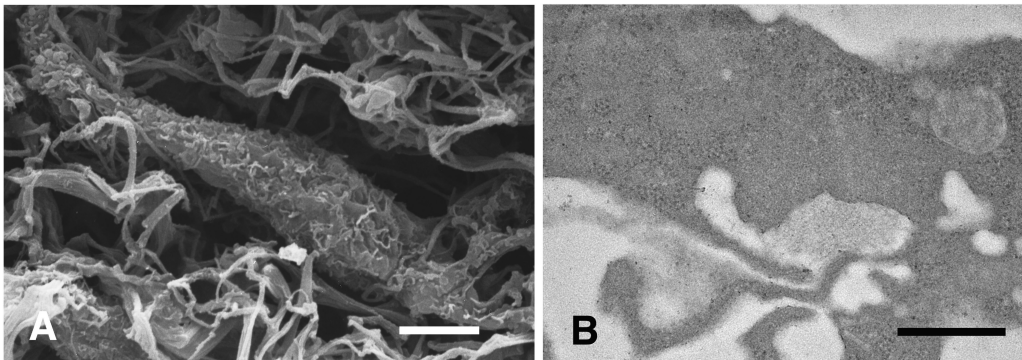


Figure 35. Cell-BCS & NaYal interaction at 24 hours after cell seeding. (A), SEM image showing the strongly interaction with the material, Bar 5 μm . (C), TEM micrograph of filopodia and lamellipodia in close contact with collagen fibers, Bar 500 nm.

At 24 hours after cell seeding, fibroblasts have interact more strongly with collagen matrix of the BCS&NaYal sample (Fig. 35A). At this time interval, cells had firmly adhered to the collagen matrix by means of the numerous filopodia processes highly intermingled with some frayed collagen fibers. Some of the details of this interaction can be seen in Figure 35B where filopodia and lamellipodia appear in close contact with the collagen fibers and have already incorporated some portion of the collagen lying nearby.

Three days after cell seeding, cells and the BCS & NaYal collagen have interacted even more strongly. The collagen matrix started to lose its laminar appearance both in vertical and horizontal development showing a predominantly fibrillar structure. This was probably a condition or a consequence of the cell ability to wrap the collagen fibers with several extended cellular processes (Figs. 36A and 36B).

The observation by TEM provided evidence that many fibroblasts become progressively more vacuolated in the cytoplasm, exhibit an increasing number of filopodia extensions along the cell surface and present of numerous microvesicles along the cell contour (Figs. 36C and 36D). In all likelihood these features are indicative of intense endo- and exocytic activities that result from fibroblasts interacting with the collagen fibers. The possibility to distinguish where cells are actually incorporating or

releasing material into external milieu would require the use of extracellular markers and specific radioisotopes. However, the presence of cytoplasmic vacuoles with contents of varying electro density was a clear indication of an intense endocytic activity of the collagen, most likely followed by an intracellular degradation (Figs. 36E and 36F).

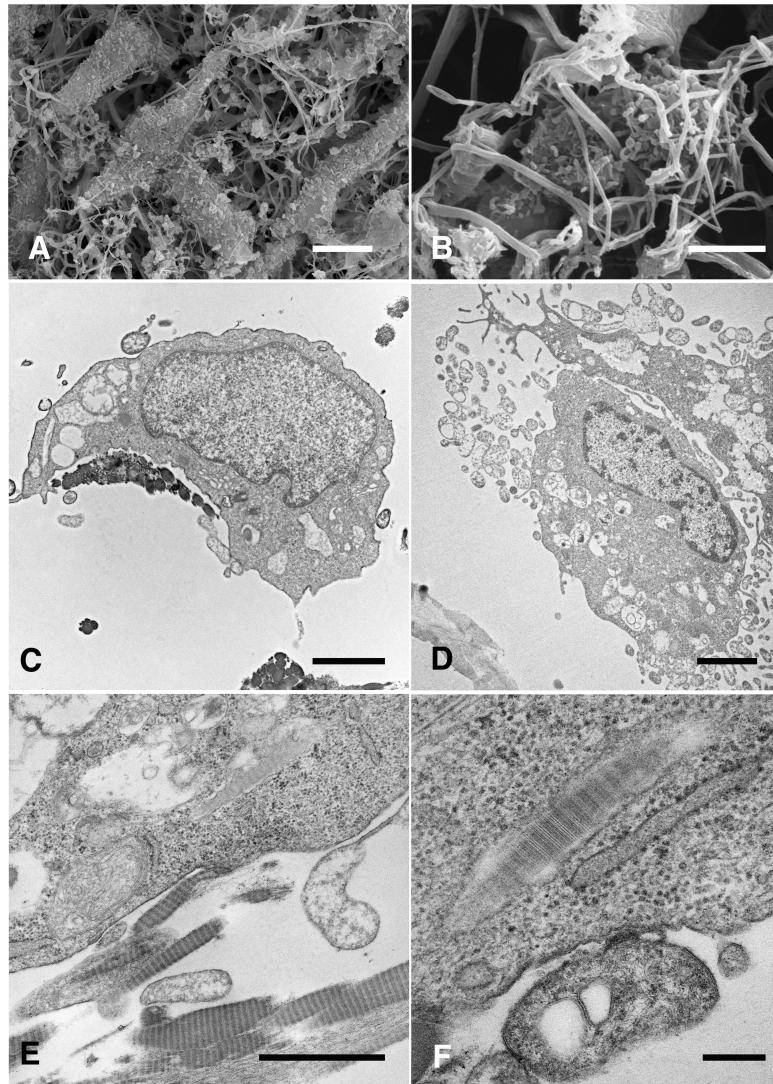


Figure 36. Cell-BCS&NaYal interaction at three days after cell seeding.(A) and (B), SEM micrographs showing how the the cells are increasing the interactions with the matrix, which begins to lose its laminar structure.(A), Bar 10 μm ; (B), Bar 5 μm . (D) and (E), TEM images showing the cytoplasm become more vacuolated, while the contour of the cell exhibits number filopodia and an increasing number of microvesicles. (E), Bar 500 nm; (F), Bar 200 nm. (E) and (F) hight magnification TEM micrographs showing the intracellular degradation of collagen.(E), Bar 500 nm; (F), Bar 200 nm.

The cells continued to be active prolonging the co-culture time at 7 (Figure 37) and 14 days (Figure 38). From SEM observations it was possible to highlight how the interactions with the substrate have become more complex (Figs. 37A and 38A). While the ultrastructural details showed the loss of laminar organization of collagen that appeared more fibrillar (Fig. 37B), as well as the level of collagen phagocytosis process was increased (Fig. 38B).

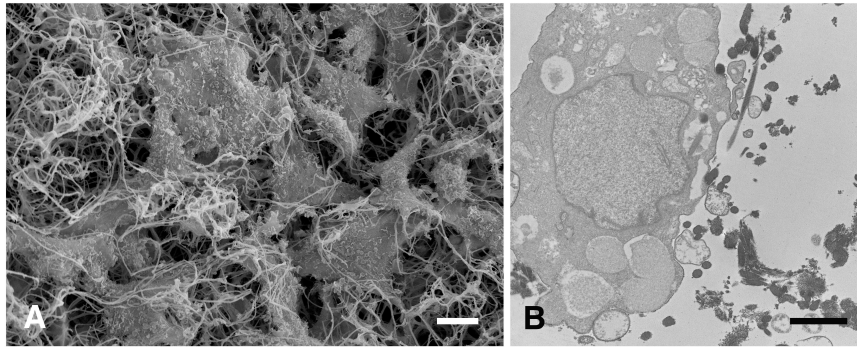


Figure 37. Cell-BCS & NaYal interaction at 7 days after cell seeding, Bars 2 μm .

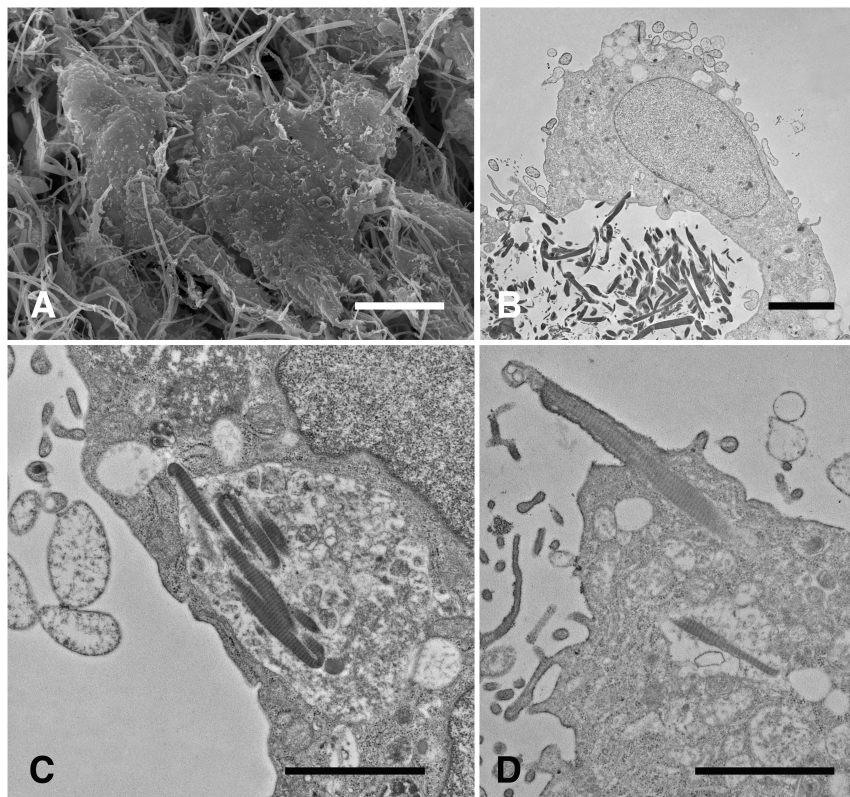


Figure 38. Cell-BCS & NaYal interaction at 14 days after cell seeding. (A), Bar 10 μm ; (B) and (C), Bars 5 μm ; (D), Bar 2 μm .

Four hours after seeding the number of cells present on the BCS surface was lower than that found on the same surface of the BCS&NaYal (Fig. 39A). Cells appeared morphologically intact and highly intertwined with the collagen fibrillar material. Many of these cells were still roundish in shape (Fig. 39B), while others had already assumed a typical fibroblast morphology, consisting of a flat and extended shape firmly adherent to the collagen substrate (Fig. 39C). An extensive TEM analysis demonstrated that cells had already gained access inside the collagen matrix and have adhered to the substrate even if some had retained a roundish shape (Figs. 39D).

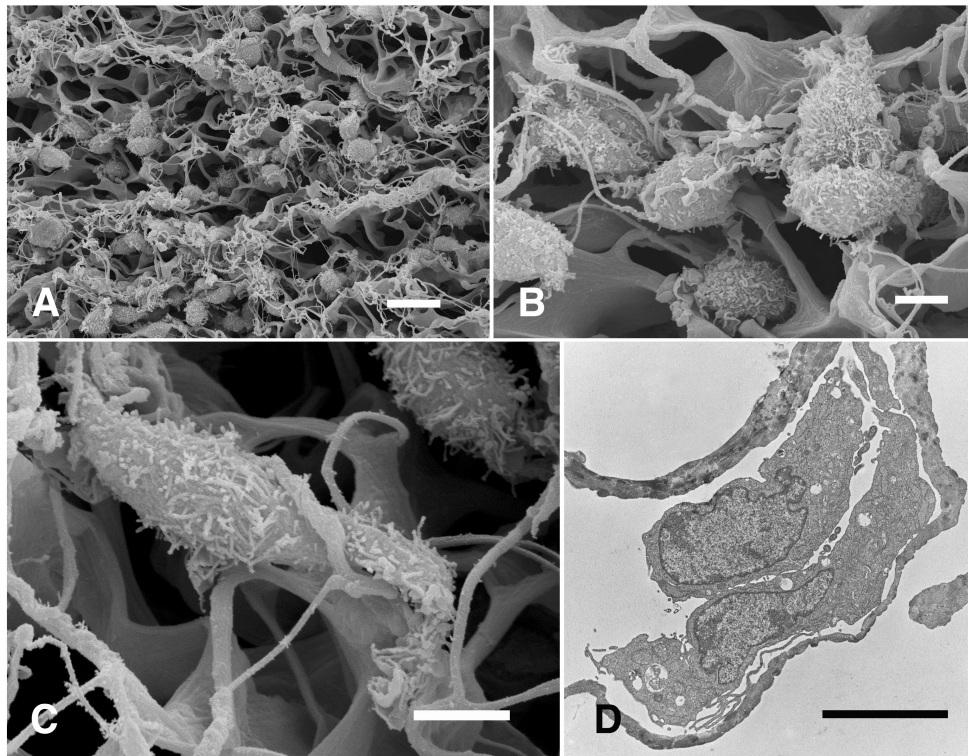


Figure 39. Cell-BCS interaction at 4 hours after cell seeding. (A), (B) and (C), progressively high magnification of cells on BCS surface at SEM level. (A), Bar 20 μm ; (B) and (C), Bars 5 μm . (D) TEM image of fibroblasts inside the collagen matrix, Bar 5 μm .

Several SEM observations indicated that cells had already started to interact more intimately with the BCS after 24 hours of co-culturing (Fig. 40A), having in the mean time assumed a firmer adhesion to the collagen and a more flattened and elongated shape than that expressed at 4 hours from the seeding time (Fig. 40B). A strong adhesion to the collagen substrate becomes even more pronounced at 3 days after cell seeding. At this incubation time the cells appeared metabolically active with numerous cytoplasmic offshoots enveloping collagen fibers. In response many fibrils may perhaps dissociate as a prelude for a subsequent molecular disaggregation (Fig. 40D). A detailed description of the cell-to-BCS interaction has already been reported in Chapter III.

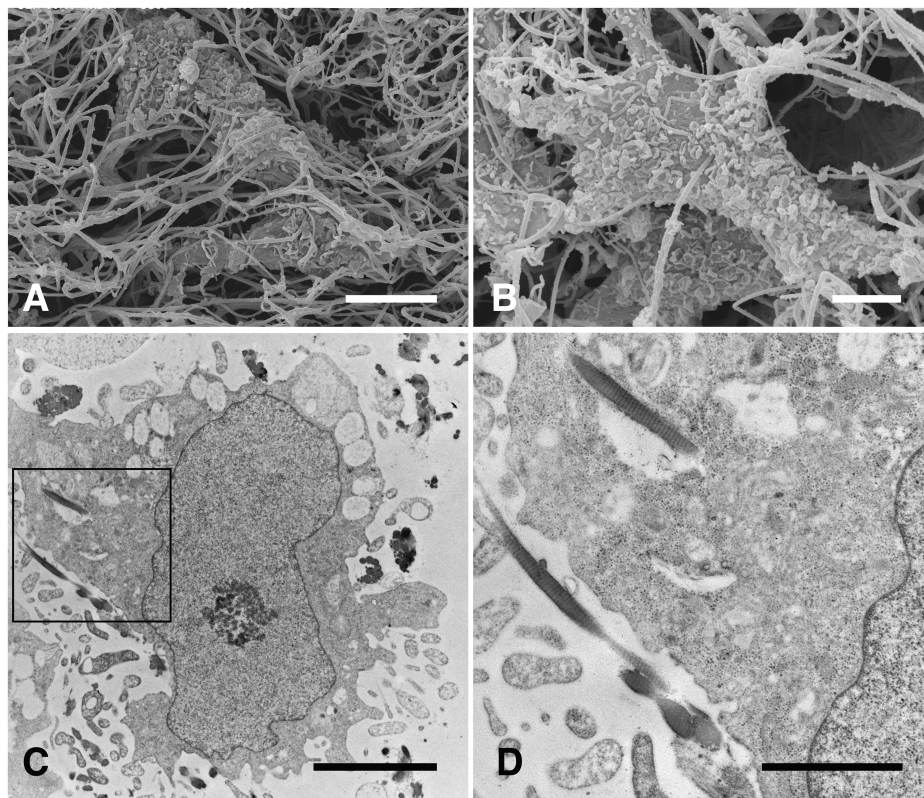


Figure 40. (A) and (B), SEM micrographs showing the Cell-BCS interaction at 24 hours of co-culture. (A), Bar 10 µm; (B), Bar 5 µm. (C) and (D) TEM images showing the intense activity of the cells at this incubation time, the cytoplasm revealed portion of collagen inside. (C), Bar 5µm; (D), Bar 1 µm.

The Figures 41 and 42 are an example of how complex is this cell interaction at 7 and 14 days of co-culture. Numerous microvesicles have already been released into the surrounding medium (Fig. 41B) and collagen fibrils appeared frayed into a mass of fibrillar material (Figs. 42B).

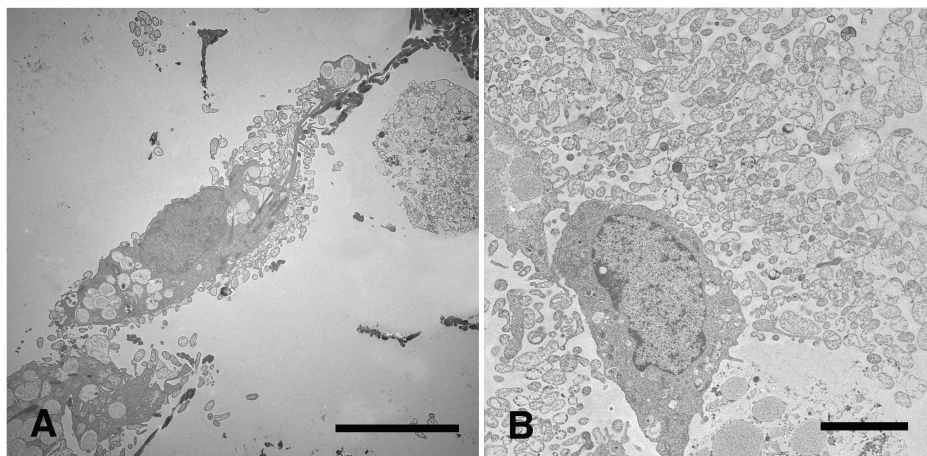


Figure 41. TEM images of Cell-to-BCS interaction at 7 days of co-culture, Bars 5 µm.

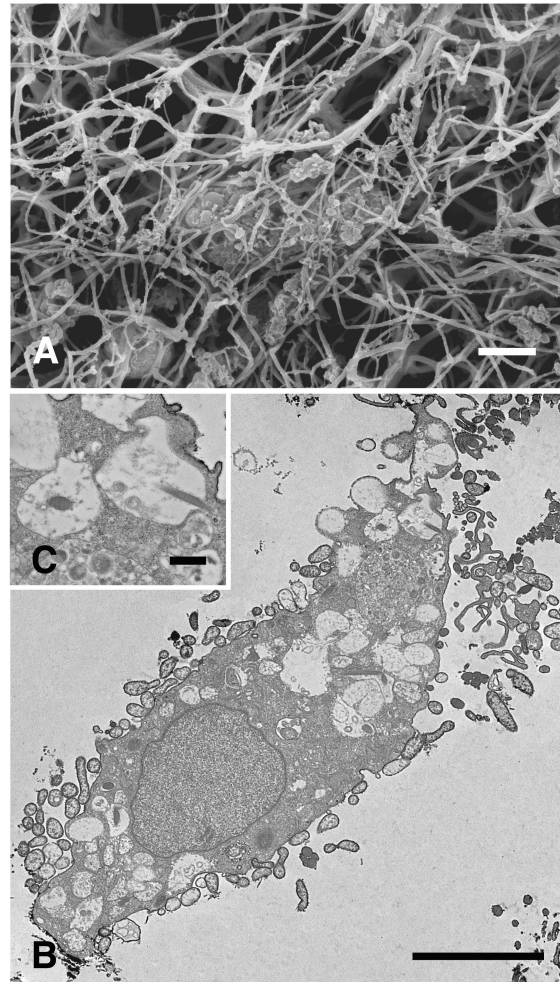


Figure 42. Cell-BCS interaction at 14 days after seeding. (A), at SEM level the cell appear completely enveloped into a mass of fibrillar material, Bar 5 μm . (B) and (C), TEM micrographs that highlight how the cytoplasm appears rich of vacuoles at different electron density, which clearly show portions of elaborated collagen inside. Bars: (B), 5 μm ; (C), 500 nm.

As far as the Coll&ORC sample is concerned, numerous SEM micrographs show that at 4 hours after seeding the number of cells was much lower than in other collagen supports previously examined. Furthermore, cell distribution was not uniform (Fig. 43A), fibroblasts clustering preferentially in some regions of the collagen scaffold (Fig. 43B). At these times of incubation, all cells were still roundish-shaped and did not exhibit any adhesion process onto the matrix. The overall tendency was for a prevalence of intercellular interactions as respect to the cell-to-collagen relationship (Fig. 43B).

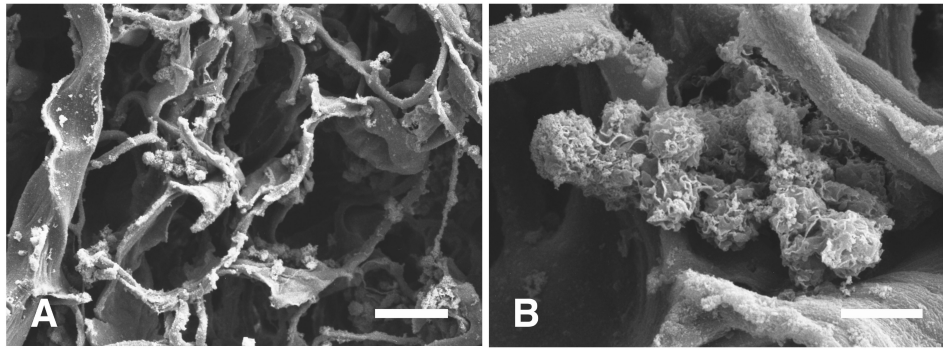


Figure 43. Cell-Coll&ORC interaction at 14 days after seeding. Bars: (A), 50 μm ; (B), 10 μm .

After 1 day of incubation cells lying on the upper surface of the Coll&ORC collagen scaffold were not yet polarized and still round-shaped (Fig. 44A). However, other cells that have migrated more deeply into the matrix, had already took contact with collagen fibers and assumed an elongated flat shape (Figs. 44B - D). The interaction with the matrix is documented by protrusion of numerous filopodia that contact the collagen fibers inside the matrix (Fig. 44D).

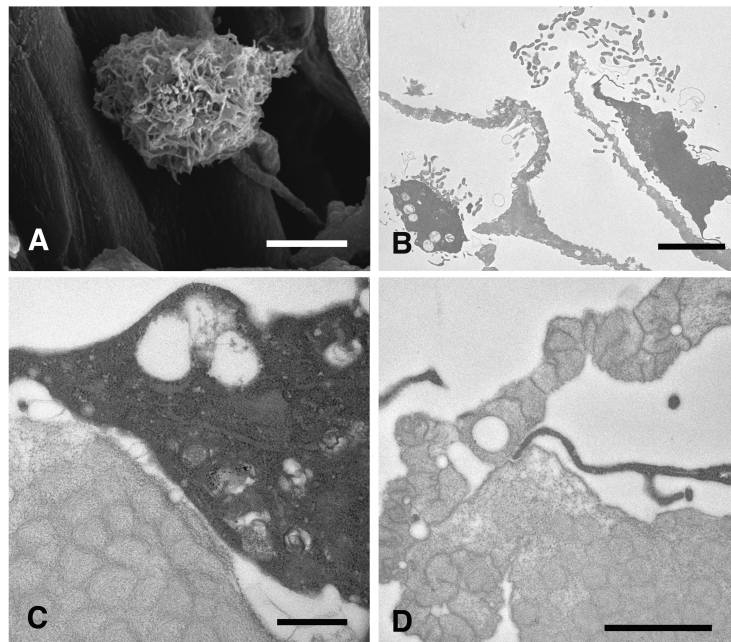


Figure 44. Cell-Coll&ORC scaffold interaction at 24 hours after cell seeding. (A) SEM image of a roundish fibroblast on the material, Bar 5 μm . (B), (C) and (D), the cell interactions with the material at TEM level. Bars: (B), 5 μm ; (C), 500nm; (D), 1 μm .

Even after three days of co-culture in Coll&ORC the number of cells was significantly lower than in other types of collagen scaffolds already observed. Although many more fibroblasts have, in the mean time, adhered to the collagen matrix, the majority of them still retained a flat shape (Fig. 45A). When observed by TEM, these cells did not appear superficially so elaborated as when placed in contact with other collagen scaffolds, even if the cytoplasm was highly enriched with many vacuoles and the plasma membrane involved in microvesicle shedding (Figs. 45B and 45C). The laminar organization of the matrix appeared more loose than that of the collagen-Biopad based scaffolds when observed within similar incubation times. Even after 7 and 14 days of culture, cells did not appear as active as when seeded on other collagen matrices.

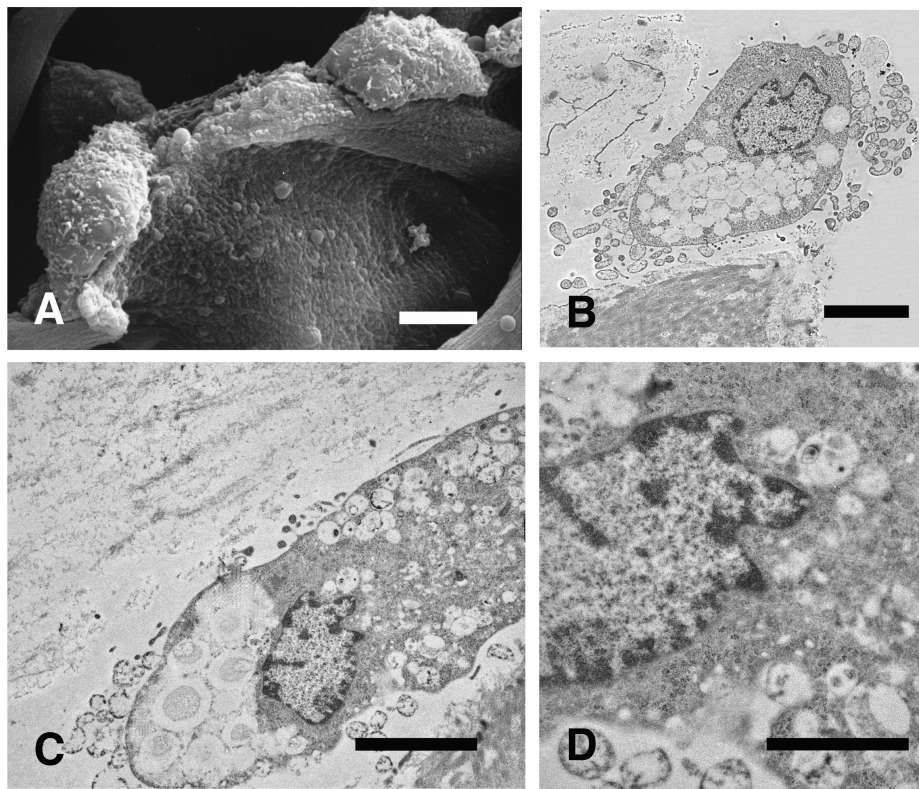


Figure 45. Cell-Coll&ORC scaffold interaction at three days of co-culture. (A), SEM image of fibroblasts that start to be more anchored to the material, Bar 5 μm . (B), (C) and (D), the cell interactions with the material at TEM level. (B) and (C), Bars 5 μm ; (D), Bar 2 μm .

5.3. Cell kinetics

To determine quantitatively how fibroblast behave under the experimental conditions adopted in this study, their cell metabolic activity was assessed with the MTT assay. This is based on the principle that the amount of NAD(P)H-dependent oxidoreductase - as measured through the reduction of a MTT tetrazolium dye - is directly correlated with the cell metabolic activity. Because of this correlation, it can be used to evaluate the number of viable cells present in the culture. As reported in Figure 46, cellular activity raised up to reach the maximum expression after 1 day of culture in all samples tested in this study. However, the extent of this increase differed from sample to sample. At 4 hours after seeding, the BCS&NaYal+3t3 sample had cell activity greater than that of the BCS+3t3 and Coll&ORC+3t3 samples. At 1 day, cell viability was significantly greater in BCS+3t3 and BCS&Nayal+3t3 than in Coll&ORC+3t3 and controls (3T3 cells, 2D cell culture). For longer incubation times (up to 14 days), both BCS and BCS&Nayal maintained a rather high cell activity, while in Coll&ORC cell activity lowered to the level of control.

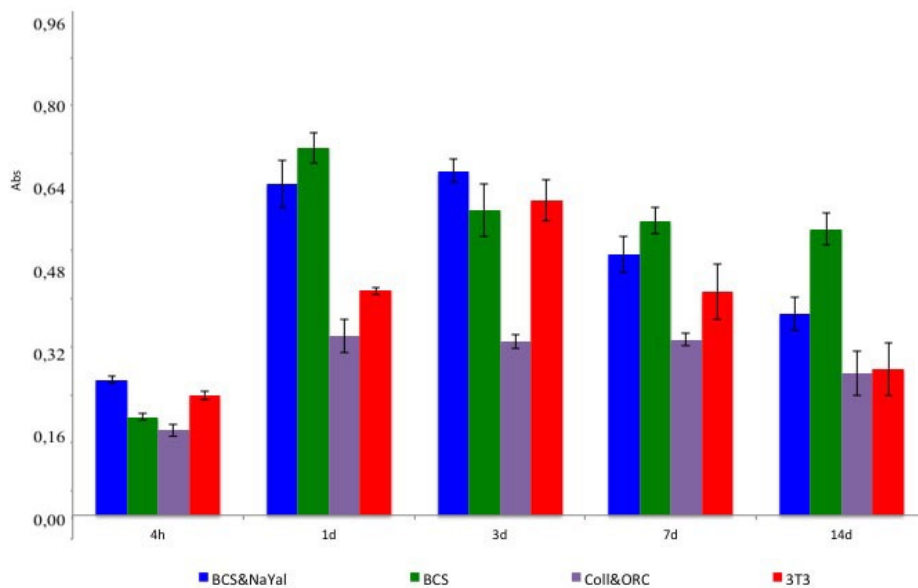


Figure 46. Results of MTT assay.

5.4. Discussion

The morpho-functional analysis conducted in this study demonstrate that the collagen-Biopad based scaffolds exhibit structural and architectural characteristics similar to those of the BCS. This is a clear indication that addition of such substances as NaYal to the BCS matrix does not interfere with the assembly of collagen fibers in the scaffold matrix.

BCS collagen fibers interact with sodium hyaluronate (NaYal), silver nanoparticles (Ag) or complex substances as Bisecure® in such a way as to maintain the same range of pore size and sufficient integrity in the internal laminar scaffold. Both of these structural characteristics are important in favoring cell interactions with the collagen matrix and the host tissue. In all likelihood they are similar to the ones expressed *in vivo* when the collagen support is applied onto the wound.

Amongst the factors promoting wound healing, a key role is played by the capacity to absorb blood and fluids and to sustain cell migration from the surrounding areas. It's clear that a good porosity of a collagen scaffold is a necessary, although not sufficient, condition for blood and fluids to be efficiently absorbed and removed. Therefore an appropriate matrix porosity does certainly contribute to increase the chances of healing, especially in chronic wounds. Moreover, a regular porosity of about 100 and 150 μm facilitates cell adhesion onto the collagen fibers and, at the same time, allows cells to migrate deeper into regions of the matrix (Lawrence and Madhally, 2008).

On the whole, the study of cells interacting with collagen-Biopad based scaffolds demonstrates that, within the first 4 hours of incubation, 3T3 fibroblasts assumed cell shapes and behaviors that are clearly indicative of optimal conditions for cell interaction. The ultrastructural analysis carried out in this thesis provides additional evidence that cells migrating within the cell matrix are highly extended in length and capable protruding with many filopodia. By contrast, cells co-incubated with the Coll&ORC sample for the same incubation times, maintain a rather roundish shape and do not migrate significantly into the collagen matrix.

For progressively longer incubation times, 3T3 cells interact more strongly with the collagen-Biopad based scaffold by migrating into larger portions of the matrix. As these cells migrate, they exhibit numerous filopodia in close contact with the collagen fibers, besides being actively involved in exo-endocytosis along the plasma membrane.

In conclusion, of the varying collagen scaffolds compared in this study, the BCS and the BCS&NaYal proved to be the best *in vitro* substrate to simulate such processes as adhesion, migration and endocytic incorporation, processes which are routinely expressed *in vivo* by all cells that accede to the extracellular matrix.

Of the cellular properties tested in this chapter, proliferation is perhaps the most relevant. For instance, the MTT assay has demonstrated that cell viability increases during the co-culture period both in BCS and BCS&NaYal. On the contrary, for the same time period, cell viability remained essentially invariant in the Coll&ORC sample. This fact can be interpreted in two different ways: (1) if the number of cells remains constant throughout the culturing period, any increase in MTT activity can be attributed to the acquisition of a more differentiated state such as to require higher energetic costs. Alternatively, (2) if cells are not undergoing differentiation *in vitro*, then any increase in MTT activity can be related exclusively to an increase in the cell proliferation rate.

The morphological analysis conducted in this study has shown that cells differentiate to some extent, but probably their differentiation is not such as to account for the entire increase in MTT activity. It remains possible therefore that cells placed in co-culture with BCS and BCS&NaYal, unlike those seeded on Coll&ORC, have been effectively increased in number simply because induced to proliferate. This aspect of the regenerative medicine should be further studied and probably subject to more stringent tests to improve the conditions that make wound healing more efficient, especially in chronic wounds.

CHAPTER VI

6. The BCS and Lipidots™

The results exposed in this section were obtained at the Nano-Bio section of CEA-Leti, Grenoble (FR). The aim of the project was to create a new collagen-Biopad based scaffold loaded with lipid nanoparticles (the Lipidots™) and determine its structural characteristics under *in vitro* conditions. Development of this new dermal substitute is part of the SWAN-iCare European Project (<http://swan.exodussa.com>) to produce an integrated autonomous device for monitoring and treating leg and foot ulcers in diabetic patients. This is an outpatient therapy that has the advantage of combining the application of a negative pressure with a collagen dressing in direct contact with the wound. While the negative pressure provides a moist environment to reduce bacterial colonization, edema and dead space, besides promoting blood circulation, new granulation tissues and epithelialization, the applied collagen dressing is used to release bioactive compounds to accelerate the healing process itself (Texier *et al.*, 2013).

In 2005 the CEA-Leti patented a lipid nanoemulsion - Lipidots™ (LNPs) - that can be employed *in vivo* for vectorization and drug delivery. LNPs are composed by an outer lipid shell stabilized by PEGylated surfactants and phospholipids that may be loaded with hydrophobic active substances (Figure 47). The viscosity of the particles core mixture (portion of wax) controls the release kinetics of the encapsulated principles. Delmas *et al.*, (2011, 2012) showed that any increase in LNPs core viscosity facilitates uptake of active principles in the particle core and slow down their release. The release of drugs by lipid nanoparticles of various nature and structure (liposomes, lipid micelles, nanoemulsions lipid, lipoprotein, solid lipid nanoparticles) has been extensively studied (Sawant *et al.*, 2008; Kenneth *et al.*, 2011; Allen *et al.*, 2013).

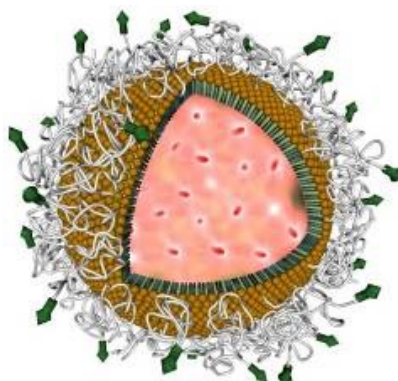


Figure 47. Schematic view of Lipidots™.

In view of the knowledge acquired on Biopad Collagen Scaffold (BCS) and on the experimental models to test it, I was asked to have a direct role in the production of Lipidots™ (BCS/LNPs) and in the characterization of their chemical/physical properties and their cellular responses. The results obtained from these experiments are currently under patent submission.

The physical-chemical properties of BCS/LNPs were determined through the assessment of their morphological and ultrastructural characters such as pore sizes, swelling ratio, thermal stability and rheological properties. In addition, their behavior was determined by exposing samples under *in vitro* conditions in the presence of cells as NIH3T3 fibroblasts and HaCat keratinocytes.

6.1. Material characterization

The material was prepared by loading two different sizes of nanoparticles, F50 (50 nm) and F120 (120 nm) at the concentration of 10/1, 5/1, 3.3/1, 2.5/1, 2/1 w/w dry collagen/lipids, in a 1% collagen gel w/w in acetate buffer (from Euroresearch srl) as described in Materials & Methods. The BCS and the BCS/Myrj™ s40 (or BCS/PEG) were used as controls.

6.1.1. Structural characterization

Scanning Electron Microscopy (SEM) explored the morphology and porosity of the material so obtained. The overall organization of the collagen fibers consists of a trabecular pattern characterized by vertically oriented laminae interconnected by thin filaments. This organization was similar to the one present in pure collagen (BCS) and in collagen/particles materials (BCS/LNPs) with collagen/lipid ratio 10/1 w/w (Figure 48). The structure tended to become slightly less regular with increasing amounts of lipid nanoparticles, for BC/LNPs ratios below 3.3/1 (Figures 49 and 50).

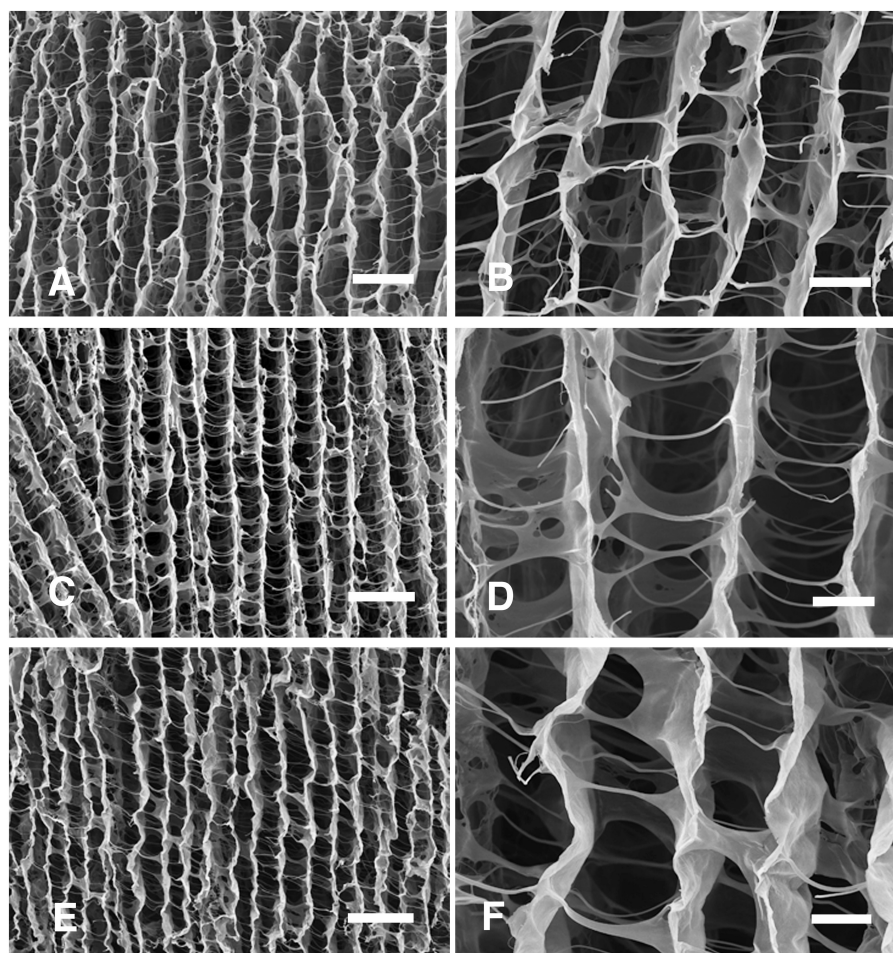


Figure 48. SEM micrographs of BCS, BCS/LNPs-F50 ratio 10/1 and BCS/LNPs-F120 ratio 10/1 dry material. (A) and (B), the BCS; (C) and (D), the BCS/LNPs-F50-10/1. (E) and (F), the BCS/LNPs-F120-10/1. (A), Bar 50 μm ; (C) and (E), Bars 100 μm ; (B), (D) and (F), Bars 20 μm .

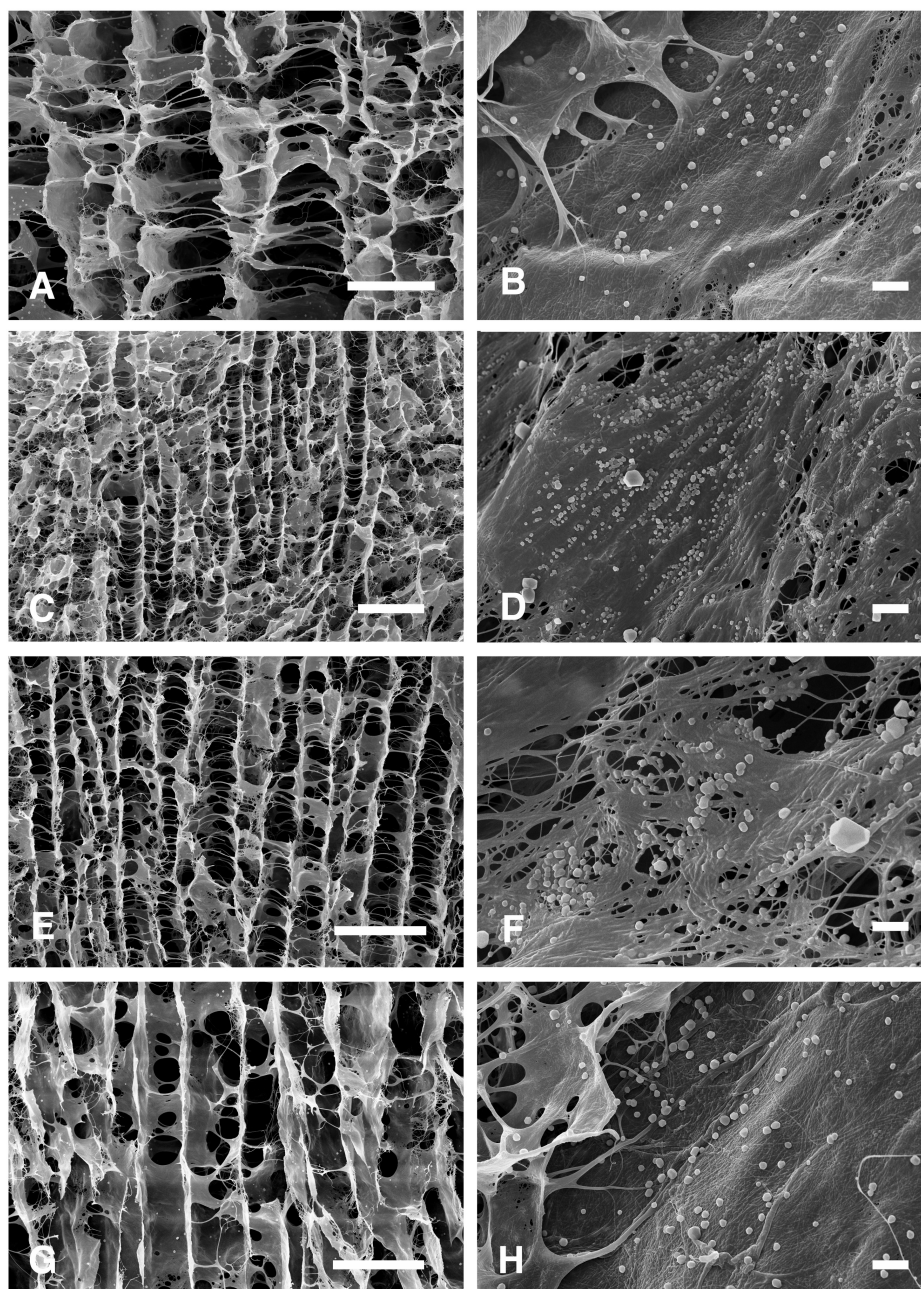


Figure 49. SEM micrographs of BCS/LNPs-F50 at different ratio (weight particles/weight polymer). (A) and (B), 5/1; (C) and (D), 3.3/1; (E) and (F), 2.5/1; (G) and (H), 2/1. (A), (C), (E) and (G), Bars 100 μm ; (B), (D), (F) and (H), Bars 10 μm .

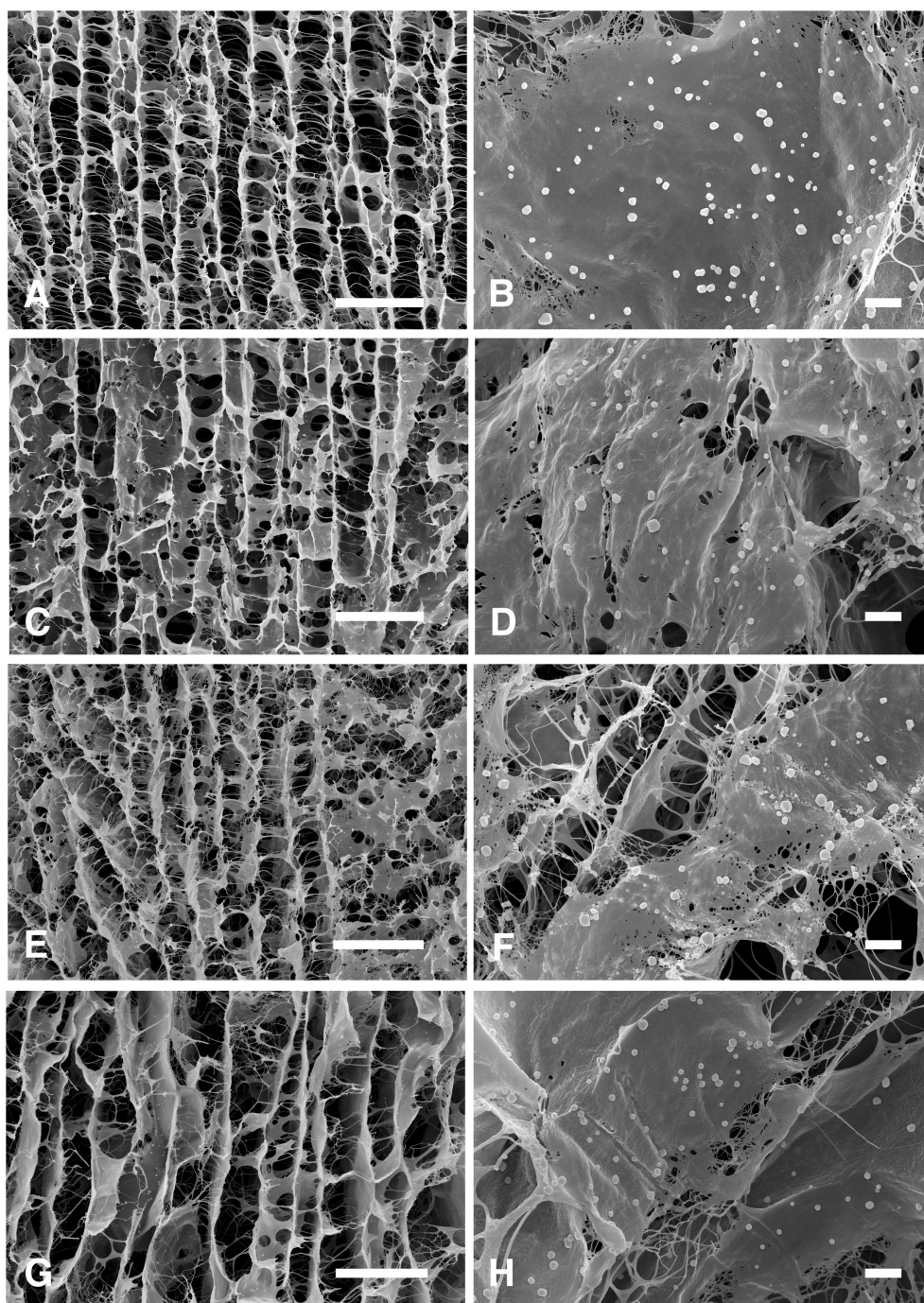


Figure 50. SEM micrographs of BCS/LNPs-F120 at different ratio (weight particles/weight polymer). (A) and (B), 5/1; (C) and (D), 3.3/1; (E) and (F), 2.5/1; (G) and (H), 2/1. (A), (C), (E) and (G), Bars 100 μm ; (B), (D), (F) and (H), Bars 10 μm .

The pore sizes were analyzed by using SEM images elaborated with ImageJ program (Figure 51). Results are listed in Table 3.

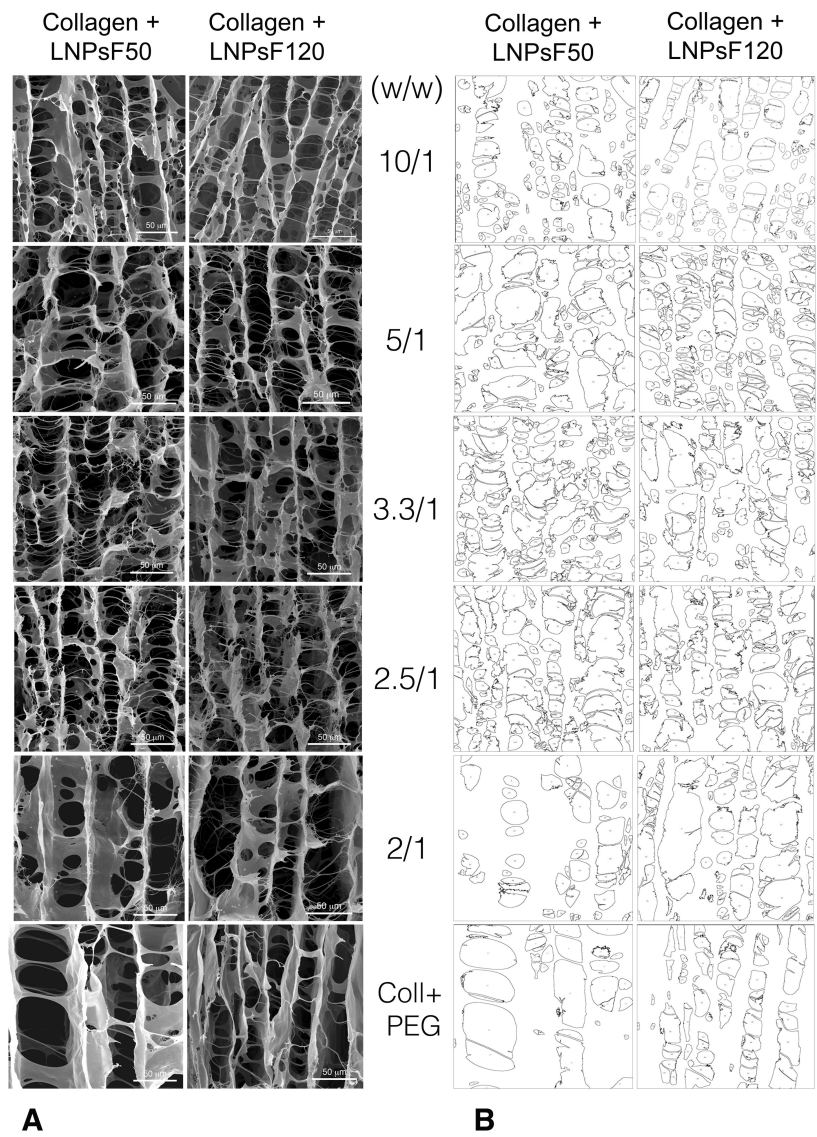


Figure 51. (A), SEM micrographs of BCS/LNPs cross sections used for pore size analysis. (B) Drawing of pores obtained from imageJ program.

	number of pores	Evaluated Area (μm^2)	% Area occupied by pores	Feret's diameter (μm)
Coll F50 10/1	121	$4 \cdot 10^4$	37.78	99 ± 62
CollF120 10/1	133	$4 \cdot 10^4$	40.43	98 ± 60
CollF50 5/1	83	$4 \cdot 10^4$	56.91	84 ± 58
CollF120 5/1	170	$4 \cdot 10^4$	49.11	98 ± 59
CollF50 3.3/1	140	$4 \cdot 10^4$	52.86	94 ± 59
CollF120 3.3/1	93	$4 \cdot 10^4$	55.11	98 ± 61
CollF50 2.5/1	108	$4 \cdot 10^4$	59.13	90 ± 57
CollF120 2.5/1	94	$4 \cdot 10^4$	53.85	95 ± 62
CollF50 2/1	56	$4 \cdot 10^4$	25.97	119 ± 57
CollF120 2/1	75	$4 \cdot 10^4$	56.08	107 ± 62
Coll/Myrj 21/1	37	$4 \cdot 10^4$	43.88	96 ± 61
Coll/Myrj 40/1	60	$4 \cdot 10^4$	33.71	82 ± 43

Table 3. Pore size analysis of the BCS/LNPs at different radio weight particle/weight polimer. Collagen/MyrjTM s40 was used as control, ratio 21/1 control F50 and ratio 40/1 control F120.

The ultrastructure of the BCS/LNPs was analyzed by Transmission Electron Microscopy (TEM). The collagen matrix of this sample comprised filamentous and laminar structures both of which are made of rather compact fibrils. At higher magnification these fibrils reveal the presence of the typical repeating banding patterns of native collagen, due to the staggered arrangement of the constituting polypeptides. Lipid spherical nanoparticles appeared regularly distributed either inside the fibers and along their surface (Figure 52). Evidence of such nanostructures could not be found in materials prepared by mixing the collagen gel with only the MyrjTMs40 co-surfactant (Figure 53).

The ultrastructure of collagen was well preserved, as the nanoparticle coating did not alter the collagen fiber integrity up to high concentrations (Figures 54; 55 and 56). For low collagen/lipid ratios (below 2.5/1) (Figures 55 and 56) the collagen fibers

appeared more loose. Nanoparticle analysis confirmed that their sizes remained within the range of 50 nm diameter (Fig. 57A) or 120 nm diameter (Fig. 57B).

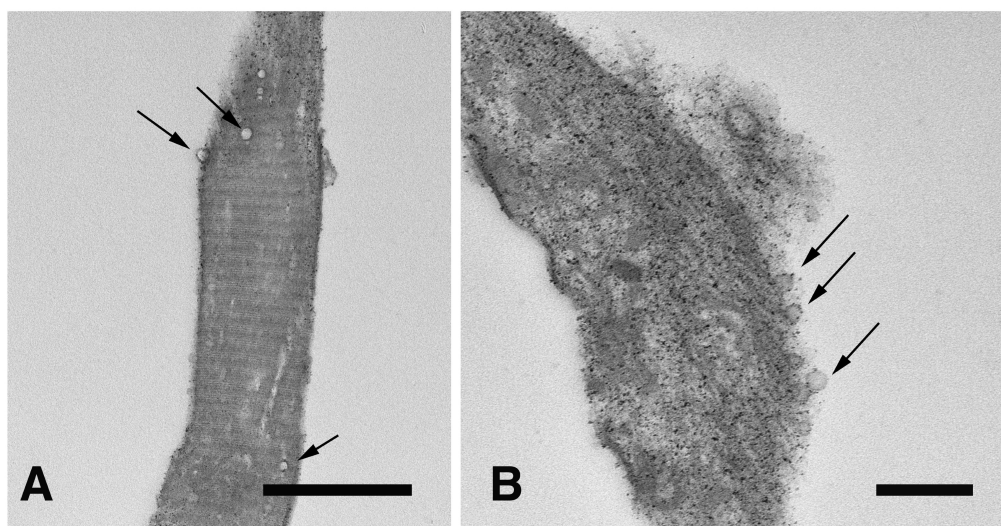


Figure 52. TEM micrographs of BCS/LNPs-F50 ratio 10/1. (A), Collagen fiber with LNPs (arrows), Bar 500 nm; (B), Collagen fibrils with LNPs (arrows), Bar 200 nm.

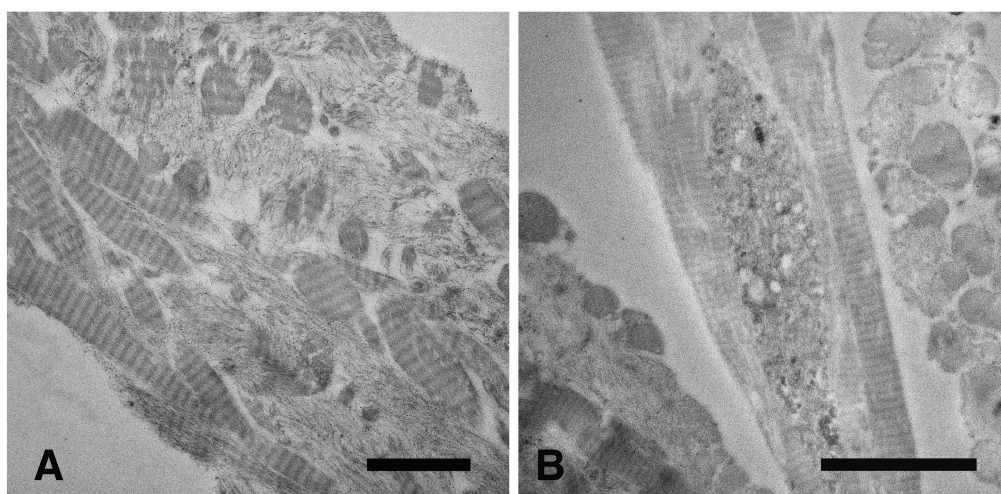


Figure 53. TEM micrographs of BCS/Myrj™s40 (BCS/PEG). (A), BCS/PEG ratio 21/1, control for F50 nanoparticles, Bar 500 nm; (B), BCS/PEG ratio 40/1, control for F120 nanoparticles, Bar 1 μ m.

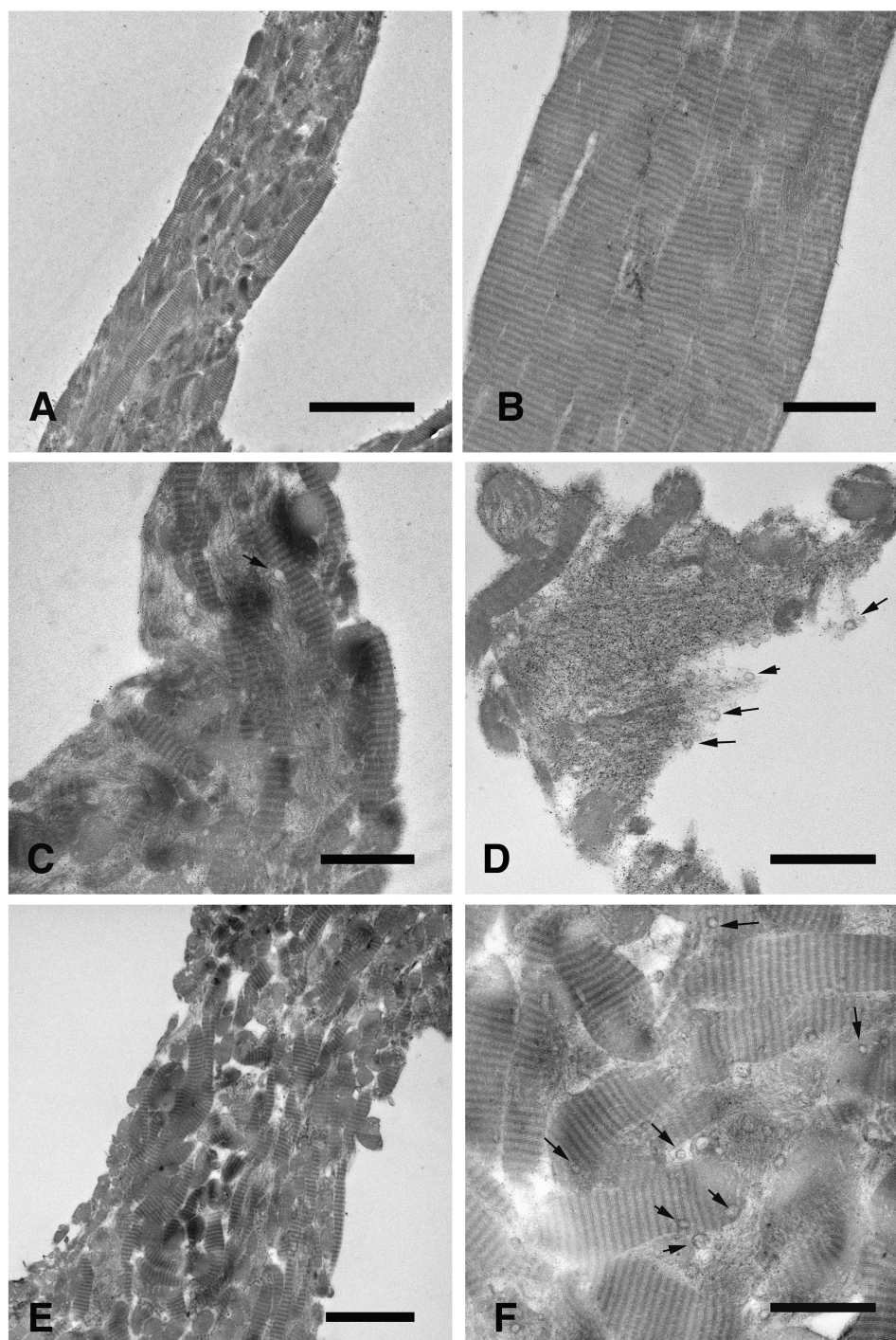


Figure 54. TEM micrographs of BCS, BCS/LNPs-F50 and BCS/LNPs-F120 ratio 10/1. (A) and (B), the BCS. (C) and (D), the BCS/LNPs-F50. (E) and (F), the BCS/LNPs-F120. The arrows revealed the LNPs in the collagen matrix. (A), Bar 2 μm ; (B), (C), (D) and (F), Bars 500 nm; (E), Bar 1 μm .

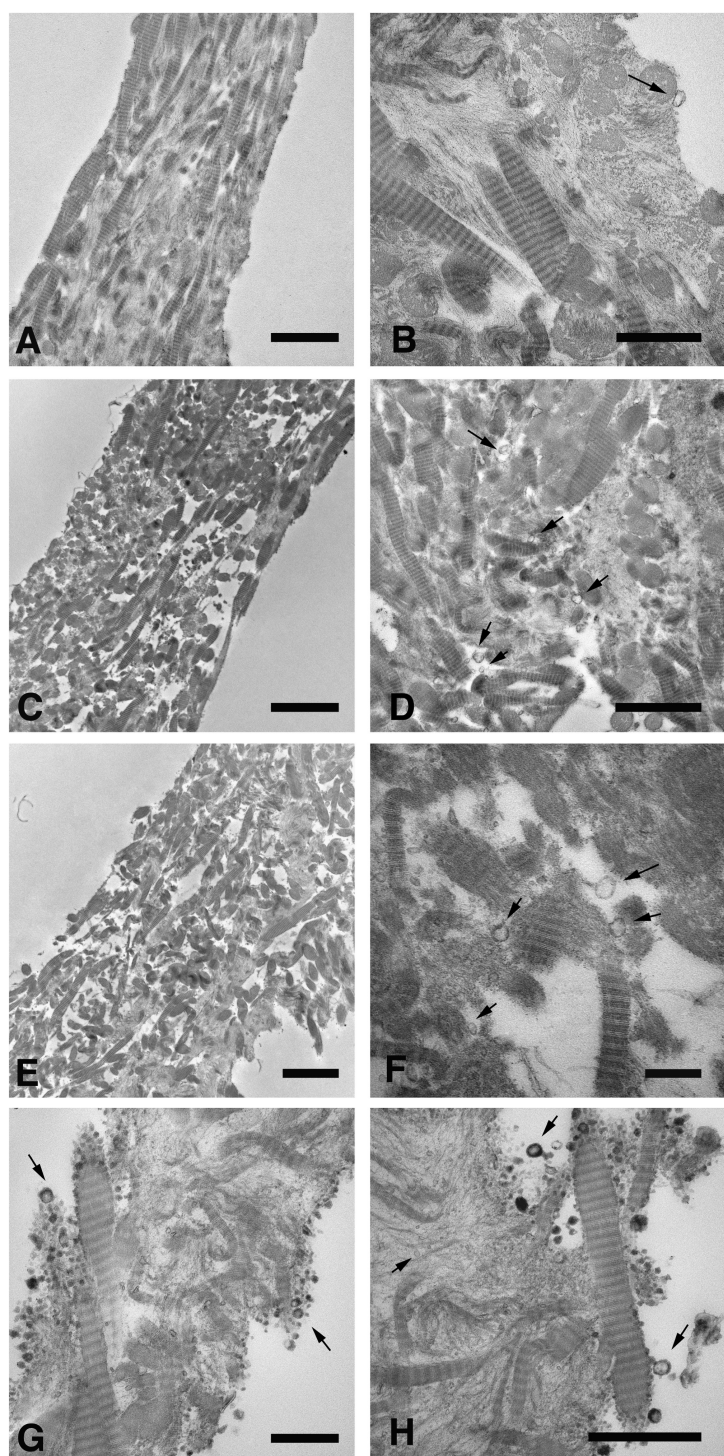


Figure 55. TEM micrographs of BCS/LNPs-F50 at different ratio (weight particles/weight polymer). (A) and (B), 5/1; (C) and (D), 3.3/1; (E) and (F), 2.5/1; (G) and (H), 2/1. The arrows revealed the LNPs in the collagen matrix. (A), (B), (D) and (G), Bars 1 μ m; (B) and (H), Bars 500 nm; (C) and (E), Bars 2 μ m; (F), Bar 200 nm.

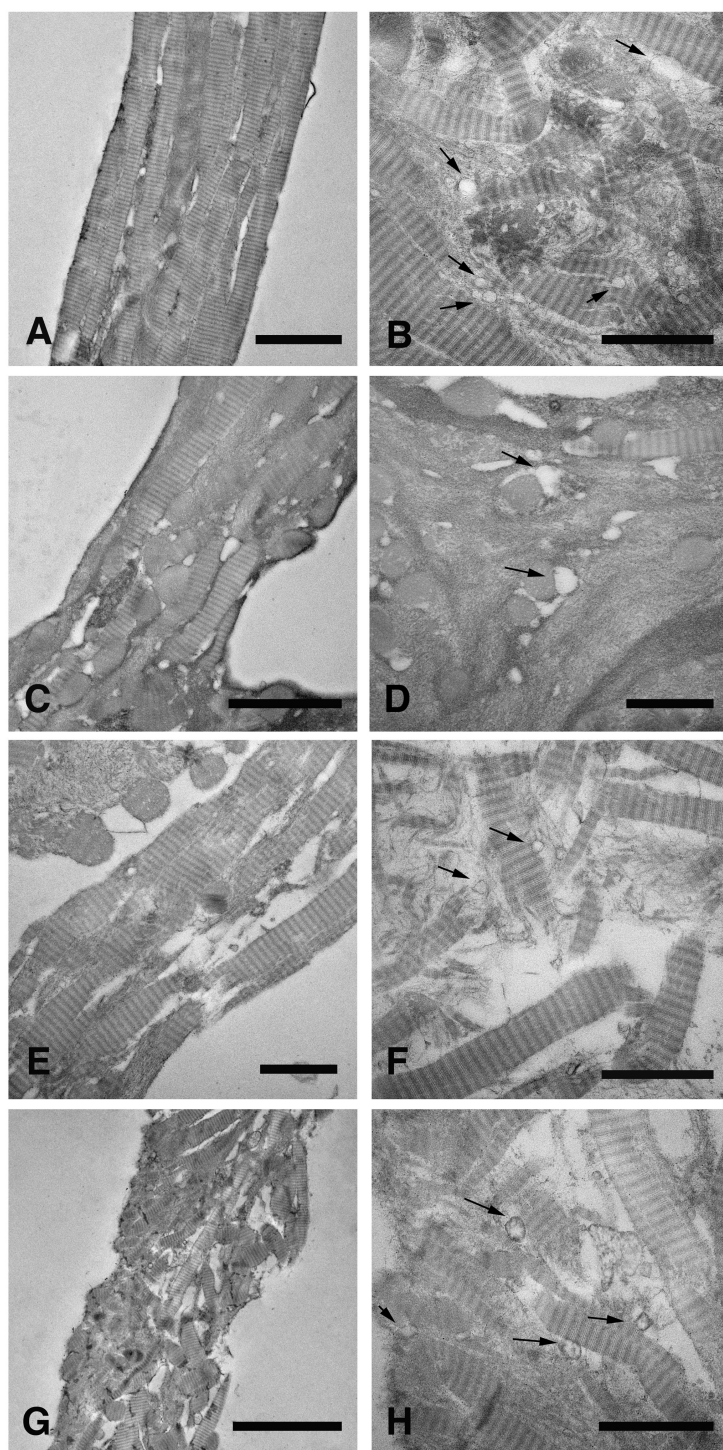


Figure 56. TEM micrographs of BCS/LNPs-F120 at different ratio (weight particles/weight polymer). (A) and (B), 5/1; (C) and (D), 3.3/1; (E) and (F), 2.5/1; (G) and (H), 2/1. The arrows revealed the LNPs in the collagen matrix. (A), (C) and (D), Bars 1 μ m; (B), (E), (F) and (H), Bars 500 nm; (G), Bar 2 μ m.

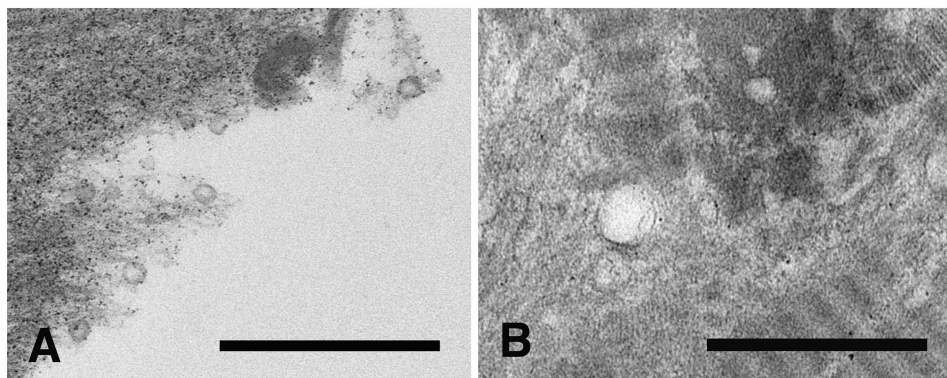


Figure 57. TEM micrographs of LNPs in collagen matrix. (A), F50 (50 nm), Bar ; (B), F120 (120 nm), Bars 500 nm .

To verify whether nanoparticles were indeed associated with the collagen fibers BCS was mixed with FRET nanoparticles loaded with either DiI or DiD dyes and the resulting sample examined by Confocal Laser Scanning Microscopy (CLSM) (Figure 58). The fluorescence patterns envisioned by this technique provide a clear indication that nanoparticles are uniformly distributed within the collagen matrix. Moreover, the observation that the topological distribution of fluorescent signals overlaps with the collagen fiber network as evidenced by SEM, is an additional evidence that nanoparticles have actually adhered to the collagen fibers, having formed a uniform coat all over the scaffold matrix.

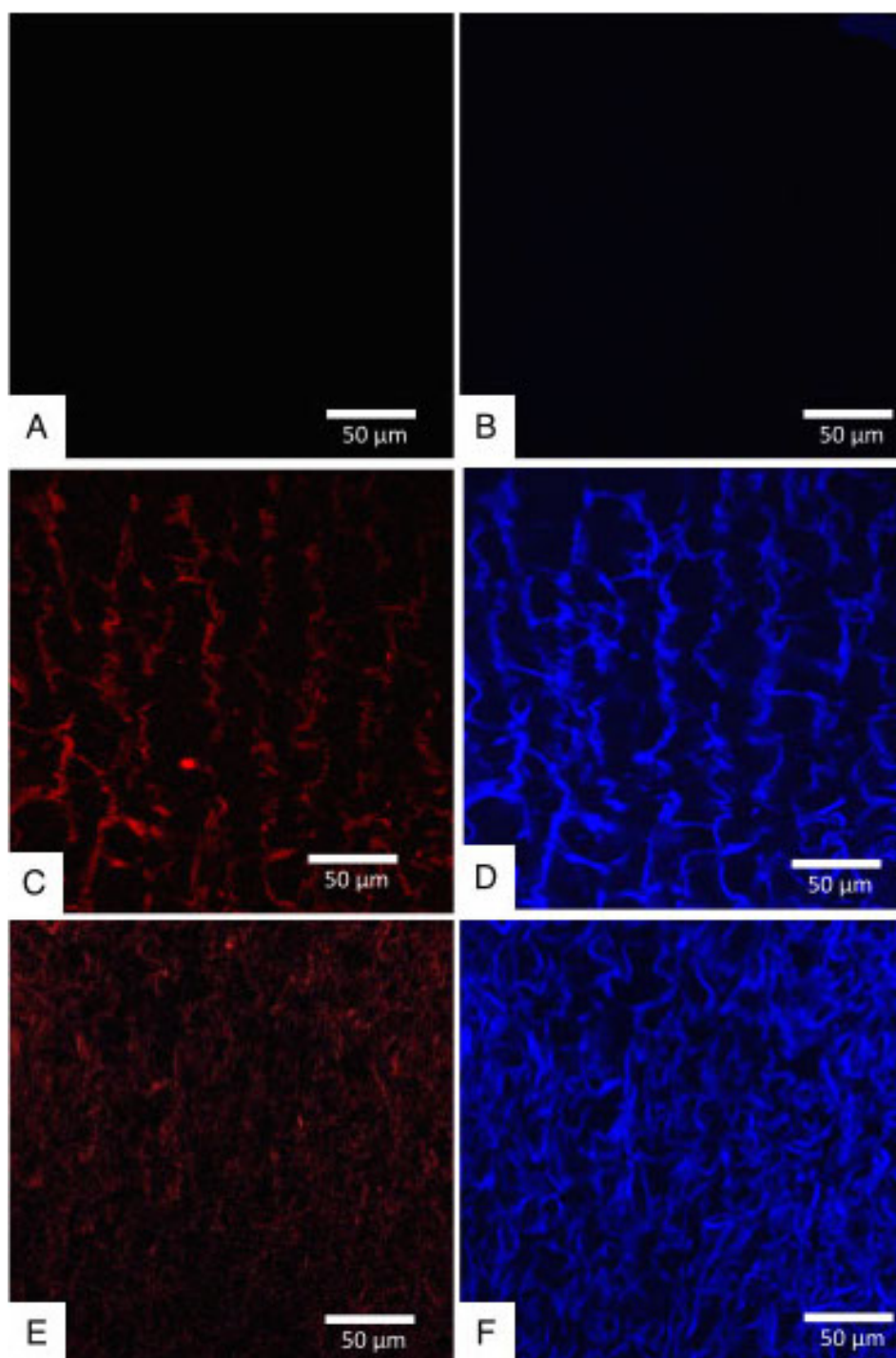


Figure 58. CSLM images of BCS and BCS/LNPs ratio 10/1. (A) and (B), the BCS used as control. (C), TRICH excitation (555-620 nm); (D), CY5 650-750 nm, for the BCS/FRET-LNPs-F50. (E), TRICH 555-620 nm; (F) CY5 650-750 nm, for the BCS/FRET-LNPs-F120.

6.1.2. Particles stability

To assess the stability nanoparticles in the lyophilized collagen/lipidots samples, a protocol for particles recovery from matrix were developed as described in materials and methods. Table 4 reports the results obtained by DLS measurements of the diameter and polydispersity (PDI) of nanoparticle extracted from the collagen matrix and evaluated both before and after sterilization. Data showed that nanoparticles analyzed after their embedment in the collagen matrix have maintained diameter size and PDI values higher than those obtainable from the same sample in solution.

	BEFORE sterilization		AFTER sterilization	
	SIZE	PDI	SIZE	PDI
BCS/LNPs-F50	107,0 ± 0,702	0,206 ± 0,008	109,2 ± 0,458	0,266 ± 0,012
BCS/LNPs-FRET F50	113,2 ± 1,513	0,150 ± 0,008	97,63 ± 0,90	0,262 ± 0,010
BCS/LNPs-F120	138,7 ± 1,15	0,188 ± 0,011	157,4 ± 3,089	0,282 ± 0,010
BCS/LNPs-FRET F120	129,7 ± 0,873	0,181 ± 0,015	130,7 ± 0,472	0,275 ± 0,004
LNPs-F50	59,01 ± 0,7	0,152 ± 0,01	55,15 ± 0,33	0,151 ± 0,01
LNPs-FRET F50	67,90 ± 0,91	0,071 ± 0,01	62,93 ± 0,29	0,084 ± 0,01
LNPs-F120	129,5 ± 0,7	0,126 ± 0,01	118,7 ± 1,04	0,119 ± 0,01
LNPs-FRET F120	122,5 ± 0,01	0,073 ± 0,02	114,6 ± 0,56	0,088 ± 0,01

Table 4. Results of DLS measurements

6.2 Material properties

6.2.1. Swelling ratio

To verify whether the BCS water absorbing capacity has undergone any change of following addition of nanoparticles, its swelling ratio was measured as described in materials and methods. The results of this analysis are presented in Table 5. When compared with pure BCS, the swelling ratio of BCS/LNPs in water was much lower - having a slightly more pronounced effect for F120 than for F50 nanoparticles – most likely in relation to hydrophobic effect induced by the lipid coating of the nanoparticles. No significant effect could be observed in 1XPBS, probably in relation to the presence of higher salt concentrations. However, in both these conditions the BCS/LNPs sample maintained good absorbing properties, as it is normally required for dressing wounded skin areas.

Materials	SR in MilliQ water	SR in 1X PBS
BCS	428 ± 13	528 ± 16
BCS/F50 10/1	329 ± 28	522 ± 22
BCS/F50 3.3/1	342 ± 15	531 ± 64
BCS/F50 2/1	361 ± 31	516 ± 44
BCS/F120 10/1	287 ± 12	502 ± 25
BCS/F120 3.3/1	334 ± 19	520 ± 40
BCS/F120 2/1	333 ± 15	507 ± 2

Table 5. Swelling ratio after 24 hours immersion in either MilliQ water or PBS 1X, the BCS were used as control, BCS/F50 nanoparticles with collagen/lipid ratios 10/1, 3.3/1, and 2/1, BCS/F120 nanoparticles with collagen/lipid ratios 10/1, 3.3/1, and 2/1.

The enhanced hydrophobic character of the BCS/LNPs (10/1) sample becomes detectable since the very early phases of hydration. In fact, if a drop of water (10 µL) is deposited on scaffold matrix made of only pure collagen, it is immediately absorbed. If,

on the other hand, it is poured on the BCS/LNPs/F50 (10/1) sample, it requires ten seconds to be fully absorbed. Even longer periods are required for the BCS/LNPs/F120 (10/1) sample. The angle formed by the water drop while in contact with the upper surface of the collagen sample can be measured by using a Digidrop instrument. The speed of liquid absorbance inside the varying collagen samples may instead be monitored by recording drop images at different times after the initial contact (Figure 59). Results are presented in Table 6.

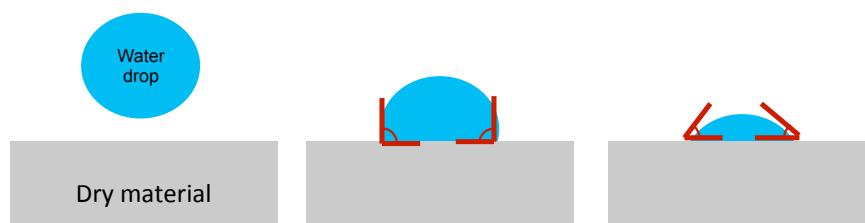


Figure 59. Schematic view of contact angle between dry materials and the aqueous phase (water drop).

Samples	Water drop contact angle 10 seconds after drop deposit	Speed of water absorbance
BCS	Left angle: 0° Right angle: 0°	<0,5 sec
BCS&LNPs- F50	Left angle: 48,5° Right angle: 47,5°	21 sec
BCS&LNPs-FRET F50	Left angle: 86,0° Right angle: 84,3°	>90 sec

Table 6. Results of contact angle and speed of water absorbance obtained by Digidrop instrument.

6.2.2. Thermal stability

The thermal stability of the collagen scaffold with or without nanoparticles added was determined by Differential Scanning Calorimetry (DSC). The heating rate was maintained constant at 3°C/min throughout the measurement while the temperature was gradually increased from 20 °C to 110°C. Data are summarized in Table 7.

The shrinkage temperature could be determined as the initial value attained by the endothermic peak. The thermal denaturation temperature (for helix-to-coil transition of the collagen fibers) was taken at the endothermic peak maximum. The value of denaturation enthalpy was calculated with respect to the weight of the collagen in the dried matrix. Several samples of each material were analyzed.

Sample	Shrinkage temperature (onset) (°C)	Thermal denaturation temperature (peak) (°C)	Denaturation enthalpy (J/g)
BCS	49.8	59.3	42.9
BCS&LNPs/F50 (10/1)	53.6	60.1	53.2
BCS&LNPs/F50 (2/1)	53.3	58.5	39.3
BCS&LNPs/F120 (10/1)	55.3	59.3	58.4
BCS&LNPs/F120 (2/1)	53.2	58.4	33.8

Table 7. Results of DSC.

6.2.3. Mechanical properties

Uniaxial compression tests were performed on samples of lyophilized collagen (BCS, BCS/F50 10/1, BCS/F120 10/1) that were shaped as cylinders measuring 4.1 mm in diameter and 5 mm in height using the AR2000EX rheometer. A preload of 0.03 N was initially applied, followed by a compression speed of 10 µm/s (Figure 60). The stress (σ) was calculated as the normal force divided by the surface area of the sample, whereas the strain (ϵ) was the % of compression of the material. The stress-strain plots obtained were typical of an elastic foam-like material (Gibson, 2005), and the Young

modulus E^* of the materials could be calculated as the slopes of the stress-strain curves for low strains (linear domain). Values are presented in Table 7.

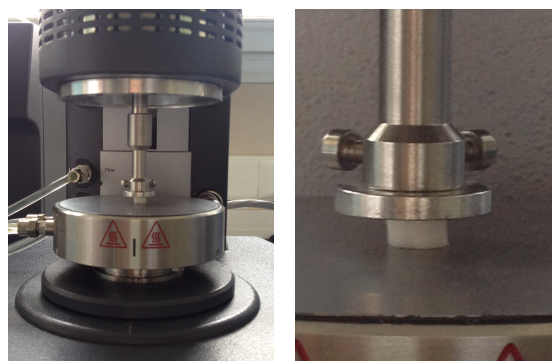


Figure 60. Rheometer during the compression tests.

E^* [kPa]	Sample 1	Sample 2	Mean $\pm$ SD
BCS	115.676	106.958	111.4 ± 6.2
BCS/F50 10/1	95.202	51.359	73.3 ± 31.0
BCS/F120 10/1	54.985	59.205	57.1 ± 3.0

Table 7. Young modulus (E^*) of BCS (control), BCS/F50 and BCS/F120 ratio 10/1.

6.3. Cell-matrix interaction

To characterize the cell/matrix interaction aliquots of NIH-3T3 fibroblasts or HaCat keratinocyte were seeded on the surface of BCS/F50 and BCS/F120 ratio 10/1 samples.

6.3.1. Biocompatibility

The possible toxic effect of samples was monitored by measuring the mitochondrial enzyme succinate dehydrogenase activity. Cells cultured in the presence of BCS (3t3+coll) increased their MTT activity quite rapidly after 48 hours, while in BCS/LNPs (3t3+collF50 and 3t3+collF120) the MTT activity remained constant for the entire the incubation period. Cells cultured *in vitro* without collagen-based scaffold (3t3 in 2D cell-culture) and with zinc material (3t3+zn) were used as controls. The results are showed in Figure 61.

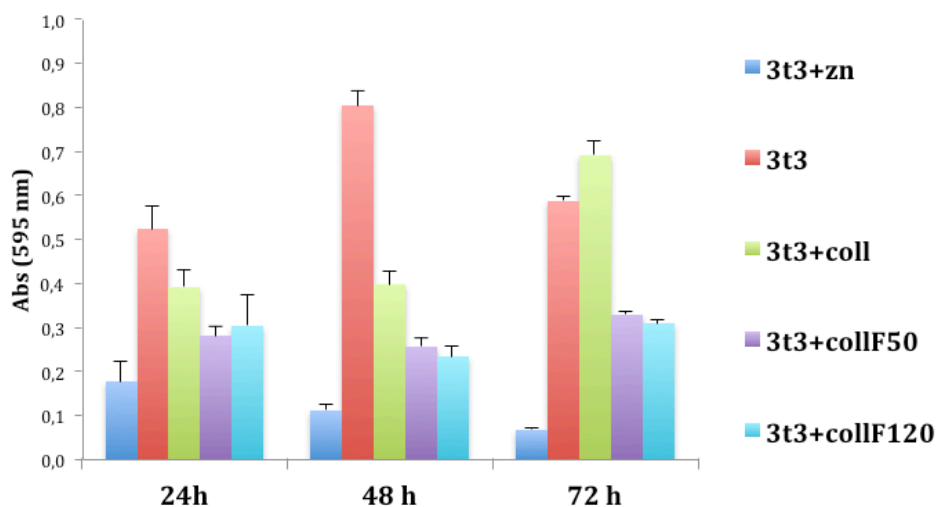


Figure 61. Results of MMT assay.

6.3.2. HaCat keratinocytes-matrix interaction

SEM images taken at 3 and 7 days after cell seeding show that cells cultured on BCS/LNPs (10/1) can proliferate normally as in control samples (Figure 62).

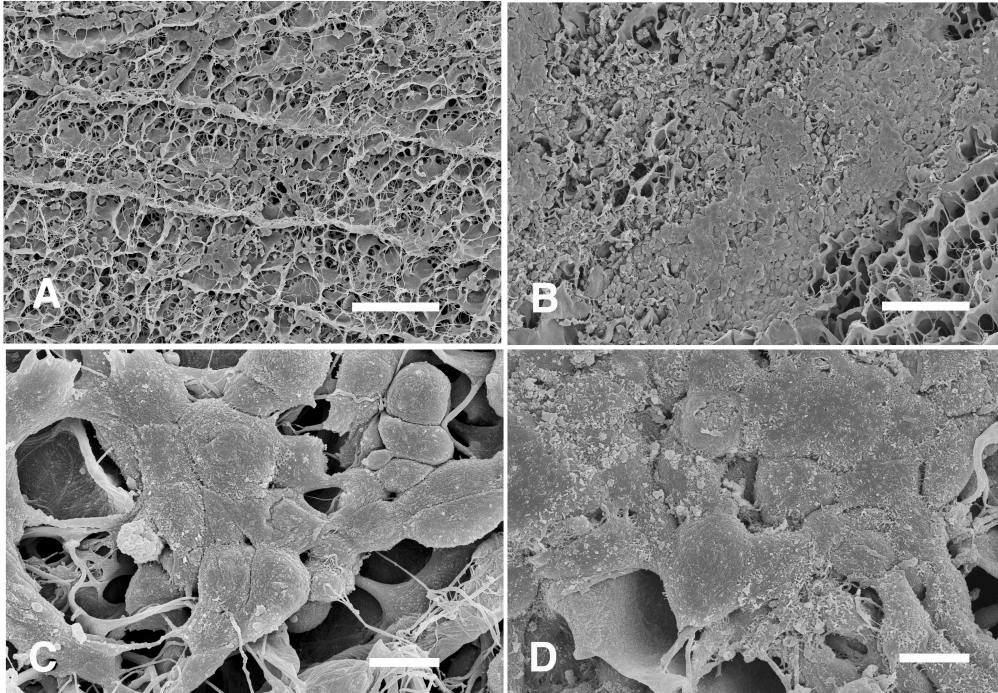


Figure 62. SEM micrographs of HaCat keratinocyte cultured on BCS/LNPs-F120. (A) and (C), at 3 day after cell seeding. (B) and (D) at 7 days after cell seeding. (A) and (B), Bars 100 µm; (C) and (D), Bars 10 µm.

However, while the BCS underwent extensive degradation in the presence of cells (Figs. 63A and 63B), the collagen fibers and the collagen matrix of the BCS/F50 (Figs. 63C and 63D) and BCS/F120 samples remained integral for periods up to 3 days (Figs. 63E and 63F), and even after 7 days. From these observations it may be concluded that addition of nanoparticles to the collagen matrix does not constitute a major hindrance for cell interaction, even though the collagen matrix itself appears to persist longer in the presence of these cells.

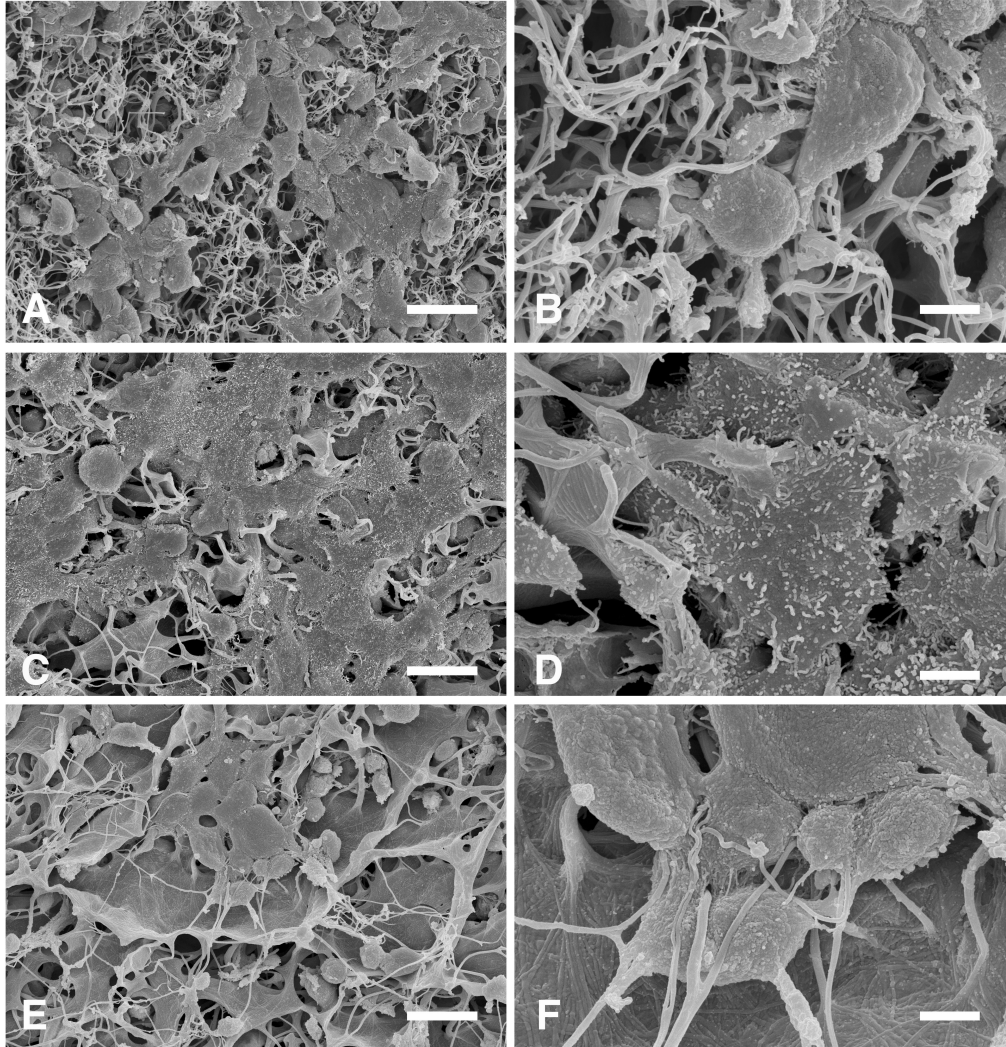


Figure 63. SEM micrographs of HaCat keratinocyte at 3 day after cell seeding. (A) and (C) BCS+HaCat. (B) and (D), BCS/F50+HaCat. (E) and (F), BCS/F120+HaCat. (A), (C) and (E), Bars 5 μm ; (B), (D) and (F), Bars 5 μm .

The results obtained by Transmission Electron Microscopy (TEM) have confirmed the observation at SEM level. After 4 hours of culture, HaCat cells had already attained a good adhesion (Figs. 64A and 64B). At 3 days of culture cells were still actively interacting with the collagen/LNPs fibers as demonstrated by the appearance of several of some collagen fragments in their cytoplasm (Figs. 64C and 64D).

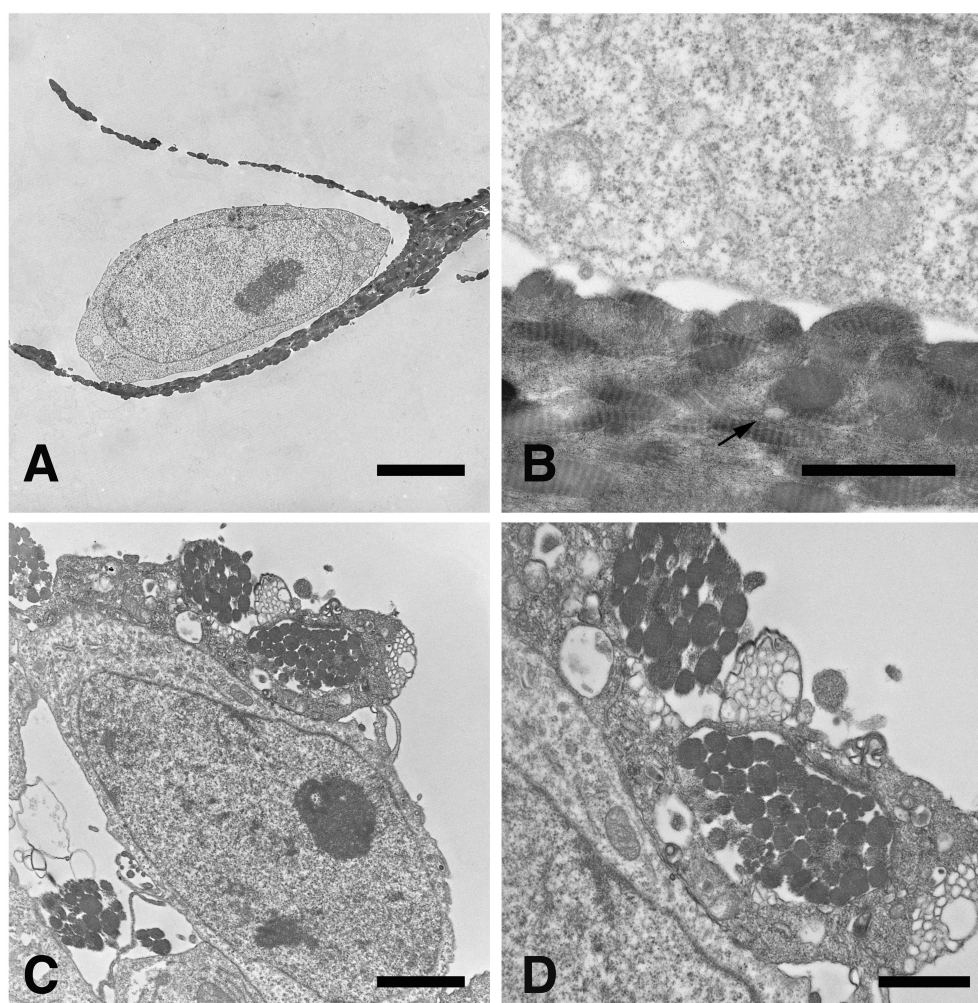


Figure 64. TEM micrographs of HaCat keratinocytes seeding on BCS/F50; (A) and (B), after four hours of co-culture; (C) and (D), at three days from cell seeding. (A), Bar 5 μm ; (B) and (D), Bars 1 μm ; (C), Bar 2 μm .

6.3.3. NIH3T3 fibroblasts-matrix interaction.

Four hours after seeding cells had adhered to BCS/LNPs matrix as already observed for the BCS sample (Figure 65). After three days of culturing cells had interacted to such an extent to incorporate portions of collagen/LNPs fibers of the surrounding matrix (Figure 66).

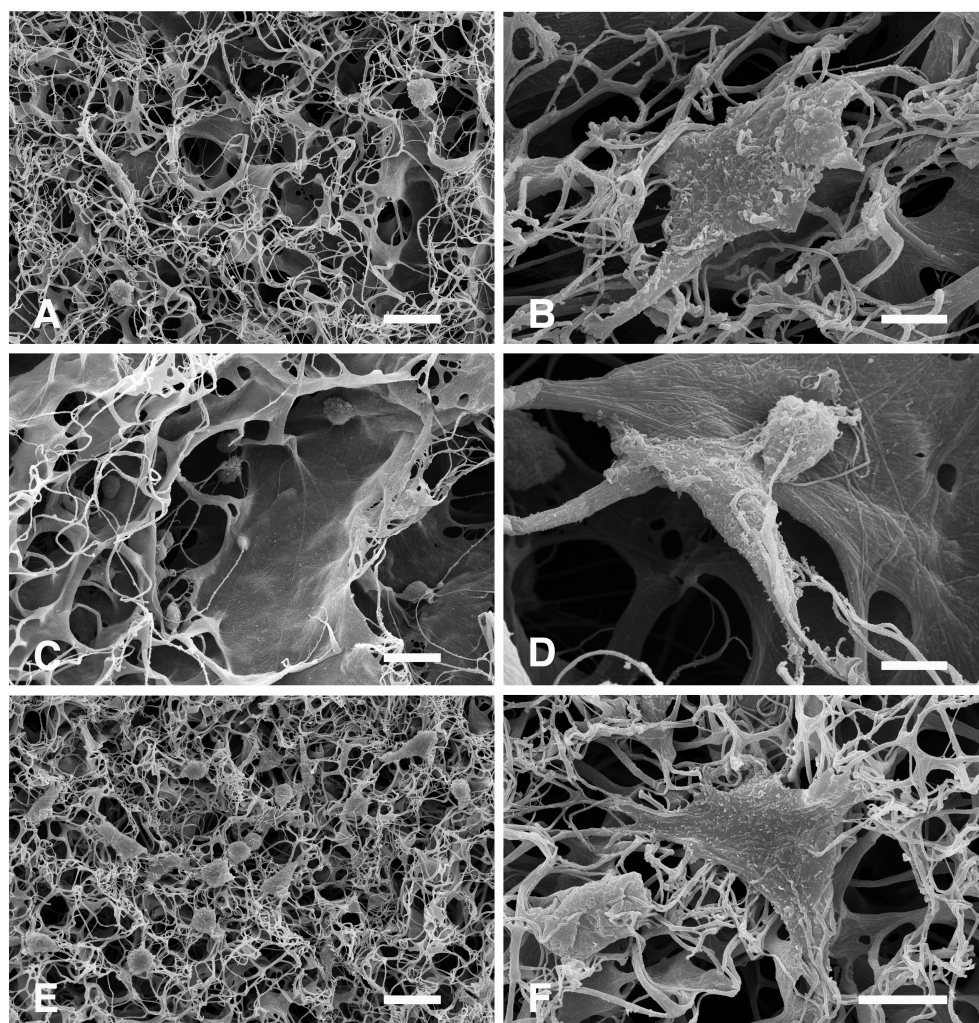


Figure 65. SEM micrographs of NIH-3T3 fibroblasts 4 hours after incubation on material. (A) and (B), BCS+NIH3T3. (C) and (D), BCS/F50+NIH3T3. (E) and (F) BCS/F120+NIH3T3. (A), (C) and (E), Bars 20 µm; (B) and (D), Bars 5 µm; (F), Bar 10 µm.

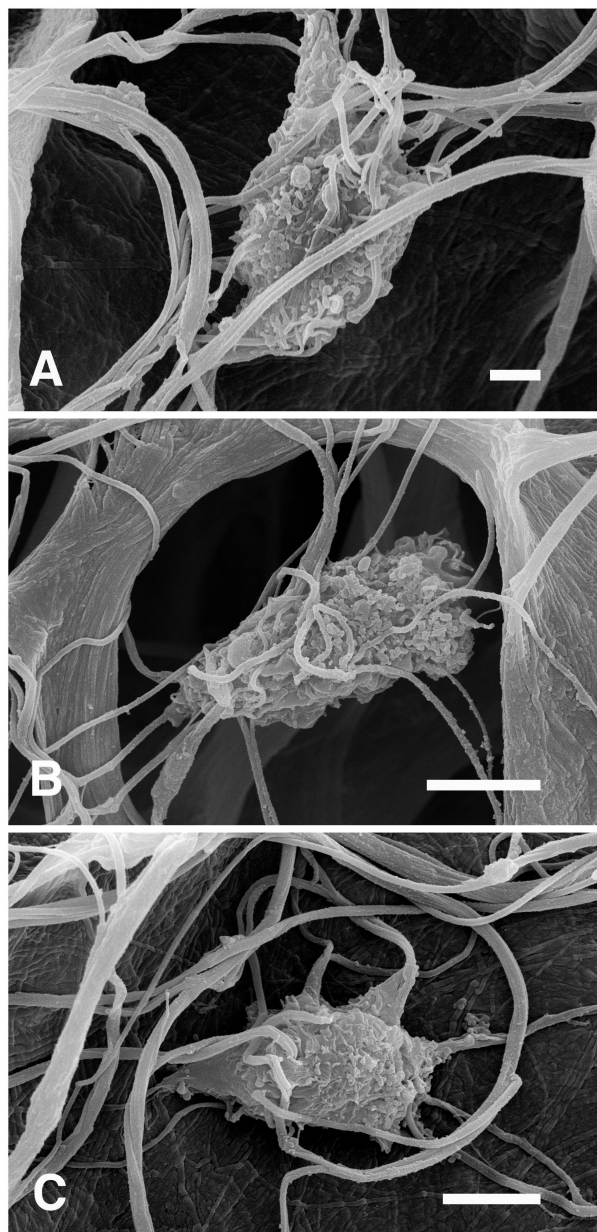
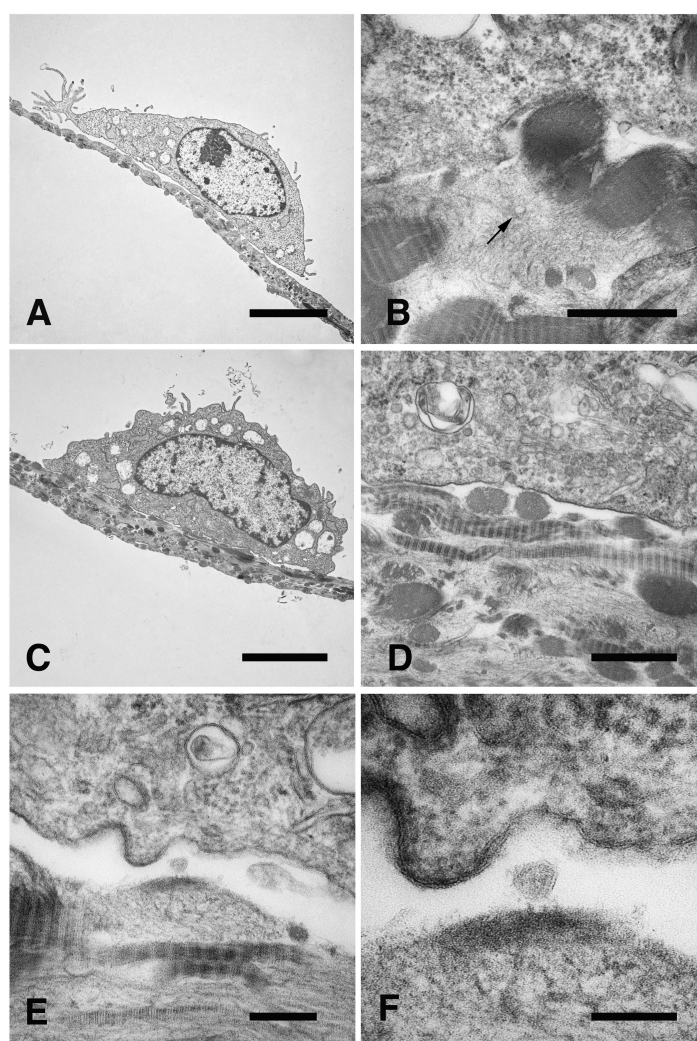


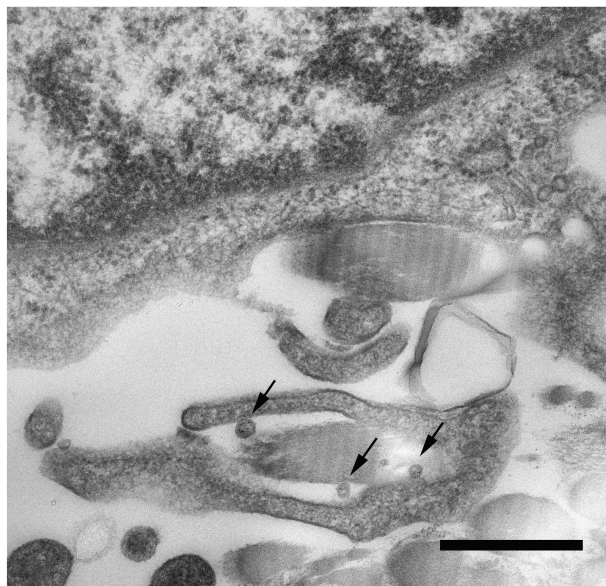
Figure 66. SEM micrographs of NIH-3T3 fibroblasts three days of co-culture. (A), the BCS+NIH3T3, Bar 2 μm ; (B), the BCS/F50+NIH3T3, Bar 5 μm ; (C), the BCS/F120+NIH3T3, Bar 5 μm .

Several TEM micrographs confirmed that cells were already well anchored to the collagen matrix both in the BCS/F50 (Figs. 67A and 67B) as well as in the BCS/F120 (Figs. 67C and 67D) as early as 4 hours of co-culturing. Figure 67E (enlarged in Fig. 67F) highlights a potential phagocytosis process of 120 nm nanoparticle placed on the collagen fiber.



Figures 67. Cell-BCS/LNPs interaction at 4 hours after cell seeding, (A) and (B), on BCS/F50; (C) and (D), on BCS/F120. As well as for the collagen/F50 material, nanoparticles can be evidenced in the fibrillar portion of the material (arrow). (E) and (F), TEM micrographs showing a beginning of phagocytosis process of 120 nm nanoparticle placed on the collagen fiber. (A) and (C), Bars 5 μm; (B) and (D), Bars 500 nm; (E), Bar 200 nm; (F), Bar 100 nm.

Three days after seeding cells had interacted more strongly with the collagen matrix for having incorporate portions of the collagen fibers (data not shown). Interestingly some protruding filopodia can be seen enveloping both fragments of the collagen fibers and several F50 nanoparticles (arrows in Figure 68).



Figures 68. Cell - BCS/F50 interaction at three days of co-culture, Bar 500 nm.

6.4. Discussion

To reduce healing time and to improve the quality of the regenerating tissues, several new wound care dressings have been recently designed (Murphy *et al.*, 2012). Of the various additives used to pursuit this goal, the type of nanoparticles explored in this study seem to be most promising. The first step to realize this objective was to devise a procedure for preparing a dry collagen sample coated with nanoparticles that would not alter the overall structural organization of BCS. The evidence provided in this study on the BCS/LNPs ultrastructure demonstrates that the collagen scaffold is not

modified by the addition of lipid nanoparticles. In fact, the overall structure of the collagen matrix started to become slightly irregular and the fibers more relaxed only if the collagen/lipid ratio was brought below 3.3/1 (see Chapter V). In all other conditions, there were no apparent alterations within the resolutions used in this study (!).

Nanoparticles were shown to retain their nominal size when coated with the collagen fibers. The observation that they increase slightly in size and distribution following extraction and DLS measurements is probably due to the fragmentation of collagen and the role that collagenases may have played in this process. Particle coating had no impact on the denaturation temperature of the collagen/F50 10/1 and collagen/F120 10/1 samples. Only a very small shift of less than 1°C could be monitored for higher lipid nanoparticle concentrations (BCS/F50 2/1 and BCS/F120 2/1). This indicates that the collagen structure can be preserved unaltered if maintained in a proper balance with the concentration of nanoparticles. However, the shrinkage temperature was significantly augmented if particle coating was performed at the +3°C temperature. Similarly, the higher denaturation enthalpy observed for the BCS/F50 10/1 and BCS/F120 10/1 samples, is taken to indicate that coating with nanoparticles does not interfere with the helical structure of the collagen fibers. A force of definite value (stress) and deformation (strain) was apparently more effective on the collagen/lipid nanoparticle samples than the collagen itself. On the whole the effect was more pronounced on the BCS/F50 than on the BCS/F120 sample. These measurements demonstrate that the collagen/lipid nanoparticle samples are apparently more elastic than BCS.

When co-cultured with either keratinocytes or fibroblasts, all collagen matrices (BCS/F50 nanoparticle 10/1, BCS/F120 nanoparticle 10/1) tested in this study displayed good adhesion properties. In addition, the collagen fibers from these samples were apparently more resistant to the enzymatic degradation, whereas cells retained their capacity to internalize both the collagen fibers and the nanoparticles. Therefore in case nanoparticles could be used as vehicles for delivering a drug cargo to target tissues, the possibility that the drug itself is released intracellularly should be taken into account.

This is the main reason why association of lipid nanoparticles and polymeric matrices are so extensively studied in recent literature (Sawant and Dodiya, 2008;

Schlapp and Friess, 2003; Ruszczak and Friess, 2003; Alarcon *et al.*, 2012). The primary goal of these studies is to search for hydrophobic drugs that could be uploaded in a vehicle suited for topical or mucosal delivery in a form of gel (Ding *et al.*, 2011; Eap *et al.*, 2013; Craciunescu *et al.*, 2014). Lipid nanoparticles could certainly be used for this scope. When combined with nanoparticles collagen becomes less hydrophilic, exhibits higher resistance to degradation, while it maintains unaltered its overall structure and porosity.

Future developments of these studies should explore the possibility of loading Lipidots™ with drugs designed for targeting specific injured sites. The drug delivery system should be endowed with such a specificity as to guarantee a local drug release in high concentrations. It is hoped that, in a not too distant future, such medicine chapters as tissue regeneration and wound healing could be approached therapeutically with these expectations fully satisfied.

CHAPTER VII

7. Conclusion

The morpho-functional analysis reported in this thesis has clearly demonstrated that the equine collagen Type I scaffold (BCS) is comprised of a regular 3D structure of sufficient integrity to sustain cell adhesion and cell migration. The laminar organization of this scaffold and the pore sizes - within the range of 100/150 μm - are in fact optimal parameters for rendering cell-to-cell and cell-to-matrix interactions as close as possible to those realized *in vivo* between the host tissue and the collagen support. The capacity to keep longer this native structure on the wound bed provides favorable conditions for fibroblasts to infiltrate and proliferate within the matrix itself, and consequently for accelerating the formation of new granulation tissues.

That BCS does indeed provide optimal conditions for cell interaction is clearly demonstrated by the observation that 3T3 cells adhere onto the collagen matrix soon after seeding. As these migrating cells adhere on the matrix they also change shape by becoming elongated, with the leading edge extended into numerous filopodia. It is thus by virtue of its regular inner architecture that this collagen matrix makes it possible for cells to first adhere on the substrate and then begin an effective migration inside it. With effect from 4h after seeding, cell-to-matrix interactions become progressively more pronounced and extended to larger and larger portions of the scaffold. Under these conditions, 3 days of *in vitro* culturing suffice to make cells highly intertwined with the collagen fibers and their plasma membranes enriched with numerous exo- and endocytic pits.

Co-culturing with fibroblasts for up to 7 days causes the collagen matrix to be gradually transformed from an initial laminar structure to a prevailing fibrillar architecture. In an attempt to unravel the molecular mechanisms controlling this transformation the present thesis has searched for two potential causal correlations. First, 3D co-culturing in the presence of a collagen matrix causes fibroblasts to shed numerous microvesicles into the surrounding medium. Since these vesicles outnumber the ones released in 2D *in vitro* cultures, it is the presence of collagen that, in all

likelihood, stimulates their shedding. Second, co-culture with the collagen matrix is a condition necessary, though not sufficient, for fibroblasts to release MMP-9 extracellularly and to spread it on the matrix itself. A number of experiments aimed at localizing MMP-9 on the fibroblasts and on the associated collagen fibers do clearly demonstrate that microvesicle shedding and metalloproteinase release are not independent phenomena. On the contrary, all evidence gathered in this thesis points to their temporal and topological correlations, such as vesicles act as potential carriers for the diffusion and activation of the metalloproteinases onto the matrix. Interestingly, my ultrastructural analysis demonstrates that such a causal correlation is actually realized along the filopodia tips, where microvesicle shedding coincides with metalloproteinase conveying through an inner vesicular traffic. This interpretation is in line with the role played by microvesicles in the extracellular release of MMP-9 and in its diffusion onto the collagen matrix. At the same time, it accounts for the degradation of the prosthetic collagen *in vitro* and presumably as a precondition for the deposition of a new extracellular matrix in wound healing.

The present thesis has extended the morphological analysis to different BIOPAD®-collagen based scaffolds to verify whether addition of active substances or nanoparticles to the matrix could alter the overall collagen organization. Comparison of these varying collagen scaffolds demonstrates that, if the gel/solution ratios are maintained within certain limits, the overall collagen architecture is not at all altered. When compared with other types of collagen scaffolds, i.e., Coll&ORC (Promogran®), the same morphological analysis demonstrated that such cell processes as adhesion, interstitial migration and uptake from the extracellular milieu are not as good as in BIOPAD®-collagen based scaffolds. Amongst the activities considered in this study, cell proliferation is perhaps the most important one for wound healing. With the use of the MTT assay I have shown that the BCS and BCS&NaYal samples are by far better than others in guaranteeing cell viability throughout the culturing times.

In conclusion, the collagen equine type I tested in this study has the advantage of maintaining a considerable architecture stability even when added with other substances. This result may be considered as a first significant step toward the

possibility of developing new collagen scaffolds that could in a not too distant future improve the quality of wound management in chronic and normal situations.

Chapter VIII

8. Materials and methods

8.1. Material preparation

8.1.1. The BIOPAD[®] Collagen Scaffold (BCS)

The collagen scaffold used in this study is a heterologous Type I collagen obtained from equine Achilles tendon. The extraction and purification procedures are such as to maintain the native structure of the collagen fibers. The collagen based scaffolds compared in this study are obtained loading the different element in the collagen gel before the freeze-drying process. 2,5% of sodium hyaluronate was added to the collagen gel for BCS&NaYal; 10ppm of silver nanoparticles for BCS&Ag and with 2% of Biosecure for BCS&Biosecure.

8.1.2. The BCS and Lipidots[™]

The material was prepared loading in collagen gel 1% w:w in acetate buffer (Euroresearch) two different sizes of nanoparticles: F50 and F120 nanoparticles, with and without Dil/DiD, at the concentration of 10/1 w/w dry collagen/lipids. More precisely, 25 g of collagen gel (containing 250 mg of dry collagen), 2.25 mL of milliQ water and 250 μ L of either F50 emulsion, or F120 emulsion, or FRET F50 emulsion, or FRET F120 emulsion were mixed at 100 rpm for 1 hour at room temperature (20°C).

A directional freeze-drying procedure under thermally constant freezing conditions was then carried out to provide a sponge like material with a uniform porous microstructure. Either 3 g of gel material were distributed into a 35 mm Petri dish, or 2 g of gel material were distributed into each well of 12 Well Cell Culture Plate. The air or upper surface was covered with a polypropylene membrane before the freezing. The Petri dish and the 12 Well Plate were placed into a glass container and quickly frozen in liquid nitrogen for 5 min. The freeze-drying process was done for 24 hours with a Cryotec V8.11 plate lyophilizer. After freeze-drying process the polypropylene membrane was removed with a tweezer and the Petri dish or micro-well plate were

packed and sealed in a bag for the sterilization process. The sterilization process was done with gamma irradiation, exposed to a nominal dose of 25 kGy.

Materials with higher nanoparticle payloads were also prepared loading in collagen gel 1% w/w in acetate buffer (Euroresearch) the two different sizes of nanoparticles, F50 and F120 nanoparticles at the concentration of 10/1, 5/1, 3.3/1, 2.5/1, 2/1 w/w dry-collagen/lipids. More precisely, respectively 25 g of collagen gel, 2.25 mL of milliQ water and 250 μ L of either F50 emulsion or F120 emulsion at 10% w/v lipids (for concentration 10/1), 25 g of collagen gel, 2.0 mL of milliQ water and 500 μ L of either F50 emulsion or F120 emulsion at 10% w/v lipids (for concentration 5/1), 25 g of collagen gel, 1.75 mL of milliQ water and 750 μ L of either F50 emulsion or F120 emulsion at 10% w/v lipids (for concentration 10/1), 25 g of collagen gel, 1.5 mL of milliQ water and 1000 μ L of either F50 emulsion or F120 emulsion at 10% w/v lipids (for concentration 2.5/1), and 25 g of collagen gel, 1.25 mL of milliQ water and 1250 μ L of either F50 emulsion, or F120 emulsion (for concentration 2/1), were mixed at 100 rpm for 1 hour at room temperature (20°C). The gel materials were processed as described above through freeze-drying to obtain dried collagen/lipid nanoparticle materials containing respectively 10/1, 5/1, 3.3/1, 2.5/1 and 2/1 w/w dry collagen/lipids.

Control collagen/MyrjTM s40 (collagen/PEG) materials were prepared as well, with collagen/MyrjTM s40 ratio 21/1 and 40/1 weigh/weight. These ratios correspond respectively to the collagen/PEG ratio of the collagen/F50 10/1 material and collagen/F120 10/1 material.

8.1.3. The LipidotsTM

Neutral F50 (50 nm diameter) and F120 (120 nm diameter) lipid nanoparticles.

An oil premix was prepared. The amount of refined soybean oil (Croda Uniquema, France), Suppocire NCTM (Gatéfossé, France), and Lipoid S75TM (Lipoid, Germany) used in the oil premix are, respectively, 85, 225, and 65 mg for F50, and 150, 450, and 45 mg for 120. After homogenization at 60°C, the continuous aqueous phase, composed of 345 mg of Myrjis40TM (Croda Uniquema, France), for F50, 215 mg for F120, and of the appropriate amount of aqueous medium (154 mM NaCl if not stated

otherwise, qsp 2 mL), was introduced. The vial was placed in a 60°C water bath and the mixture was sonicated for 5 min using a VCX750 ultrasonic processor (power output 190 W, 3-mm probe diameter, Sonic). Lipidots were dialyzed overnight at room temperature against 1000 times their volume in the appropriate aqueous buffer (12-14.000 Da MW cut of membranes, ZelluTrans). Finally, the lipidot dispersion was filtered through a 0.22 µm Millipore membrane for F50 and through a 0.45 µm Millipore membrane for F120.

Neutral F50 and F120 loaded with Dil/DiD FRET fluorophore pair.

A FRET dye pair was constituted of a donor dye (here Dil) which emission spectrum overlaps with an acceptor dye (here DiD) absorption spectrum. When the two fluorophore were in close proximity (ie a few nm), the emission of the donor (Dil) was quenched and transferred to the acceptor (DiD), when the excitation was in the donor range. The co-encapsulation of a FRET dye pair in the lipid core of the nanoparticles can therefore be used to monitor the particle core integrity, measuring the FRET signal, quantified as the acceptor/donor (Dil/DiD) fluorescence ratio. Neutral FRET F50 and F120 nanoparticles were prepared as described previously, adding 40 µl of Dil at 10 mM in EtOH (Life Technologies, France) and 160 µl of DiD at 10 mM in EtOH (Life Technologies, France) for F120, and 20 µl of Dil at 10 mM in EtOH and 80 µl of DiD at 10 mM in EtOH for F50 in the lipid phase of the formulations, prepared otherwise exactly as previously described. The absorbance and fluorescence measurements were performed in 1X PBS, respectively, using a Cary 300ScanUV-Visible spectrophotometer (Varian) and a Perkin Elmer LS50B fluorimeter.

8.2. Morphological and ultrastructural analysis.

8.2.1. Scanning Electron Microscopy (SEM)

The scaffold architecture of the collagen matrix was structurally characterized by Scanning Electron Microscopy (SEM). To this end, collagen scaffolds were cut with a sterile razor blade into a number of small blocks and attached to aluminum stubs with a carbon tape. Blocks were oriented in such a way as to expose either the upper or the

lower surfaces, while others were glued on their longitudinal cut profile. They were sputter-coated with gold in a Balzers MED 010 unit and observed in a JEOL JSM 6010LA electron microscope. The native structure of collagen was also checked by Transmission Electron Microscopy as specified below.

To assess the collagen-matrix interactions, scaffolds inclusive of their cellular loads were cut into a number of small blocks with a sterile razor blade and fixed overnight at 4 °C with 3% glutaraldehyde in phosphate buffer (PB) at pH 7.2. Following extensive rinsing with the same buffer at 4 °C, they were immersed for 1h in 0.5% tannic acid in PB at 4 °C. Then they were rinsed again, four times in the same buffer for 15 min at 4 °C, and post-fixed with 1% osmium tetroxide for 1h in PB at 4 °C. Specimens were finally washed in distilled water and then dehydrated in a graded series of ethanols. The samples were first dried by the critical point method in a Balzers Union CPD 020, and then sputter-coated with gold in a Balzers MED 010 unit and then observed in a JEOL JSM 6010LA electron microscope.

8.2.2. Transmission Electron Microscopy (TEM)

To check the collagen scaffold ultrastructure and the cell-matrix interactions, the samples were cut into a number of small blocks with a sterile razor blade and fixed as described for SEM analysis, after post-fixation in osmium tetroxide the specimens were washed in distilled water, block-stained with 1% uranyl acetate in distilled water and then dehydrated in a graded series of ethanol. The dehydrated samples were infiltrated for two days with graded mixtures of LRWhite resin/ethanol. By the end of this procedure, samples were embedded in fresh LRWhite resin and cut with Reichert Ultracut ultramicrotome equipped with a diamond knife. Ultrathin sections (60-80 nm thick) were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX II electron microscope. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software.

8.2.3. Pore size analysis

The porous matrix ultrastructure was examined by SEM images of cross-sections, and the pore sizes were calculated by analysis of the same area for each samples. The images were subjected to thresholding to convert matrix pores and collagen fibrils into black and white images. This procedure allows adjusting the pixel density in such a way that collagen fibrils are eliminated from the image background. The 2D black and white matrix representations that are so obtained are then evaluated as particle sizes and their distribution established using an image analysis program (Image J).

8.2.4. Confocal Scanning Light Microscopy (CSLM)

The organizations of DiI/DiD LNPs in the collagen fiber were investigated with Confocal Laser Scanning Microscopy (CLSM or LSCM). The materials, were cut in a small blocks with a sterile bistoury and embedded for 4 hours, at room temperature, within OCT (compound for Cryostat Sectioning, Tissue-Tek®). The materials were transferred, avoiding the bubbles, in fresh OCT and quickly placed and orientated on cryostat adapter. The samples were directly frozen into the Cryostat (Cryostat Slicing Leica CM 3050S) at -20°C for 3/4 minutes. The sections (10 µm) were collected on microscope slides Superfrost® plus (Menzel-Glaser, Thermo scientific, J1800AMNZ) and covered with one or two drops of FluoroSave™ Reagent (Calbiochem) before placement of coverslip. The images were acquired with three different wavelengths: FITC (500-535 nm); TRITC (555-620 nm) and CY5 (650-750 nm).

8.2.5. Colloidal stability

To perform analysis on the lipid nanoparticles embedded in the collagen matrix, a protocol for particle recovery from the matrix was developed. 15 mg of collagenase (Sigm) and 10 mg of calcium chloride (Redi-Dri, Aldrich) were dissolved in 10 mL of PBS (1X, pH 7.4) in a 10 mL glass bottle. 1 mL of this collagenase solution is added to 4 mg of lyophilized material and incubated at 37°C for 3 hours. Non-digested materials, precipitated calcium chloride and collagenase are eliminated by centrifugation at 10 000

g for 2 minutes. The supernatant, containing digested collagen chains and lipid nanoparticles, was filtered and analyzed by DLS measurements.

8.2.6. Swelling ratio

The swelling ratio of the BCS&LNPs materials, reflecting their water absorbing abilities, was quantified. The materials (BCS as control, BCS&F50 nanoparticles with collagen/lipid ratios 10/1, 3.3/1, and 2/1, BCS&F120 nanoparticles with collagen/lipid ratios 10/1, 3.3/1, and 2/1), prepared how previously described, were cut with a bistoury in small pieces (about 1 cm x 1 cm), weighed and immersed for 24 hours in 5 mL of water or phosphate-buffered saline (PBS) buffer, at room temperature. Then, the unabsorbable solution was removed by aspiration and the materials were drained with a filter paper for 3 minutes. Finally, the wet materials were weighed and the swelling ratio (SR) calculated according to the following equation:

$$SR = 100 (W_w - W_d) / W_d$$

in which W_d is the weight of dry material (i.e. of the lyophilized material before water or PBS contact) and W_w is the weight of wet drained material.

This hydrophobic character of BCS/LNPs materials in comparison to BCS was observed at early times of contact between the material and an aqueous phase. The water drop (10 μ L) contact angle with the material was measured by using a Digidrop instrument.

8.2.7. Thermal stability

Thermal stability of BCS&LNPs materials was investigated by Differential Scanning Calorimetry (DSC) using the Angele et al. (2004) protocol. 33-45 mg of dry material was immersed for 1h in deionized water, then drayed on filter paper for 1 minute before being sealed in aluminium pans and analyzed using a micro DSC III SETARAM. An aluminium pan with the same weight of deionized water than the wet sample was used as reference. The heating rate was constant at 3°C/min and the temperature was increased from 20 °C to 110°C. Shrinkage temperature was determined as the onset value of the endothermic peak. The thermal denaturation temperature (for

helix-to-coil transition of the collagen fibers) was taken at the endothermic peak maximum. The value of denaturation enthalpy was calculated with respect to the weight of collagen in the dried material. Several samples of each material were analyzed.

8.2.8. Compressive stress test

Uniaxial compression tests were performed on lyophilized cylinder shaped materials (BCS, BCS&F50 10/1, BCS&F120 10/1) of 4.1 mm diameter and 5 mm height using an AR2000EX rheometer (TA instrument) with an initial preload of 0.03 N and a compression speed of 10 $\mu\text{m/s}$. The stress, σ , was calculated as the normal force divided by the surface area of the material, whereas the strain, ϵ , was the % of compression of the material. The stress-strain plots obtained were typical of an elastic foam-like material (Gibson, 2005), and the Young modulus E^* of the materials could be calculated as the slopes of stress-strain curves for low strains (linear domain).

8.3. Cell culturing

Stabilized murine fibroblasts (NIH 3T3) and human keratinocytes (HaCat) were used throughout this study. To collect enough cells for plating on collagen matrices, NIH 3T3 fibroblasts and HaCat keratinocytes were first cultured in 75 cm² flasks containing DMEM (Dulbecco's Modified Eagle's Medium) culture medium, supplemented with 10% of newborn calf serum (NCS) and fetal bovine serum (FBS) respectively, 1% glutamine and 1% penicillin/streptomycin, and maintained in an incubator at 37 °C with 5% CO₂ and controlled humidity. Upon reaching confluence, cells were passed and the medium changed every three days.

3D collagen scaffolds were cut with a sterile razor blade into blocks of approximately 1 cm² of surface area each and inserted into 24-well plates. Aliquots of 2×10^5 cells were seeded on the upper surface of every collagen block and incubated at 37 °C in 1.5 ml of culture medium under a continuous flow of 5% CO₂ for time intervals ranging from 4 h to 14 days. Medium was then renewed every three days.

For 2D control experiments, NIH 3T3 fibroblasts were seeded on 24 well plates and incubated in 1 ml of culture medium at the same conditions as the 3D cell-cultures and assayed at regular time intervals for cell viability and microvesicle release.

8.4. MTT assay

Cell viability was monitored by measuring the activity of the mitochondrial enzyme succinate dehydrogenase by using the MTT test. Since reduction of MTT is correlated with cell metabolic activity, cells metabolically active are expected to yield high MTT values. At each incubation time, 1 ml aliquots of culture medium, containing both collagen scaffolds and cells, were withdrawn from each well. Subsequently 0.1 ml MTT were added to every aliquot and left to incubate for 4h in the dark at 37 °C under a 5% CO₂ flow. By the end of this incubation time, the resulting formazan crystals were completely dissolved in DMSO glycine buffer at pH 10.5 and the relative absorbance measured at a wavelength of 570 nm. Absorbance values for NIH 3T3 and HaCat cells were measured at each time point of the entire culture period in both 2D plates and 3D matrix.

8.5. Immuno Electron Microscopy (IEM)

Samples were fixed with a mixture of 4% paraformaldehyde and 0.25% glutaraldehyde in 0.05 M PIPES, pH 7.3 for 2 h at room temperature. After rinsing for 10 min in the same buffer, samples were dehydrated in a graded ethanol series and embedded in medium grade LRWhite resin, by adding the LRWhite accelerator (London Resin Company, Berkshire, England). The resin was polymerised in tightly capped gelatine capsules for 20 min at room temperature. Ultrathin sections were obtained using a Reichert Ultracut ultramicrotome with a diamond knife and collected on nickel grids.

For immunogold staining, an incubation protocol suggested by Aurion was used (Aurion, Vageningen, the Netherlands). Residual aldehyde activity was suppressed by using PBS with 0.05 M glycine, pH 7.4 for 20 min. A subsequent block step was made with 5% BSA, 5% normal goat serum and 0.1% cold water fish skin gelatine for 30 min.

Sections were washed in incubation buffer (PBS and 0.1% BSA-cTM, pH 7.4) (3x5 min). Incubation was made overnight in a moist chamber with the rabbit polyclonal antiserum anti-MMP-9 (Ab 38898, Abcam) and anti-collagen Type I (Ab34710, Abcam). All the antibodies were diluted 1:100 and in incubation buffer. Sections were then washed in incubation buffer (6x5 min) and incubated for 1 h with a secondary goat anti-rabbit antibody conjugated to 10 nm gold particles (British BioCell International, UK), diluted 1:10 in incubation buffer. After rinsing in incubation buffer (6x5 min), the grids were washed in PBS (6x5 min). Sections were subsequently stained with uranyl acetate and observed with a Jeol JEM EXII transmission electron microscope at 100 kV. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software. Control sections in which the primary antibodies were omitted were also analyzed.

8.6. Immuno Histo-Chemistry (IHC)

Immunohistochemistry (IHC) was performed by the ABC-peroxidase reaction with nickel enhancement (Abelli *et al.*, 1996). Collagen and 3T3 samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C and embedded in paraffin wax. Serial sections of 5-7 microns were incubated for 18 hours at 4 °C with a rabbit polyclonal antiserum anti-MMP-9 (Ab 38898, Abcam) diluted 1:500. The primary antibody was omitted in the negative control. Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgG serum for 60 min (Vector Labs, Burlingame, USA), diluted 1:1000 with PBS containing 0.1% sodium azide and 1% BSA, followed by incubation with the avidin-biotinylated peroxidase complex for 60 min (ABC Elite Vectastain, Vector). Following rinsing and staining (diaminobenzidine and nickel enhancement), the sections were dehydrated, mounted and examined under bright field illumination. Images were acquired using Zeiss microscope equipped with a color camera (Axio Cam MRC, Arese, Milan, Italy) and a software (AxioVision and KS 300).

8.7. Western Blot

At 1 day, 3 and 7 days from cell seeding the samples, BCS+3T3 and 3T3, were washed twice in sterile PBS(1X). The collagen matrices with their cellular load were frozen in liquid nitrogen for 2 min, pulverized and incubated for 30 minutes at room temperature in Ripa buffer with proteases inhibitors. The protein concentration in extracts was determined by using the Bradford microassay method (Sigma) with bovine serum albumin as a standard. 50 µg of protein for each samples were first separated by SDS-polyacrylamide gel (10%) electrophoresis and then transferred onto a nitrocellulose membranes (Millipore, Burlington, MA) using a wet transfer system (Biorad). The membranes were blocked in TBST (Tris-buffer saline with 0.01% Tween-20, pH 7.4) with 5% BSA for 1h at room temperature, washed and incubated overnight at 4°C with a rabbit polyclonal antiserum anti-MMP-9 (Ab 38898, Abcam) diluted 1:1000. The membrane were then washed and incubated for 2–4 h with secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:1000 dilution. The blots were developed using the ECL chemiluminescence detection system (ChemiDoc, Biorad).

8.8. Gelatin Zimography

Gelatinolytic activity in cell lysate and culture medium from serum free cell cultures in 3D (BCS+3T3) and 2D (3T3) was detected by gelatin zymography following the protocol of Toth and Fridman (2001). The solutions containing MMPs were prepared in order to maintain the enzyme functionality, without reducing agents and without being boiled before loading on the gel.

The samples were subjected to SDS-PAGE with a 10%(w/v) polyacrylamide gel containing 0.1 mg/ml of porcine gelatin (Sigma). After 90 minutes of run at constant voltage (125V), the gels were incubated for 30 min at room temperature in 2.5% (v/v) Triton X-100 to remove SDS, then rinsed once in doubly distilled water. After renaturation the gels were incubated at 37 °C for a further 20 h in developing buffer (20 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35, 50 mM Tris/HCl buffer (pH 7.8)⁹, then stained with 0.1% Coomassie Brilliant Blue R-250, followed by destaining in 10% acetic acid

and 30% methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background and the analysis of data was done by acquiring the gel image with the ChemiDoc instrument (Biorad). The images were analyzed by ImageJ program for a semiquantitative evaluation (Xueyou and Beeton, 2010).

CHAPTER IX

9. References

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