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HELPING WOUND HEALING PROCESS THROUGH DRUG DELIVERY SISTEMS

by

OVIDIO CATANZANO

APPROVED BY

Supervisor: Antonio Calignano, PhD
PhD Program Coordinator: Maria Valeria D'Auria, PhD

University of Napoli Federico II, Napoli, Italy
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INTRODUCTION

ALGINATE – HYALURONIC ACID HYBRID HYDROGELS AD SPONGES FOR WOUND HEALING

MELT-SPUN BIOACTIVE SUTURES CONTAINING NANOHYBRIDS FOR LOCAL DELIVERY OF ANTINFLAMMATORY DRUGS

CHAPTER I

INTRODUCTION

Wounds

A wound can be described as a defect or a break in the skin, resulting from physical or thermal damage or as a result of the presence of an underlying medical or physiological condition[1]. According to the Wound Healing Society, a wound is the result of 'disruption of normal anatomic structure and function with consecutive loss of function'[2].

Of all the body tissues, the skin is definitely the most exposed to damage and easily prone to injury, abrasions and burns due to trauma or surgery. The rapid restoration of homeostatic physiological condition is a prerequisite for a complete lesions repair, because a slow and incorrect repair can cause a series of damage including the loss of skin, hair and glands, the onset of infection, the occurrance of skin diseases, a injury to the circulatory system and, in severe cases, death of the tissue[3]. To prevent these undesirable effects it is essential that the body activates a series of fine regulated mechanisms for a quick and efficient wounds repair in order to reduce the possibility of irreversible damage to the skin structure. For all these reasons, wound repair is one of the main biological processes in humans.

Given the complexity of the wound repair process, it is remarkable that it rarely becomes uncontrolled and that malignant transformation is an uncommon event in the wound environment[4,5]. For most injuries, repair results in once functional tissue becoming a patch of cells (mainly fibroblasts) and disorganized extracellular matrix (mainly collagen) that is commonly referred to as a scar (Figure 1). Surprisingly, in some eukaryotic organisms, the response to injury can completely recapitulate the original tissue architecture through a process of regeneration. Humans have this ability during prenatal development, but this is lost during adult life[6]. How regeneration occurs and why humans lose this ability remain a mystery[7].

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[1] {Boateng, 2008 635 /id}
[2] Lazarus G.S., Cooper D.M., Knighton D.R., Percoraro R.E. *et al.* Wound Repair Regen. 1994
[3] Broughton G., Janis J.E. and Attinger C.E. Plast.Reconstr.Surg. 2006
[4] Konigova R. and Rychterova V. Acta Chir Plast. 2000
[5] Trent J.T. and Kirsner R.S. Adv.Skin Wound Care. 2003
[6] Colwell A.S., Longaker M.T. and Lorenz H.P. Front Biosci. 2003
7 Gurtner G.C., Callaghan M.J. and Longaker M.T. Annu.Rev.Med. 2007

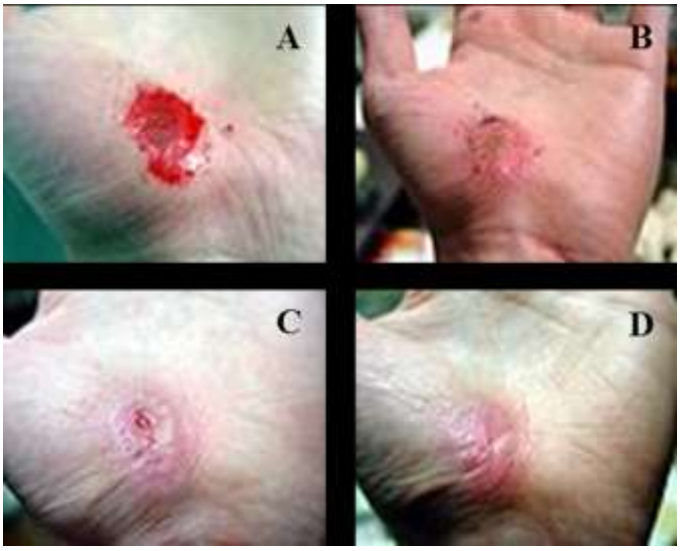

Figure 1. Macroscopic wound healing of hand abrasion. A: 0 Day; B: 2 Days; C: 17 Days; D: 30 Days. Source Wikipedia (http://en.wikipedia.org/wiki/Abrasion_%28medical%29).

The closure of a skin wound can be realized by regeneration or repair. While regeneration describes the specific substitution of the tissue, i.e. the superficial epidermis, mucosa or fetal skin, skin repair displays an unspecific form of healing in which the wound heals by fibrosis and scar formation. The latter, unfortunately, represents the main process in adult skin wound healing. The knowledge gained through studies carried out on cells that can regenerate itself, might help to unlock latent regenerative pathways in humans and to transform a fibrotic healing process in a regenerative one, restoring the original tissue functions. This will greatly improve the quality of life and it would change medical practice, as happened with the introduction of antibiotics in the twentieth century[8].

In general, the wound repair process occurs in almost all tissues after exposure to almost any destructive stimulus. Integumental injuries are defined as open or outer wounds, whereas inner or closed wounds describe injuries or ruptures of inner organs and tissues with a still intact skin. Thus, the sequence of events that follows a myocardial infarction (heart attack), for example, is remarkably similar to that following a spinal-cord injury, a burn or a gunshot wound, despite the different types of insult and the different organs affected. Likewise, scar formation that occurs during wound repair leads to similar tissue dysfunction wherever it takes place. In the case of myocardial infarction, the formation of myocardial scar tissue is thought to

[8] {Gurtner, 2008 516 /id}

result in congestive heart failure (a condition in which the heart cannot supply the body's tissues with enough blood) and/or abnormal heart rhythms (arrhythmias), which together account for nearly 100,000 deaths each year in the United States alone[9]. In addition, cirrhosis of the liver and some forms of fibrosis of the lungs are thought to result from fibrotic responses to toxin-mediated injury[10]. Interestingly, in other circumstances, the liver is one of the few organs in the human body that can regenerate up to 70% of itself without scar formation. Why the liver's regenerative capacity manifests only in some cases remains incompletely understood. A leading hypothesis is that the immune system is involved in the switch between regeneration and fibrotic healing, because human fetuses, which heal without scarring, have immature immune systems[11].

Based on the nature of the repair process, wounds can be classified as acute or chronic wounds. Acute wounds are usually tissue injuries that heal completely, with minimal scarring, within the expected time frame, usually 8–12 weeks[12]. The primary causes of acute wounds include mechanical injuries due to external factors, such as abrasions, which are caused by frictional contact between the skin and hard surfaces. Mechanical injuries also include penetrating wounds caused by knives and gun shots and surgical wounds caused by incisions, for example to remove tumors. Another category of acute wounds include burns and chemical injuries, which arise from a variety of sources such as radiation, electricity, corrosive chemicals and thermal sources. The temperature of the source and the exposure time influence the degree of a thermal burn[13]. Chronic wounds on the other hand arise from tissue injuries that heal slowly, that have not healed in 12 weeks[14] and often reoccur. Such wounds fail to heal due to repeated tissue insults or underlying physiological conditions[15] such as diabetes and malignancies, persistent infections, poor primary treatment and other patient related factors. These result in a disruption of the orderly sequence of events during the wound healing process (see later). Chronic wounds include decubitus ulcers (bedsores or pressure sores) and leg ulcers

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[9] National Heart, Lung and Blood Institute. Morbidity & Mortality: 2002 Chart Book on Cardiovascular, Lung, and Blood Diseases. 2002
[10] Selman M., King T.E. and Pardo A. Ann.Intern.Med. 2001
[11] Mescher A.L. and Neff A.W. Adv.Biochem.Eng Biotechnol. 2005
[12] Percival JN. 2002. Classification of wounds and their management. Surgery 20:114–117.
[13] Naradzay FX, Alson R. 2005. Burns, thermal.Web MD.
[14] Harding KG, Morris HL, Patel GK. 2002. Science, medicine and the future: Healing chronic wounds. Br Med J 324:160–163.
[15] Moore K., McCallion R., Searle R.J., Stacey M.C. *et al.* Int Wound J. 2006

(venous, ischemic or of traumatic origin). Wounds are also classified based on the number of skin layers and area of skin affected[16].

Injury that affects the epidermal skin surface alone is referred to as a superficial wound, whilst injury involving both the epidermis and the deeper dermal layers, including blood vessels, sweat glands and hair follicles is referred to as partial thickness wound. Full thickness wounds occur when the underlying subcutaneous fat or deeper tissues are damaged in addition to the epidermis and dermal layers. Ferreira *et al.*[17] have described both acute and chronic wounds that are difficult to heal as 'complex wounds' with unique characteristics. The properties of complex wounds can be summarized as: (a) extensive loss of the integument which comprises skin, hair, and associated glands; (b) infection (e.g. Fournier's gangrene) which may result in tissue loss; (c) tissue death or signs of circulation impairment; (d) presence of underlying pathology.

[16] Krasner D., Kennedy K.L., Rolstad B.S. and Roma A.W. Ostomy Wound Manage. 1993
[17] Ferreira M.C., Tuma P., Jr., Carvalho V.F. and Kamamoto F. Clinics (Sao Paulo). 2006

1.1. Wound healing

Wound healing is a specific biological process related to the general phenomenon of growth and tissue regeneration. The process of wound healing can artificially be divided into three to five phases which overlap in time and space [18]

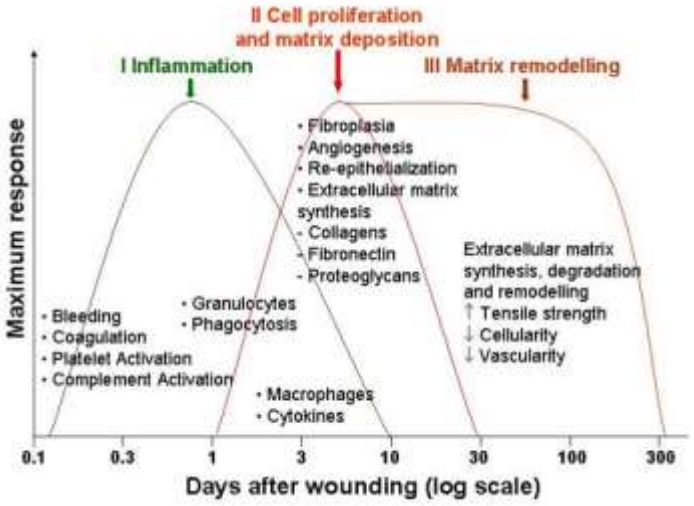

Figure 2. Evolution in time of physiological stages of wound repair

[18] Reinke J.M. and Sorg H. Eur.Surg.Res. 2012

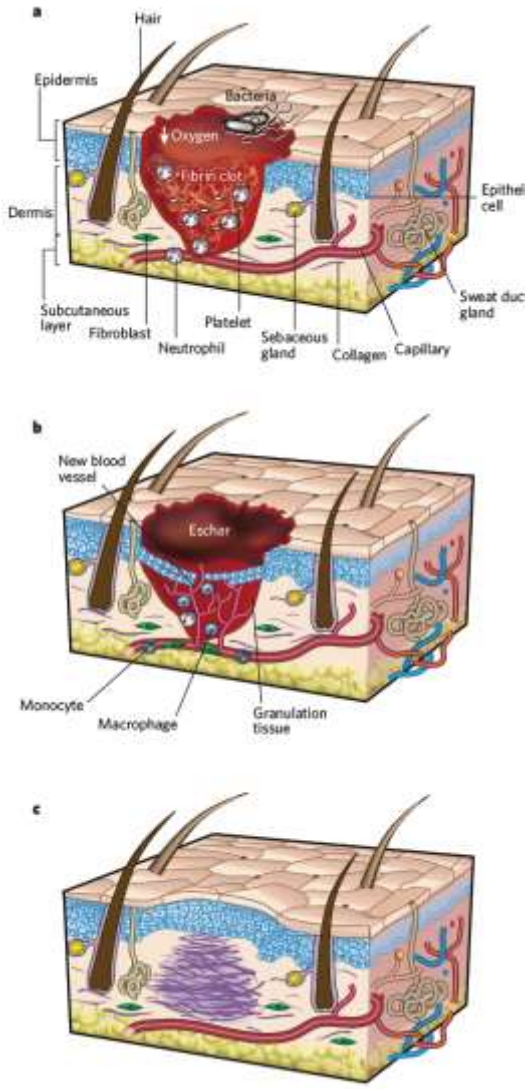

Figure 3. Classical stages of wound repair. There are three classical stages of wound repair: inflammation (a), new tissue formation (b) and remodelling (c). Inflammation (a) lasts until about 48 h after injury. Depicted is a skin wound at about 24–48 h after injury. The wound is characterized by a hypoxic (ischaemic) environment in which a fibrin clot has formed. Bacteria, neutrophils and platelets are abundant in the wound. Normal skin appendages (such as hair follicles and sweat duct glands) are still present in the skin outside the wound, New tissue formation (b) occurs about 2–10 days after injury. Depicted is a skin wound at about 5–10 days after injury. An eschar (scab) has formed on the surface of the wound. Most cells from the previous stage of repair have migrated from the wound, and new blood vessels now populate the area. The migration of epithelial cells can be observed under the eschar. Remodelling (c) lasts for a year or longer. Depicted is a skin wound about 1–12 months after repair. Disorganized collagen has been laid down by fibroblasts that have migrated into the wound. The wound has contracted near its surface, and the widest portion is now the deepest. The re-epithelialized wound is slightly higher than the surrounding surface, and the healed region does not contain normal skin appendages. Adapted from Gurtner G.C. *et al.*[19].

[19] Gurtner G.C. *et al.* Wound repair and regeneration. Nature 2008

1.1.1. The Vascular Response: Hemostasis and Coagulation

The first stage of physiological or acute wound healing is dedicated to hemostasis and the formation of a provisional wound matrix, which occurs immediately after injury and is completed after some hours (fig. 1). Furthermore, this phase initiates the inflammatory process. Sometimes this phase is also described as the 'lag-phase', in which the organism has to manage the recruitment of many cells and factors for the healing process in the absence of the mechanical strength of the wound.[20] The different clotting cascades are then initiated by clotting factors from the injured skin (extrinsic system), and thrombocytes get activated for aggregation by exposed collagen (intrinsic system). At the same time the injured vessels follow a 5- to 10-min vasoconstriction, triggered by the platelets, to reduce blood loss and fill the tissue gap with a blood clot comprising cytokines and growth factors[21]. Furthermore, the blood clot contains fibrin molecules, fibronectin, vitronectin and thrombospondins, forming the provisional matrix as a scaffold structure for the migration of leukocytes, keratinocytes, fibroblasts and endothelial cells and as a reservoir of growth factors. The life-saving vasoconstriction with clot formation accounts for a local perfusion failure with a consecutive lack of oxygen, increased glycolysis and pH-changes[22]. The vasoconstriction is then followed by a vasodilation in which thrombocytes invade the provisional wound matrix. In addition, platelets and leukocytes influence the infiltration of leukocytes by the release of chemotactic factors[23].

1.1.2. The Cellular Response: Inflammation

The inflammatory phase of the wound healing cascade gets activated during the hemostasis and coagulation phase and can roughly be divided into an early phase with neutrophil recruitment and a late phase with the appearance and transformation of monocytes (figure 4). The inflammatory response to injury is essential for supplying growth factor and cytokine signals that are responsible for cell and tissue movements and considered crucial for the subsequent repair mechanisms in adult mammalians[24]. Due to the response of the activated complement pathway, degranulated platelets and by-products of bacterial degradation, neutrophils are recruited to the site of the skin injury and are present for 2–5 days unless the wound gets infected. The work of the neutrophils is crucial within the first days after injury

[20] Robson M.C., Steed D.L. and Franz M.G. Curr.Probl.Surg. 2001
[21] Martin P. Science. 1997
[22] Woo Y.C., Park S.S., Subieta A.R. and Brennan T.J. Anesthesiology. 2004
[23] Werner S. and Grose R. Physiol Rev. 2003
[24] Leibovich S.J. and Ross R. Am.J.Pathol. 1975

because their ability in phagocytosis and protease secretion kills local bacteria and helps to degrade necrotic tissue. Furthermore, they act as chemoattractants for other cells that are involved in the inflammatory phase[23]. Approximately 3 days after injury, macrophages enter the zone of injury and support the ongoing process by performing phagocytosis of pathogens and cell debris[25,26] as well as secretion of growth factors, chemokines and cytokines (figure 4). Macrophages have many functions including host defense, the promotion and resolution of inflammation, the removal of apoptotic cells and the support of cell proliferation and tissue restoration following injury[27].

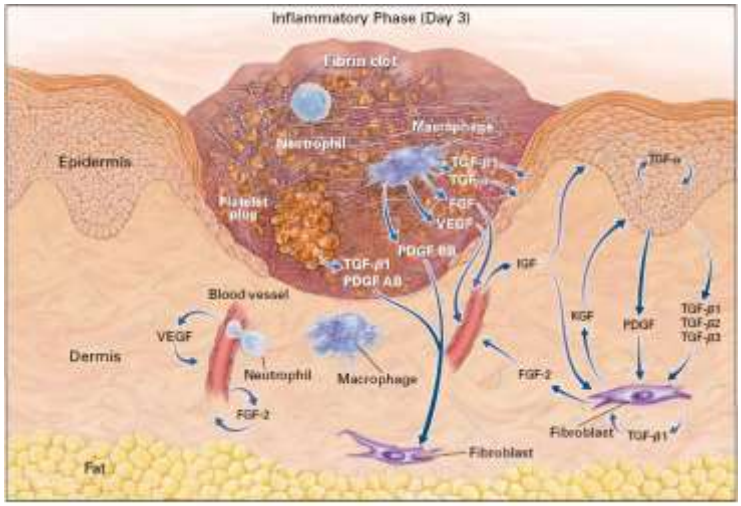

Figure 4. Cutaneous wound healing three days after the injury

1.1.3. Proliferation and Repair

In the phase of proliferation (approx. 3–10 days after injury) the main focus of the healing process lies in covering the wound surface, in the formation of granulation tissue and in restoring the vascular network. Therefore, next to the migration of local fibroblasts along the fibrin network and the beginning of reepithelization process from the wound edges, neovascularization and angiogenesis get activated by capillary sprouting.[28]

[25] Profyris C., Tziotzios C. and Do V., I. J.Am.Acad.Dermatol. 2012
[26] Tziotzios C., Profyris C. and Sterling J. J.Am.Acad.Dermatol. 2012
[27] Koh T.J. and DiPietro L.A. Expert Rev.Mol.Med. 2011
[28] Bauer S.M., Bauer R.J. and Velazquez O.C. Vasc.Endovascular Surg. 2005

The last step in the proliferation phase is the development of the acute granulation tissue (figure 5). At the same time the remodeling phase is already initiated. The granulation tissue is characterized by a high density of fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen bundles (figure 5). Since the angiogenesis is not completed yet, this tissue is highly vascularized and for this reason it appears with a classic redness and might be easily traumatized. However, the dominating cell type in this phase is the fibroblast, which fulfils different functions such as the production of collagen and ECM substances (i.e. fibronectin, glycosaminoglycans, proteoglycans and hyaluronic acid). Subsequently, the synthesis of collagen increases throughout the wound, while the proliferation of fibroblasts declines successively, adjusting the balance between synthesis and degradation of the ECM[29].

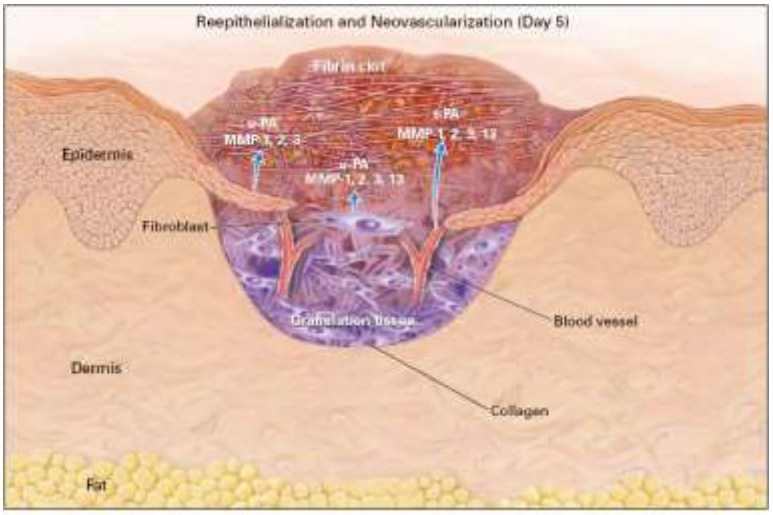

Figure 5. Cutaneous wound healing five days after the injury

1.1.4. Remodeling

Remodeling is the last phase of wound healing and occurs from day 21 to up to 1 year after injury. The formation of granulation tissue stops through apoptosis of the cells. A mature wound is, therefore, characterized as avascular as well as acellular[30]. During the maturation of the wound the components of the ECM undergo certain changes. Collagen III, which was produced in the proliferative phase, is now replaced by the stronger collagen I. This type of

[29] Madden J.W. and Peacock E.E., Jr. Ann.Surg. 1971
[30] Greenhalgh D.G. Int J.Biochem.Cell Biol. 1998

collagen is oriented in small parallel bundles and is, therefore, different from the basket-weave collagen in healthy dermis[31]. Later on, myofibroblasts cause wound contractions by their multiple attachment to collagen and help to decrease the surface of the developing scar[25;]**Errore. Il segnalibro non è definito.**[31]. Furthermore, the angiogenic processes diminish, the wound blood flow declines, and the acute wound metabolic activity slows down and finally stops. The epidermis of the resultant scar differs from uninjured skin after wound healing due to the lack of rete pegs that are normally anchored into the underlying connective tissue matrix and are responsible for the tight connection of the epidermis to the dermis

1.1.5. Molecular mechanisms of wound repair

Following the observation that genes regulated in response to skin injury are functionally important for the wound repair process, DNA-microarray showed that the gene-expression pattern of healing skin wounds strongly resembles that of highly malignant tumors[32], highlighting the importance of functional genomics studies for research into wound repair and cancer. The proteins involved in re-epithelialization have been described by Raja *et al.*[33] and include various extracellular-matrix proteins and their receptors, proteases (including matrix metalloproteinases), cytoskeletal proteins, and enzymes that regulate the cellular redox balance (Table x).

Table 1. Soluble mediators of re-epithelialization. Adapted from Gunter *et al.* Nature 2008.

Ligand	Receptor	Type of receptor	Signaling proteins	Role in re-epithelialization
HGF	MET	Receptor tyrosine kinase	Unknown, possibly ERK1 and ERK2, AKT, GAB1, PAK1 and/or PAK2	Stimulation of keratinocyte migration and probably proliferation
FGF7, FGF10 and FGF22	FGFR2-IIIb, possibly FGFR1-IIIb	Receptor tyrosine kinase	Unknown, possibly ERK1, ERK2, AKTand/or STAT3	Stimulation of keratinocyte proliferation and migration
Heparin-binding EGF and other EGFfamily members	EGFR (also known as ERBB1), possibly ERBB2, ERBB3 and/or ERBB4	Receptor tyrosine kinase	Unknown, possibly ERK1 and ERK2, AKT and/or STAT3	Stimulation of keratinocyte proliferation and migration
TGF-β	TGF-β receptor I and TGF-β receptor II	Receptor serine/threonine	SMAD3 and others, including SMAD2 and MAPK	Inhibition of keratinocyte proliferation and survival

[31] Gurtner G.C. and Evans G.R. Plast.Reconstr.Surg. 2000
[32] Chang H.Y., Sneddon J.B., Alizadeh A.A., Sood R. *et al.* PLoS Biol. 2004
[33] Raja, Sivamani K., Garcia M.S. and Isseroff R.R. Front Biosci. 2007

		kinase		
Acetylcholine	M3 receptor	G-protein-coupled receptor	Ca^{2+}-dependent guanylyl cyclase, cyclic GMP and PKG, leading to inhibition of RHO	Inhibition of keratinocyte migration
	M4 receptor	G-protein-coupled receptor	Adenylyl cyclase, cyclic AMP and PKA, leading to activation of RHO	Stimulation of keratinocyte migration
Catecholamines, Including adrenaline	β_2-Adrenoceptor	G-protein-coupled receptor	Activation of phosphatase PP2A, resulting in dephosphorylation and inhibition of ERK1 and ERK2	Inhibition of keratinocyte migration
Polyunsaturated fatty acids	PPAR-α and PPAR-β*	Nuclear receptor	Direct activation of target genes by binding to the promoter/enhancer of these genes	Stimulation of keratinocyte migration and survival

EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor; FGFR1-IIIb, IIIb isoform of FGF receptor 1; GAB1, growth-factor-receptor-bound protein 2 (GRB2)-associated binding protein 1; HGF, hepatocyte growth factor; M3, muscarinic receptor subtype 3; PAK, p21-activated kinase; PKA, cyclic-AMP-dependent protein kinase; PKG, cyclic-GMP-dependent protein kinase; PPAR, peroxisome-proliferator-activated receptor; SMAD3, SMAD-family member 3; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-β. *PPAR-β ligands might be fatty acids.

1.2. Wound Exudate

Thomas[34] has described wound exudate as: 'a generic term given to liquid produced from chronic wounds, fistulae or other more acute injuries once hemostasis has been achieved'. It is essentially formed by blood from which most of the red cells and platelets have been removed. Exudate is a key component in all the stages of wound healing, irrigating the wound continuously and keeping it moist.[35] The maintenance of a moist wound bed is widely accepted as the most ideal environment for effective wound healing.[36] Exudate also supplies the wound with nutrients and provides favorable conditions for migration and mitosis of epithelial cells[37] and leucocytes, which helps to control bacteria and reduce the incidence of infection at the wound surface.

1.2.1. Composition of exudate

Exudate contains a variety of substances including water, electrolytes, nutrients, inflammatory mediators, white cells, protein-digesting enzymes (e.g. matrix metalloproteinases – MMPs), growth factors and waste products. Exudate is derived from the fluid that has leaked

[34] Thomas S. J.Wound Care. 1997
[35] Gray D and White RJ. Wounds UK:Applied wound management Suppl. 2004
[36] Eaglstein W.H., Davis S.C., Mehle A.L. and Mertz P.M. Archives of Dermatology. 1988
[37] Quick A. Nurs.Times. 1994

out of blood vessels and closely resembles blood plasma. Fluid leaks from capillaries into body tissues at a rate that is determined by the leakiness (permeability) of the capillaries and the pressures (hydrostatic and osmotic) across the capillary walls. The relationship between the factors that determine how much fluid leaks out is known as Starling's hypothesis. In general, most (about 90%) of the leaked fluid is reabsorbed into capillaries, but small amount that is not reabsorbed (about 10%) is returned to the central circulation via the lymphatic system. As a result, in the steady state, leakage from capillaries is balanced by the reabsorption and drainage of fluid. In a wound, the initial injury initiates inflammation, an early stage of the healing process. Mediators involved in inflammation, e.g. histamine, increase capillary permeability so that white blood cells can escape and the blood vessels leak more fluid. The excess fluid enters the wound where it forms the basis of exudate (Figure 6).

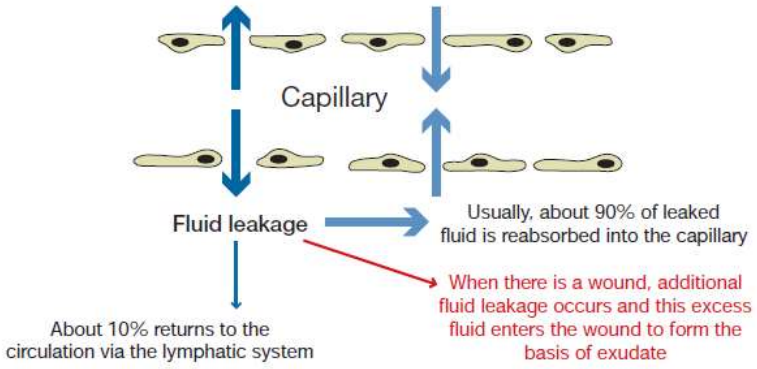

Figure 6. exudate homeostasis in wound healing

In the healing wound, exudate appears to promote healing in a number of ways, including stimulating cell proliferation. MMPs, which break down the cell-supporting extracellular matrix, are present mainly in an inactive form. In wounds not healing as expected (chronic wounds), exudate appears to have opposite effects. This exudate contains elevated levels of inflammatory mediators and activated MMPs.

1.2.2. Exudate management

Nowadays the wound exudate is generally recognized as a key factor in wound healing. Although a moist environment is necessary for optimal wound healing, conditions of extreme wetness or dryness may adversely affect healing. In a healing wound, exudate production generally reduces over time, but in certain conditions, such as chronic wounds, there is

excessive amounts of exudates which can lead to complications. In a wound that is not healing as expected, exudate production may continue and be excessive due to ongoing inflammatory or other processes.

The amount of exudate produced by a wound is partly dependent on surface area. Consequently, the larger the surface area, the greater the likely volume of exudate. Some wound types are perceived to have high rates of exudate production, e.g. burns, venous leg ulcers, skin donor sites and inflammatory ulcers (rheumatoid and pyoderma gangrenosum). However, these are often large wounds and so would be expected to produce higher volumes of exudate[38]

Traditionally, information about exudate is gained from examination of colour, consistency, odor and amount (Figure 7). These characteristics may indicate components, contaminants or underlying cause (Tables 2).

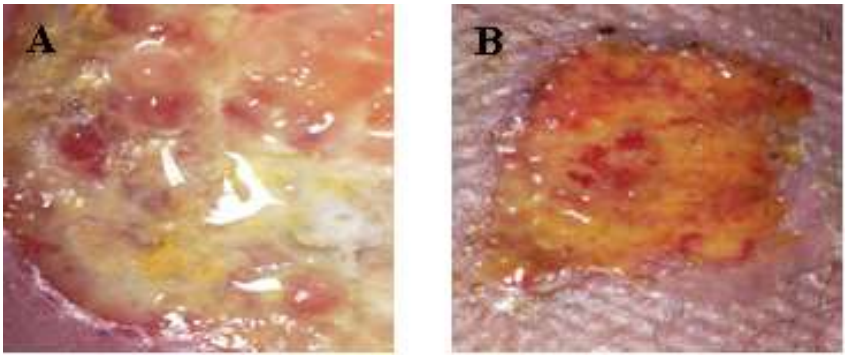

Figure 7. A) Mainly clear, serous, thin exudate with granulation tissue visible in the wound bed; B) Thick haemopurulent exudate. (Photos copyright of the Cardiff and Vale NHS Trust – Professor Keith Harding)

Table 2. Colour, consistency and odor of exudate[39].

[38] Thomas S., Fear M., Humphreys J., Disley L. *et al.* Wounds-A Compendium of Clinical Research and Practice. 1996

[39] Adapted from: Wound exudate and the role of dressings A consensus document World Union of Wound Healing Societies, 2007

Significance of exudate color*	
Characteristic	**Possible cause**
Clear, amber	▪ Serous exudate, often considered 'normal', but may be associated with infection by fibrinolysin-producing bacteria such as Staphylococcus aureus; may also be due to fluid from a urinary or lymphatic fistula
Cloudy, milky or creamy	▪ May indicate the presence of fibrin strands (fibrinous exudate– a response to inflammation) or infection (purulent exudatecontaining white blood cells and bacteria)
Pink or red	▪ Due to the presence of red blood cells and indicating capillary damage (sanguineous or haemorrhagic exudate).
Green	▪ May be indicative of bacterial infection, e.g. Pseudomonas aeruginosa
Yellow or brown	▪ May be due to the presence of wound slough or material from an enteric or urinary fistula.
Grey or blue	▪ May be related to the use of silver-containing dressings
Significance of exudate consistency	
High viscosity (thick, sometimes sticky)	▪ High protein content due to infection or inflammatory process ▪ Necrotic material ▪ Enteric fistula ▪ Residue from some types of dressings or topical preparations
Low viscosity (thin, 'runny')	▪ Low protein content due to venous or congestive cardiac disease or malnutrition ▪ Urinary, lymphatic or joint space fistula
Significance of exudate odour	
Unpleasant	▪ Bacterial growth or infection ▪ Necrotic tissue ▪ Sinus/enteric or urinary fistula

In local wound management, dressings are the main option for managing exudate. A key characteristic of modern wound dressings is the removal of excess exudate while maintaining moisture at the wound bed.[40]

[40] Sibbald R.G., Goodman L., Woo K.Y., Krasner D.L. *et al.* Adv.Skin Wound Care. 2011

1.3. Factors Which Impair Wound Healing: Chronic Wounds

Although most wounds will heal uneventfully, problems can sometimes occur, that lead to failure of the wound to heal or a prolonged healing time. Failure of a wound to heal within the expected time frame usually results in a chronic wound. A chronic wound fails to heal because the orderly sequence of events is disrupted at one more of the stages of wound healing

A chronic wound is defined as a loss of integrity of a skin surface area including one or more layers of underlying structures, which fail to heal within eight weeks[41]. Different factors can affect a regular wound healing process, such as underlying pathobiology or microorganism invasion and when this happen failure to heal occurs often leading to a chronic wound (ulcer).

Furthermore, impaired wound healing can lead to an excessive production of exudates that can cause maceration of healthy skin tissue around the wound[42]

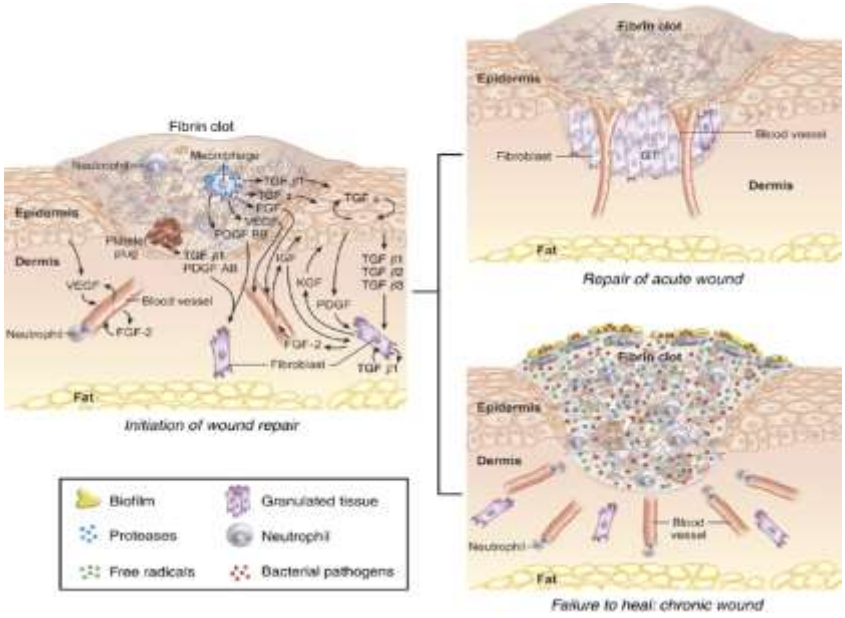

Figure 8. Tissue injury results in an acute wound healing response under normal physiologic conditions but may fail when underlying pathobiology or microbial invasion interfere with the healing process, thereby creating a chronic wound. Adapted from Clark et al.[43]

[41] Lawall H. Vasa. 2012
[42] Cutting K.F. and White R.J. J.Wound Care. 2002
[43] {Clark, 2007 560 /id}

Underlying pathobiology known to interfere with acute wound healing includes venous insufficiency that results in fluid transudation and fibrin cuffing of venules secondary to high hydrostatic pressure in the venous system; diabetes mellitus that results in high glucose and both cell and ECM dysfunction from non-enzymatic glycation; arterial occlusion or high external pressure that results in tissue hypoxia and cell dysfunction or death. Bacteria colonizing the wound often produce a biofilm composed of a wide variety of polysaccharides. The biofilm protects these colonies of mixed microorganisms, as it is relatively impervious to phagocytic cells and impermeable to antibiotics. Frustrated phagocytes release a plethora of proteases and toxic oxygen radials into the wound milieu making a bad situation worse as these agents destroy tissue cells, extracellular matrix, and growth factors in the wound. Not surprisingly, such chronic wounds lack epidermal migration and ingrowth of granulation tissue.

In addition, exudate from chronic wound differs from acute wound fluid with relatively higher levels of tissue destructive proteinase enzymes[44] and therefore more corrosive. The smell and staining caused by exudate can also have a negative impact on a patient's general health and quality of life[45]. On the same time underlying diseases such as diabetes[46] and anemia delay wound healing because compromised circulation results in the delivery of inadequate nutrients, blood cells and oxygen to the wound (table 3).

Table 3. Crucial factors in the development of chronic wounds and wound healing disorders.

Confounding factor	Examples
Circulatory disorders	PAD, chronic venous insufficiency, lymphedema, right ventricular insufficiency
Metabolic factors	Diabetes mellitus, renal insufficiency, hepatic disease, tumors, cachexia, malnutrition
Injuries and chronic-degenerative disorders of the soft tissue, muscle and skeletal systems	Fractures with mal-alignment, arthrosis, rheumatoid arthritis, diabetic neuro-osteoarthro-pathy (Charcot foot)
Nervous disorders	Polyneuropathy, stroke
Skin diseases	Allergies, infectious and immunological skin diseases, collagenosis, cancer
Local factors	Postoperative scars, radiotherapy, foreign matter, hematomas
Medication	Glucocorticoids, immunosuppressives, cytostatics
Local noxins	Incontinence, pressure stress, burns
Toxins	Nicotin, drugs, alcohol

[44] Chen W.Y., Rogers A.A. and Lydon M.J. J.Invest Dermatol. 1992
[45] Hareendran A., Bradbury A., Budd J., Geroulakos G. *et al.* J.Wound Care. 2005
[46] Falanga V. Lancet. 2005

Other problems associated with wound healing include the formation of keloid scars, rubbery lesions resulting from excess collagen production in the latter part of the wound healing process[47]. Also foreign bodies introduced deep into the wound at time of injury can cause chronic inflammatory responses delaying healing and sometimes leading to granuloma or abscess formation.

Bacterial contamination on the wound site is a negative predictor for wound healing. Pathogenic bacteria such as *S. aureus, P. aeruginosa, S. pyrogenes* and some Proteus, Clostridium and Coliform species can be detrimental to the healing process. Inadequate control measures to manage infected wounds can lead to cellulitis (cell inflammation) and ultimately bacteraemia and septicaemia, both of which can be fatal. It has been shown that the presence of *P. aeruginosa and S. aureus* significantly reduced skin graft healing and also that 94% of ulcers that were slow to heal, or recurred after discharge, contained *S. aureus*[48].

Poor nutritional status and old age[49] also reduce the ability to fight infection. Protein, vitamin (e.g. vitamin C) and mineral deficiencies impair the inflammatory phase and collagen synthesis, leading to prolonged healing times[50].

Treatment with drugs such as steroids suppress the body's inflammatory responses and thereby impede the inflammatory stage of wound healing, which eventually leads to a compromised immune response. Glucocorticoids for example have been shown to impair wound healing in both rats and humans[51], due to inhibition of the production of keratinocyte growth factor (KGF)[52]. Also some nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g., aspirin, ibuprofen) interfere with wound healing by decreasing collagen production. The precise mechanism is not fully understood.

Radiation therapy is an established modality in the treatment of head and neck cancer patients. Compromised wound healing in irradiated tissues is a common and challenging clinical issue[53]. Radiation damages the ability of the tissues to promote new blood vessel growth as well as interferes with cellular functions necessary for wound healing. These effects are not reversible once the radiation exposure has been completed and in fact may worsen with time. Because of these effects, a seemingly minor injury in an area that previously received radiation

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[47] martin p 1997
[48] Gilliland E.L., Nathwani N., Dore C.J. and Lewis J.D. Ann.R.Coll.Surg.Engl. 1988
[49] Lee J., Singletary R., Schmader K., Anderson D.J. *et al.* J.Bone Joint Surg.Am. 2006
[50] Patel G.K. Int J.Low Extrem.Wounds. 2005
[51] Beck L.S., DeGuzman L., Lee W.P., Xu Y. *et al.* J.Clin.Invest. 1993
[52] Chedid M., Hoyle J.R., Csaky K.G. and Rubin J.S. Endocrinology. 1996
[53] Haubner F., Ohmann E., Pohl F., Strutz J. *et al.* Radiat.Oncol. 2012

may result in a chronic, open wound. Vitamin E has been shown to improve wound strength in areas exposed to radiation but often the entire wound may need to be excised to remove the damaged, radiated tissue.

In a long-standing wound that looks clean but still will not heal, the wound may be harboring an underlying cancer.[54] The concern about an underlying cancer is especially applicable in chronic wounds in elderly patients and on sun-exposed areas of the body. We tend to expect basal cell or squamous cell skin cancers to be relatively small (< 2–3 cm), but, if left untreated, they can grow to be quite large. Cancer of the breast and soft tissue sarcomas can erode through the skin to create a chronic open wound.[55]

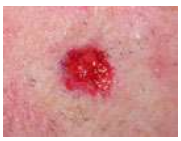
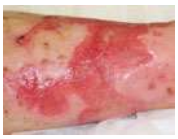
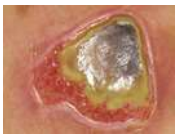
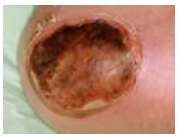
Common chronic skin and soft tissue wounds include the diabetic foot ulcers, pressure ulcers, and the venous stasis ulcers.

[54] Alexander S. J.Wound Care. 2009
[55] Seaman S. Semin.Oncol.Nurs. 2006

WOUNDS DRESSINGS

A dressing is defined as a piece of material used to cover and protect a wound.[56] Natural skin is considered the perfect wound dressing and therefore an ideal wound dressing should try to replicate its properties[57]. Once the cause is identified and properly treated, the basic principle of wound treatment is essentially the same for all kind of injuries, that is use of a proper wound dressing. Different types of wounds and the different stages of a healing wound require different dressings or combinations of dressings. The following table shows suitable dressings for particular wound types.

Table 4. suitable dressing for wound types

	Wound type	Dressing type
	Clean, medium-to-high exudate (epithelialising)	• Paraffin gauze • Knitted viscose primary dressing
	Clean, dry, low exudate (epithelialising)	• Absorbent perforated plastic film-faced dressing • Vapour-permeable adhesive film dressing
	Clean, exudating (granulating)	• Hydrocolloids • Foams • Alginates
	Slough-covered	• Hydrocolloids • Hydrogels
	Dry, necrotic	• Hydrocolloids • Hydrogels

Often the dressings may require secondary dressings such as absorbent pad and bandages. Several factors apart from the choice of wound dressings need to be considered to ensure

[56] http://www.oxforddictionaries.com/
[57] Morin R.J. and Tomaselli N.L. Clin.Plast.Surg. 2007

successful wound healing. In the case of chronic wounds, underlying factors such as disease, drug therapy and patient circumstance must all be taken into account and addressed before a particular wound dressing is selected. Table x describes factors to be considered in the choice of wound dressings based on their performance characteristics (functions).[1,,58]

Table 5. Functions (Desirable Characteristics) of Wound Dressings according to Eccleston [59]

Desirable Characteristics	Clinical Significance to Wound Healing
Debridement (wound cleansing)	Enhances migration of leucocytes into the wound bed and supports the accumulation of enzymes. Necrotic tissue, foreign bodies and particles prolong the inflammatory phase and serve as a medium for bacterial growth
Provide or maintain a moist wound environment	Prevents desiccation and cell death, enhances epidermal migration, promotes angiogenesis and connective tissue synthesis and supports autolysis by rehydration of desiccated tissue
Absorption. Removal of blood and excess exudate	In chronic wounds, there is excess exudate containing tissue degrading enzymes that block the proliferation and activity of cells and break down extracellular matrix materials and growth factors, thus delaying wound healing. Excess exudate can also macerate surrounding skin
Gaseous exchange (water vapour and air)	Permeability to water vapour controls the management of exudate. Low tissue oxygen levels stimulate angiogenesis. Raised tissue oxygen stimulates epithelialisation and fibroblasts
Prevent infection: Protect the wound from bacterial invasion	Infection prolongs the inflammatory phase and delays collagen synthesis, inhibits epidermal migration and induces additional tissue damage. Infected wounds can give an unpleasant odour
Provision of thermal insulation	Normal tissue temperature improves the blood flow to the wound bed and enhances epidermal migration
Low adherence. Protects the wound from trauma	Adherent dressings may be painful and difficult to remove and cause further tissue damage
Cost effective Low frequency of dressing change	Dressing comparisons based on treatment costs rather than unit or pack costs should be made (cost-benefit-ratio). Although many dressings are more expensive than traditional materials, the more rapid response to treatment may save considerably on total cost

Due to the distinct characteristics of the different types of wounds and of each of the wound healing stages, there is no one single dressing that can be efficiently applied in all situations. However, it is possible to develop and to optimize different biocompatible wound

[58] Thomas S, editor. 1990. Wounds and wound healing in: Wound management and dressings. 1st edition. London: Pharmaceutical Press. pp 1–14.
[59] Eccleston G.M. Wound dressings. in *Pharmaceutics: The science of dosage form design*. 2007

dressing materials in terms of their chemical and physical properties, e.g. moisture absorption and permeation capacities, in order to meet most of the needs for a particular wound stage.[60]

2.1. History of dressings

The history of wound care spans from prehistory to modern medicine. The aim of wound treatment has always been to reduce the risks caused by the wound itself and to minimize potential complications. The first written records containing medical information on clay tablets discovered in Mesopotamia and dated around 2500 B.C.. Over the centuries, such systems have become more sophisticated, but their primary goal of healing damage remains the same.

In early civilization, medicine was a mixture of magic and empiricism and was practiced by wizards.[61] Wound treatment was no more scientific in Egypt, despite the great anatomic knowledge resulting from the Egyptians' intricate embalming technique. The most of Egyptian medicine is derived from other civilization, in fact the first medical papyri from Egypt, the Smith papyrus (about 1650 B.C.), is considered to be a copy of a much older document.[62] The agents most used in Egypt were probably mixtures of honey and lard or honey and resin. The grease-soaked gauze in Egyptian times[63] can be considerate the first attempt to advanced dressing. Furthermore the Egyptians appear to have learned that a closed wound heals faster than an open one. They invented the adhesive bandage by applying gum to linen strips and using these to draw wound edges together. Severe bleeding may have been treated with cautery.

Greek medicine borrowed much from the older Egyptian, especially after the two cultures met in Alexandria from about 300 B.C., but a medical tradition had existed in Greece since the time of Asklepios, approximately five hundred years earlier. Hippocrates (460-377 B.C.) is widely considered to be the "Father of Medicine" and was the first to believe that diseases were caused naturally, not because of superstition and gods. He suggested that contused wounds should be treated with salves in order to promote suppuration, remove necrotic material and reduce inflammation. He preferred leaving wounds dry once they had been washed and he recommended the use of wine or vinegar for washing. Honey and oil as well as wine were among the most widely used ointments, though wool boiled in water or wine was considered a useful dressing.

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[60] Fonder M.A., Lazarus G.S., Cowan D.A., Aronson-Cook B. *et al.* J.Am.Acad.Dermatol. 2008
[61] Lyons A S. Medicine, an Illustrated History. 1978
[62] Forrest R.D. J.R.Soc.Med. 1982
[63] Sipos P., Gyory H., Hagymasi K., Ondrejka P. *et al.* World J.Surg. 2004

The Romans had no medical tradition and no physicians until Greeks settled in Rome about 200 B.C.. One of the most important encyclopedic opera about the medical knowledge at that time was the “De Medicina” written by Aulus (perhaps Albinovarus) Cornelius Celsus (ca 25 B.C. - ca 50 A.C.). Celsus wrote clearly on the care of wounds and was the first to define the four cardinal signs of inflammation (though not when discussing wounds): *'Notae vera inflammationis sunt quattuor; rubor et tumor cum calore et dolore*'. He described a variety of different wounds and gave detailed descriptions of their treatment, classifying topical preparations according to their effects on the wound. After Celsus this knowledge was reconsidered and improved by Claudius Galeno (c.129-200 A.C.). Although Galen was a skilled surgeon (he had served four years as a surgeon to the gladiators in Pergamon) , he relied heavily on medicines and advocated polypharmacy. He waited a large number of treatises, which were so accurate that even his hypotheses were universally accepted as fact and remained unchallenged until the Renaissance.

Table 6. natural remedies used in Roman and Greek medicine

Animal	Vegetable	Mineral
Bile	Bark	Alum
Blood	Dyes	Antimony
Butter	Fruit	Arsenic
Cobweb	Herbs	Clay
Cochineal	Honey	Copper salts
Egg-white	Leaves	Lead salts
Faeces	Oils	Mercury salts
Lard or grease	Resins	Potassium salts
Meat	Sap	Tar or pitch
Milk	Sugar	Zinc salts
	Turpentine	
	Wine or vinegar	

During the Middle Ages, following the teaching of Greek and Roman medicine, wounds were left to “rot a bit” in order to develop “laudable pus,” which meant the patient would

actually survive.[64] During this age the surgeon believed that the pus was a decisive element of healing and tried in every way to provoke its formation, by introducing foreign bodies and liquids irritating the wound. On the contrary, in the Renaissance prevailed the orientation to keep the wound clean at all costs, draining the moods, removing the necrotic and above all make sure that no foreign objects remain within.

Around 1500 the introduction of gunpowder into Europe started a violent controversy over the way in which gunshot wounds should be treated. In the seventeenth century the classical procedure in case of gunshot injury consisted in a surgical exploration to eliminate the foreign bodies penetrated into the tissues using surgical tools or substances that were believed to have this ability. After appropriate explorations, the wound was filled with tow or linen wedges and medicated with different balms. The gunshot wound treatment was the same until the First World War when the chemist Henry Drysdale Dakin invented the Dakin's solution, made of sodium hypochlorite and boric acid, to wash out the traumatic wounds of British soldiers fighting in France. During World War II, large scale production and the diffusion of penicillin made a major difference in the number of deaths and amputations caused by infected wounds among allied forces, saving an estimated 12%–15% of lives.

The first advances in wound care in modern era began with the work of Ignaz Philipp Semmelweis, a Hungarian obstetrician who discovered how hand washing and cleanliness in medical procedures prevents maternal deaths. Semmelweis's work was furthered by an English surgeon, Joseph Lister, who in 1860s began treating his surgical gauze with carbolic acid, known today as phenol, and subsequently dropped his surgical team's mortality rate by 45%. A number of new discoveries have been made throughout the years, but the real revolution in wound care occurred in 1962 when George D. Winter published his work on surface induced acute lesions on domestic pigs.[65] By covering a lesion with a film of occlusive material (polyethylene), Winter obtained a moist environment that ensured a more rapid re-epithelialization as compared to the healing of the lesion left to dry in air. This study, which was lacking in some aspects (non-standardized animal model, tests only on acute and not chronic wounds), has paved the way to a new way of managing wounds: the moist wound healing. Until then, in fact, a dry lesions was considered crucial, especially to counteract bacterial colonization and subsequent infection. Hence, the concept of advanced dressing emerged as opposed to the traditional ones. Traditional dressings were designed to cover and hide the wound by absorbing

[64] Broughton, 2006
[65] Winter G.D. Nature. 1962

excess exudate, while with Winter's work the concept of interaction between medication and ulcerative lesion began, with the ultimate goal of creating an environment suitable for the acceleration of the tissue repair process.

In the 1990s, improvements in composite and hybrid polymers expanded the range of materials available for wound dressing. Grafting and biotechnology have produced useful protective covering of actual human skin generated through cloning procedures. These improvements, coupled with the developments in tissue engineering, have given rise to a number of new classes of biological wound dressings. Among these the "Living skin equivalents" are very promising and may have the potential to serve as cellular platforms for the release of growth factors essential for proper wound healing.

2.2. Classification of dressings

Dressings are classified in a number of ways depending on their function in the wound (debridement, antibacterial, occlusive, absorbent, adherence), type of material employed to produce the dressing (e.g. hydrocolloid, alginate, collagen) and the physical form of the dressing (ointment, film, foam, gel).[66] Dressings are further classified into primary, secondary and island dressings. Dressings which make physical contact with the wound surface are referred to as primary dressings while secondary dressings cover the primary dressing. Island dressings possess a central absorbent region that is surrounded by an adhesive portion. Other classification criteria include traditional dressings, modern and advanced dressings, skin replacement products and wound healing devices. Classification criteria can be useful in the selection of a given dressing but many dressings fit all the criteria.[1] In this thesis dressings are classified as traditional or modern (moist wound environment) dressings.

2.2.1. Traditional wound dressings

These were used commonly in the past and though now less widely used, they are still of some benefit in certain clinical settings for wound treatment. Traditional wound healing agents include topical liquid and semi-solid formulations as well as dry traditional dressings.

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[66] Eccleston GM. 2007. Wound dressings. In: Aulton ME, editor. Pharmaceutics: The science of dosage form design. 3rd edition. UK: Churchill Livingstone. pp 264–271.

Topical formulations can be liquid (solutions, suspensions and emulsions) or semi-solid (ointments and creams) and their use is widespread. Solutions, such as povidone-iodine, are useful in the first stage of wound healing as antimicrobial agents, but they have short residence times on the wound site, especially where there is a measurable degree of suppuration (exuding) of wound fluid. Semi-solid preparations such as silver sulphadiazine cream and silver nitrate ointment used to treat bacterial infection remain on the surface of the wound for a longer period of time as compared with solutions. Also in this case, for highly exuding wounds semi-solid preparations are not very effective at remaining on the wound area as they rapidly absorb fluid, lose their rheological characteristics and become mobile.

Bandages and gauzes

Traditional dressings include cotton, wool, natural or synthetic bandages and gauzes. Unlike the topical pharmaceutical formulations, these dressings are dry and do not provide a moist wound environment. They may be used as primary or secondary dressings, or form part of a composite of several layers with each performing a specific function. Gauze is easy to use, often readily accessible in most clinics and surgical centers and is inexpensive. Though gauze dressings can provide some bacterial protection, this is lost when the outer surface of the dressing becomes moistened either by wound exudate or external fluids. [67] In addition gauze dressings tend to become more adherent to wounds as fluid production diminishes and are painful to remove, thus causing patient discomfort. Gauze dressings also provide little occlusion and allow evaporation of moisture resulting in a dehydrated wound bed although gauze impregnated with soft paraffin is occlusive and easier to remove from the skin. It has been suggested that traditional dressings should be employed only for wounds that are clean and dry or used as secondary dressing to absorb exudates and protect the wound.[68]

Traditional wound healing agents have been largely replaced for chronic wounds and burns by the more recent and advanced dressings since liquid and semi-solid formulations do not remain on the wound surface long enough whilst dry traditional dressings do not provide a moist environment for wound healing.

2.2.2. Modern wound dressings

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[67] Powers J.G., Morton L.M. and Phillips T.J. Dermatol.Ther. 2013†
[68] Morgan D.A. Hosp Pharmacist. 2002

Modern dressings have been developed as an improvement upon the traditional wound healing systems described above. Compared with standard ones, some advanced wound dressings may improve the proportion of wound healed and reduce time to healing, although evidence is limited.[69] Their essential characteristic is to retain and create a moist environment around the wound to facilitate wound healing. Dressing choice will be determined mainly by the ability of the dressing to achieve the desired exudate level, to assist healing and/or to prevent deterioration of wounds not expected to heal. In addition, properties such as fluid retention and sequestration may be considered.[70]

The modern dressings are mainly classified according to the materials from which they are produced including hydrocolloids, alginates and hydrogels, and generally occur in the form of gels, thin films and foam sheets.

Hydrocolloid Dressings

Hydrocolloid dressings are among the most widely used dressings. The term 'hydrocolloid' describes the family of wound management products obtained from colloidal (gel forming agents) materials combined with other materials such as elastomers and adhesives. Typical gel-forming agents include carboxymethylcellulose (CMC), gelatin and pectin.


[FQ1]

Figure 9: example of hydrocolloidal dressing.

[69] Greer N., Foman N.A., MacDonald R., Dorrian J. *et al.* Ann.Intern.Med. 2013
[70] Principles of best practice: Wound exudate and the roleof dressings. A consensus document. World Union of Wound Healing Societies (WUWHS). 2007.

Hydrocolloid dressings are useful clinically because unlike other dressings, they adhere to both moist and dry sites, and present the advantage of permitting easy visualization without disturbing the wound. Hydrocolloid dressings are used for light to moderately exuding wounds such as pressure sores, minor burns and traumatic injuries. In their intact state, hydrocolloid dressings are impermeable to water vapor but on absorption of wound exudate, a change in physical state occurs with the formation of a gel covering the wound. They become progressively more permeable to water and air as the gel forms.[71] As they do not cause pain on removal, they are particularly useful in paediatric wound care for management of both acute and chronic wounds.[72] Hydrocolloid dressings generally have an occlusive outer cover that prevents water vapor exchange between the wound and its surroundings. This can be disadvantageous for infected wounds that require a certain amount of oxygen to heal rapidly. Another disadvantage applies to dressings containing fibers that are deposited in the wound and often have to be removed during dressing change

Hydrogel Dressings

Hydrogels are insoluble, swellable hydrophilic materials made from synthetic, such as poly(methacrylates) and polyvinylpyrrolidine, or natural polymers (es. alginate). Hydrogels can be applied either as an amorphous gel or as elastic, solid sheet or film (Figure x). The sheets can absorb and retain significant volumes of water upon contact with suppurating wounds, but if they are applied in hydrogel form this kind of dressing already contains a significant amounts of water (70–90%) and as a result they cannot absorb much exudate, thus they can be used only for light to moderately exuding wounds.

[71] Thomas S. and Loveless P.A. World Wide Wounds. 1997
[72] Thomas S. Journal of Wound Care. 1992

Figura 10. An example of a polymeric hydrogel sheet wound dressing. Hydrogel sheets do not need a secondary dressing and due to their flexible nature, can be cut to fit around the wound.

Hydrogels possess most of the desirable characteristics of an 'ideal dressing'.[73] They are suitable for cleansing of dry, sloughy or necrotic wounds by rehydrating dead tissues and enhancing autolytic debridement. Hydrogel dressings are nonreactive with biological tissues, permeable to metabolites and nonirritant.[74] Hydrogels also promote moist healing, are non-adherent and cool the surface of the wound, which may lead to a marked reduction in pain and therefore have high patient acceptability. Morgan[75] has stated that hydrogels 'are suitable for use at all four stages of wound healing with the exception of infected or heavily exuding wounds'.

A particular class of hydrogels very used in clinical practice is made by alginates. Alginate dressings are comprised of cellulose-like polysaccharides derived from algae or kelp, which have impressive absorptive abilities. This type of dressing and all the clinical application will be treated in a separate chapter.

Foam Dressings

These dressings consist of porous polyurethane foam or polyurethane foam film, sometimes with adhesive borders. Some foam dressings such as Tielle™ have additional wound contact layers to avoid adherence when the wound is dry and an occlusive polymeric backing layer to prevent excess fluid loss and bacterial contamination.

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[73] Morgan DA. 1999. Wound management products in the drug tariff. Pharm J 263:820–825

[74] Wichterle O, Lim D. 1960. Hydrophilic gels for biological use. Nature 185:117–118.

[75] Morgan DA. 2002. Wounds—What should a dressing formulary include? Hosp Pharmacist 9:261–266.

Figure 11: Example of a foam dressing.

Foam dressings maintain a moist environment around the wound, provide thermal insulation and are convenient to wear.[76] They are highly absorbent, absorbency being controlled by foam properties such as texture, thickness and pore size. The porous structure of the dressings, make them suitable for partial or full-thickness wounds with minimal or moderate drainage, to highly absorbent structures for heavily exuding wounds.[77] Foam dressings are also indicated for granulating wounds where they are reported to help treat over granulation.

Biological Dressings and Skin Substitutes

These dressings are made from biomaterials that play an active part in the wound healing process and sometimes referred to as 'bioactive dressings'. Bioactive wound dressings also include tissue engineered products derived from natural tissues or artificial sources. These technologies usually combine polymers such as collagen, hyaluronic acid, chitosan, alginates and elastin.[1] Biomaterials have the advantage of forming part of the natural tissue matrix, are biodegradable and sometime play an active part in normal wound healing and new tissue formation. These characteristics make them attractive from a biocompatibility and toxicological point of view. In some cases they may be incorporated with active compounds such as antimicrobials and growth factors for delivery to the wound site.

Traditional and modern dressings though useful, cannot replace lost tissue, particularly missing dermis as occurs in severe burns. Advances in the fabrication of biomaterials and the culturing of skin cells have led to the development of a new generation of engineered skin

[76] Wound care guidelines. Medicines Management Committee Report. 2005.
[77] Thomas S, editor. 1990. Wounds and wound healing in: Wound management and dressings. 1st edition. London: Pharmaceutical Press. pp 1–14.

substitutes.[78] Such polymers act as scaffolds for tissue engineered substrates that replace lost tissue rather than just facilitate wound healing. When introduced into the body they gradually degrade, leaving behind a matrix of connective tissue with the appropriate structural and mechanical properties. Though these advanced dressings have great potential for treating chronic wounds and third degree burns, they are still limited by the high costs involved, the risk of infection and antigenicity as well as having to create a second wound in the case of harvesting patient's own cells to aid wound healing.

2.3. Medicated dressings for drug delivery

The use of topical bioactive agents in the form of solutions, creams and ointments may be advantageous, but difficult to attain due the issues described before. The introduction of dressings for controlled delivery of bioactive species can provide an excellent means to concentrate a drug at wound site in a consistent and sustained fashion over long periods of time without the need for frequent dressing change. In chronic wound management, where patients usually undergo long treatments and frequent changes of dressing, a system that delivers an active substance to a wound site in a controlled fashion for a sustained period can improve the patient compliance and therapeutical outcome.

Bioadhesive, synthetic, semisynthetic and naturally derived polymeric dressings are potentially useful in the treatment of local infections where it may be beneficial to realize increased local concentrations of antibiotics while avoiding high systemic doses thus reducing patient exposure to an excess of drug beyond that required at the wound site.[79] In addition, biodegradability can allow them to be easily washed off the wound surface, once they have exerted their desired effect.

2.3.1. Polymeric Drug Delivery Dressings

Most modern dressings are made from polymers which can serve as vehicles for the release and delivery of drugs to wound sites The polymeric dressings employed for controlled drug delivery to wounds include systems based on synthetic polymers such as poly(lactide-co-

[78] Horch R.E., Kopp J., Kneser U., Beier J. *et al.* J.Cell Mol.Med. 2005
[79] Langer R. 1980. Polymeric delivery systems for controlled drug release. Chem Eng Commun 6:1–48.

glycolide), poly(vinyl pyrrolidone), poly(vinyl alcohol), poly(hydroxyalkylmethacrylates), polyurethane, or natural hydrocolloids such as hyaluronic acid collagen and chitosan. Also synthetic polymers, such as silicone gel sheets, can be employed as swellable dressings for controlled drug delivery. Composite dressings comprising both synthetic and naturally occurring polymers have also been reported for controlled drug delivery to wound sites.[1]

By controlling the degree of swelling, crosslinking density, and degradation rate, delivery kinetics can be engineered according to the desired drug release schedule.[80] Drug release from polymeric formulations is controlled by one or more physical processes including (a) hydration of the polymer by fluids or (b) swelling to form a gel, (c) diffusion of drug through the polymer matrix and (d) eventual degradation/erosion of the polymeric system.[81,82] It seems feasible that hydration, erosion and subsequent drug diffusion kinetics will play a part in controlled drug release from these dressings when they come into contact with wound exudate. Upon contact of a dry polymeric dressing with a moist wound surface, wound exudate penetrates into the polymer matrix. This causes hydration and eventually swelling of the dressing to form a release system over the wound surface.

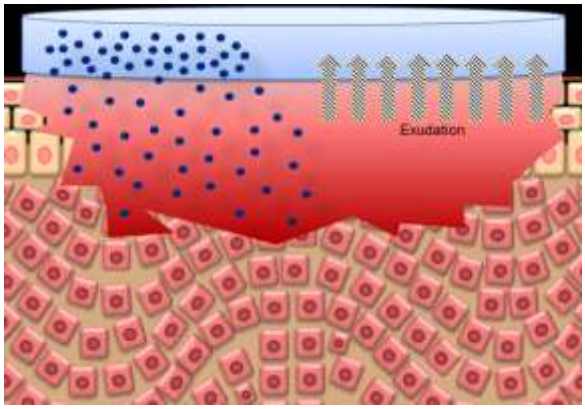

Figure 12. General scheme for the movement of exudation and drugs during wound healing

[80] Slaughter B.V., Khurshid S.S., Fisher O.Z., Khademhosseini A. *et al.* Advanced Materials. 2009
[81] Korsmeyer RW, Gurny R, Doelker E, Buri P, Peppas NA. 1983. Mechanisms of solute release from porous hydrophilic polymers. Int J Pharm 15:25–35.
[82] Martin A, Bustamante P, and Chun AHC. Diffusion and dissolution in physical pharmacy: Physico-chemical principles in the Pharmaceutical Pharmaceutical Sciences. 1993

In certain wound dressings, the mechanism for drug release has been explained by the hydrolytic activity of enzymes present in the wound exudates[83] or from bacteria in the case of infected wounds.[84]

2.3.2. Controlled drug delivery to the wound

Wound dressings can be used to deliver the bioactive molecules to the wound sites. The incorporated drugs can play an active role in the wound healing process either directly as cleansing or debriding agents for removing necrotic tissue, or indirectly as antimicrobials which prevent or treat infection or growth agents (factors) to aid tissue regeneration.

The purpose of applying antibiotics and other antibacterials is mainly to prevent or combat infections especially for diabetic foot ulcers, surgical and accident wounds where the incidence of infections can be high due to reduced resistance resulting from extreme trauma.[85] Common antibiotics incorporated into available dressings for delivery to wounds include dialkylcarbamoylchloride, povidone-iodine and silver, used with most of the modern dressings. The delivery of antibiotics to local wound sites may be a preferred option to systemic administration because doses needed to achieve sufficient systemic efficiency often results in toxic reactions. The use of dressings to deliver antibiotics to wound sites can provide tissue compatibility, low occurrence of bacterial resistance and reduced interference with wound healing process. The use of low antibiotic doses within the dressings also reduces the risk of systemic toxicity considerably. In addition, local delivery from dressings can overcome the problem of ineffective systemic antibiotic therapy resulting from poor blood circulation at the extremities in diabetic foot ulcers.

As opposite to antimicrobial agents with support function, growth factors take an active physiological role in the wound healing process. These molecules can directly stimulate fibroblasts, promote angiogenesis, and encourage cellular proliferation of keratinocytes.[1] Topical application of these growth factors to human wounds has shown some benefits in animal models and clinical trials.[86] Different dressings have been used to topically administer some of the above growth factors to wound sites. These include hydrogel dressings for

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[83] Dubose J.W., Cutshall C. and Metters A.T. J.Biomed.Mater Res.A. 2005
[84] Suzuki Y., Tanihara M., Nishimura Y., Suzuki K. *et al.* J.Biomed.Mater Res. 1998
[85] O'Meara S., Cullum N., Majid M. and Sheldon T. Health Technol.Assess. 2000
[86] Friedman A. J.Drugs Dermatol. 2011

delivering transforming growth factor-b1 (TGF-b1),[87] collagen film for delivering PDGF[88] and human growth hormone,[89] alginate dressings in the form of beads used to deliver endothelial growth factor,[90] polyurethane and collagen film dressings for delivery of EGF.[91]

Most of these growth factors are recombinant proteins and are subject to all issues related to proteins delivery (high molecular weight, short half-lives, instability, and immunogenicity).[92] A molecular genetic approach in which genetically modified cells synthesize and deliver the desired growth factor in regulated fashion has been used to overcome the limitations associated with the topical application of recombinant growth factor proteins.[93] Therapy with genes encoding for growth factor and/or cytokines used independently or with stem cells may be useful in treating wounds resistant to more traditional approaches.[94]

Another group of active compounds important to the wound healing process are vitamins and mineral supplements[95] including vitamins A, C, E as well as zinc and copper. These nutrients are mainly employed as oral supplements to improve wound healing process.

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[87] Puolakkainen P.A., Twardzik D.R., Ranchalis J.E., Pankey S.C. *et al.* J.Surg.Res. 1995
[88] Koempel J.A., Gibson S.E., O'Grady K. and Toriumi D.M. Int J.Pediatr.Otorhinolaryngol. 1998
[89] Maeda M., Kadota K., Kajihara M., Sano A. *et al.* J.Control Release. 2001
[90] Gu F., Amsden B. and Neufeld R. J.Control Release. 2004
[91] Grzybowski J., Oldak E., Antos-Bielska M., Janiak M.K. *et al.* Int J.Pharm. 1999
[92] Pisal D.S., Kosloski M.P. and Balu-Iyer S.V. J.Pharm.Sci. 2010
[93] Eming S.A., Krieg T. and Davidson J.M. J.Invest Dermatol. 2007
[94] Petrie N.C., Yao F. and Eriksson E. Surg.Clin.North Am. 2003
[95] Wallace E. Br.J.Nurs. 1994

ALGINATE DRESSINGS

3.1. Alginate overview

Alginates are generally referred to as a family of polyanionic copolymers derived from marine kelp, mainly the brown sea algae, discovered by Edward Stanford in 1883. Alginates are of growing importance in the healthcare and pharmaceutical industry. Since the first successful encapsulation of islet cells by Lim and Sun[96] (1980), alginate matrices were extensively employed as biomaterials for cell culture and transplantation. It's Their interesting chemical and physical properties resulted in many commercial applications, which led to an estimated annual production to approximately 38.000 tons worldwide.[97] The functional role of alginates in some common applications are described in several reviews [98,99], and summarized in Table 7.

Table 7. Functional role of alginates in some common applications.

Application	Function
Food and beverage industry	
Drinks	Stabilizers, thickeners
Ice-cream	Stabilizers, thickeners
Jelly	Stabilizers, thickeners
Ethanol production	Encapsulation material of yeast cells
Pharmaceutical industry	
Cell culture and transplantation	Encapsulation material
Dental impression material	Mould
Tablets	Adhesive agent, sustained-release
Wound dressing	Haemostatic and absorbent
Other industries	
Fabrics	Thickeners
Paper	Adhesive agent, filler
Paint	Stabilizer and suspending agent
Toothpaste	Stabilizers, thickeners

Commercially available alginate is typically extracted from brown algae (*Phaeophyceae*), including *Laminaria hyperborea, Laminaria digitata, Laminaria japonica, Ascophyllum*

[96] Lim F. and Sun A.M. Science. 1980
[97] Andersen T., Strand B.L., Formo K., Alsberg E. *et al.* Carbohydrate Chemistry. 2012
[98] Wee S. and Gombotz W.R. Adv.Drug Deliv.Rev. 1998
[99] Chapman V.J. and Chapman D.J. Algin and alginates. in *Seaweeds and their uses*. 1980

nodosum, and Macrocystis pyrifera[100] by treatment with aqueous alkali solutions, typically with NaOH.[101] Bacterial biosynthesis may provide alginates with more defined chemical structures and physical properties than can be obtained from seaweed-derived alginate. Bacterial alginate can be produced from *Azotobacter* and *Pseudomonas*.

Alginate is a whole family of linear copolymers containing blocks of (1,4)-linked β-d-mannuronate (M) and α-l-guluronate (G) residues. The uronic blocks are composed of consecutive G residues (GGGGGG), consecutive M residues (MMMMMM), and alternating M and G residues (GMGMGM) (figure 13). The proportion and arrangement of the uronic blocks vary with the source and are related to the physical strength and other physical properties of the alginate structure.[102]

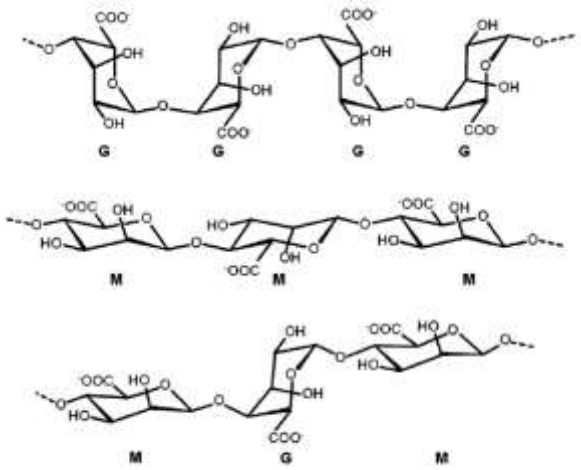

Figure 13. Chemical structure of G-block, M-block, and alternating block in alginate

The structural–property relationships of alginates are better understood, owing to extensive research and wider pharmaceutical applications in recent years. Physical properties of alginates are largely governed by the composition and arrangement of the uronate residues, molecular weight of the polymer and presence of cations that, depending on type, can cross link separate chains.[103] Alginates with a wide range of molecular weights (MWs, 50–100 000 kDa) can be dispersed in aqueous solutions giving non-Newtonian fluids, i.e. the viscosity decreases

[100] Lee K.Y. and Mooney D.J. Prog.Polym Sci. 2012
[101] . Patent n. USPatent 2036922. 1936
[102] Tonnesen H.H. and Karlsen J. Drug Dev.Ind.Pharm. 2002
[103] Martinsen A., Skjak-Braek G. and Smidsrod O. Biotechnol.Bioeng. 1989

with increasing shear rate (shear thinning). The viscosity of an alginate solution depends on the concentration of the polymer and the MW distribution.[104]

Since alginate is obtained from natural sources, various impurities such as heavy metals, endotoxins, proteins, and polyphenolic compounds could potentially be present. The immunogenic response at the injection or implantation sites might be attributed to impurities remaining in the alginate and Orive *et al.* [105] demonstrated that the alginate purified by a multi-step extraction procedure to a very high purity did not induce any significant foreign body reaction when implanted into animals. Similarly, no significant inflammatory response was observed when gels formed from commercially available, highly purified alginate have been subcutaneously injected into mice.[106] Furthermore, the immunological properties of alginates can be related to the sequential structures and molecular size of the polymer too.[107] [108] High mannuronic-containing alginate residues appeared to be involved in a receptor-mediated mechanism that stimulated and released cytokine in wounds while a high content of guluronic acid suppressed this activity.[109] Alginates with high mannuronic content were reported to show greater biocompatibility and recommended as suitable biomaterials for implants.[110] Although the biocompatibility of alginate has been extensively evaluated in vitro as well as in vivo, there is still debate regarding the impact of the alginate composition.

3.2. Alginate hydrogels

Alginate is typically used in the form of a hydrogel in biomedicine, including wound healing, drug delivery and tissue engineering applications. Hydrogels are three-dimensionally cross-linked networks composed of hydrophilic polymers with high water content. Hydrogels are often biocompatible, as they are structurally similar to the macromolecular-based components in the body, and can often be administered via minimally invasive procedures.[111] Chemical and/or physical cross-linking of hydrophilic polymers are typical approaches to form hydrogels, and their physicochemical properties are highly dependent on the cross-linking type

[104]Kong H.J., Lee K.Y. and Mooney D.J. Polymer. 2002
[105] Orive G., Ponce S., Hernandez R.M., Gascon A.R. *et al.* Biomaterials. 2002
[106] Lee J. and Lee K.Y. Pharm.Res. 2009
[107] Otterlei M., Ostgaard K., Skjak-Braek G., Smidsrod O. *et al.* J.Immunother.(1991.). 1991
[108] Espevik T., Otterlei M., Skjak-Braek G., Ryan L. *et al.* Eur.J.Immunol. 1993
[109] Otterlei M., Sundan A., Skjak-Braek G., Ryan L. *et al.* Infect.Immun. 1993
[110] Orive 2002
[111] {Sakiyama-Elbert, 2001 673 /id}

and cross-linking density, in addition to the molecular weight and chemical composition of the polymers.[112]

Alginate hydrogels can be covalently cross-linked using carbodiimide chemistry. Chemically cross-linked are more stable and have good mechanical properties, but the reagents may be toxic, and the unreacted chemicals may need to be removed thoroughly from gels. Covalent cross-linking has been widely investigated in an effort to improve the physical properties of gels for many applications, including tissue engineering. Photo cross-linking is another approach to achieve in situ gelation that exploits covalent cross-linking. Photo crosslinking can be carried out in mild reaction conditions, even in direct contact with drugs and cells, with the appropriate chemical initiators. Photo cross-linking reactions typically involve the use of a light sensitizer or the release of acid, which may be harmful to the body.

The most common method to prepare hydrogels from an aqueous alginate solution is to combine the solution with ionic cross-linking agents, such as polyvalent cations. This cations can replace and be preferentially bound to the binding sites of the residing sodium ions in the polyguluronate segments and produce a cross-linked "egg-box" model (figure x).[113,114] In general, divalent cations (Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}) are suitable crosslinking agents while monovalent cations or Mg^{2+} are not.[115] Usually, Ca^{2+} is the most commonly used divalent cations used to ionically cross-link alginate and calcium chloride ($CaCl_2$) is one of the best choices.

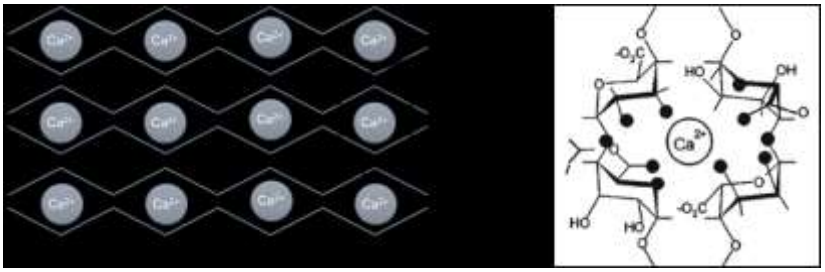

Figure 14. egg-box model

[112] Lee K.Y. and Mooney D.J. Prog.Polym Sci. 2012
[113] Rees, D. A. (1981). Polysaccharide shapes and their interactions. Some recent advances. Pure and Applied Chemistry, 53(1), 1‑14.
[114] (Gombotz & Wee, 1998;).
[115] Sutherland, L. W. (1991). Alginates. In D. Byron (Ed.), Biomaterials: Novel materials from biological sources. New York: Stockton.

The chelation at the G-residue of the alginate chains results in ionic interaction between the guluronic acid groups while the van der Waal forces between alginate segments and formation of a three-dimensional gel network[116,117]. The composition of the guluronic segments (molecular weight and M/G ratio) and hence the extent of cross-linking will largely affect the quality of the matrices formed. When gels are made from an alginate rich in guluronic acid residues, higher gels strength are obtained, which is an indicator of the mechanical stability of cross-linked matrices.[118] The proposed explanation for this behavior is that high-G gels, with their long G blocks and their short elastic segments become more of a stiff open and static network compared to the more dynamic and entangled network structure of the low-G gels with their relative long elastic segments[119] (Figure x).

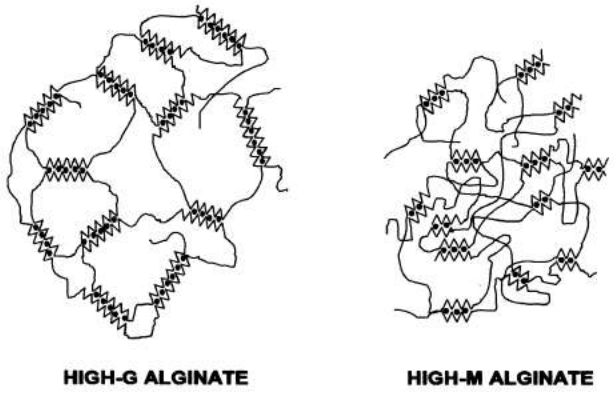

Figure 15. Proposal model for network structure in gel made from alginates with G blocks at different length. Adapted from Draget et al.[120]

In particular, the Alginate matrices made from a high content of guluronic acid tend to be rigid and brittle, while more elastic gels are produced from alginates of low α-l-guluronic acid content.[121]

[116] Gaumann et al., 2000
[117] Rees & Welsh, 1977
[118] Martinsen A., Storro I. and Skjark-Braek G. Biotechnol.Bioeng. 1992
[119] Thu B, Smidsrød O, Skja°k-Bræk, G. In: Wijffels RH, Buitelaar RM, Bucke C, Tramper J. (Eds.), Immobilized Cells; Basics and Applications Amsterdam: Elsevier Science, 1996:19.
[120] {Draget, 1997 124 /id}
[121] Goh C.H., Heng P.W.S. and Chan L.W. Carbohydrate Polymers. 2012

Cross-linking metal ions show different affinities for alginates although the extent of cross-linking may be influenced by the chemical composition of alginates.

The gelation rate is a critical factor in controlling gel uniformity and strength when using divalent cations, and slower gelation produces more uniform structures and greater mechanical integrity.[122] The gelation temperature also influences gelation rate, and the resultant mechanical properties of the gels. At lower temperatures, the reactivity of ionic cross-linkers (e.g., Ca^{2+}) is reduced, and cross-linking becomes slower.[123]

Generally, the formation of cross-linked alginate gel matrices can occur by three mechanisms, namely external gelation, internal gelation, and gelation by cooling. In the formation of large pellets or micropellets by external gelation, the drug-containing alginate solution is delivered to the cross-linking solution as extruded or atomized droplets and gel formation occurs as cross-linking cations diffuse into the alginate solution. In the internal gelation method, an insoluble calcium salt (e.g. calcium carbonate) is first added to the alginate drug solution and free calcium ions are subsequently liberated by pH adjustment.[124] For alginates formed by cooling, the alginate chains at high temperature prevented polymeric alignment and irreversibly destabilized any non-covalent intra-molecular bonding between neighboring chains.[125] Upon cooling at a lower temperature, the reestablishment of the inter-molecular bonds between the polymer chains facilitated the formation of an ordered tertiary structure and resultant homogeneous matrix.

One critical drawback of ionically cross-linked alginate gels is the limited long-term stability in physiological conditions. The ionotropic gelation of alginates is inherently a reversible process because these gels can be dissolved due to release of divalent ions into the surrounding media due to exchange reactions with monovalent cations. In addition, the calcium ions released from the gel may promote hemostasis, while the gel serves as a matrix for aggregation of platelets and erythrocytes.[126] These features may be beneficial or negative, depending on the situation.

3.3. Alginate dressings

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[122] Kuo C.K. and Ma P.X. Biomaterials. 2001.
[123] Augst A.D., Kong H.J. and Mooney D.J. Macromol.Biosci. 2006.
[124] {Chan, 2006 675 /id}
[125] {Papageorgiou, 1994 674 /id}.
[126] {Suzuki, 1998 676 /id}

The ability of calcium ions to form crosslinks with alginates makes calcium alginate dressings ideal materials as scaffolds for tissue engineering.[127] Alginate dressings occur either in the form of freeze-dried porous sheets (foams) or as flexible fibres, the latter indicated for packing cavity wounds. The use of alginates as dressings stems primarily from their ability to form gels upon contact with wound exudates (high absorbency). The high absorption occurs via strong hydrophilic gel formation, which limits wound secretions and minimizes bacterial contamination.[1] When applied to wounds, ions present in the alginate fibers are exchanged with those present in exudate and blood to form a protective film of gel. This helps to maintain the lesion at an optimum moisture content and healing temperature. Also in this case the composition of alginate gives different properties to the dressing: alginates rich in mannuronate, such as Sorbsan™ (Maersk, UK) form soft flexible gels upon hydration whereas those rich in guluronic acid, like Kaltostat™ (ConvaTec, USA), form firmer gels upon absorbing wound exudate.

Currently, the use of alginates in wound care relies largely on the clinical knowledge that alginates promote healing through a moist environment. The wide acceptance of alginates in wound healing is also related to the positive clinical advantages shown in various studies. A prospective, randomized, controlled trial involving patients with full-thickness pressure ulcers reported better clinical outcome using alginate wound dressing when compared to topical treatment with a dextranomer paste.[128] Treatments with calcium alginate dressings had shown good healing outcomes in various types of skin wounds[129,130,131,132,133,134,135,136] and animal model.[137]

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[127] Kuen Yong Leea, b, David J. Mooneya,∗ Alginate: Properties and biomedical applications
[128] Sayag J., Meaume S. and Bohbot S. J.Wound Care. 1996
[129] Attwood A.I. Br.J.Plast.Surg. 1989
[130] Bale S., Baker N., Crook H., Rayman A. *et al.* J.Wound Care. 2001
[131] Gilchrist T. and Martin A.M. Biomaterials. 1983
[132] Fraser R. and Gilchrist T. Biomaterials. 1983
[133] Gilchrist, T., Mitchell, D. C.,&Burrows, T. R. (1985). In S. R. T. D. Turner,&K.G. Harding (Eds.), Advances in wound management (p. 73). London: Wiley.
[134] Kneafsey B., O'Shaughnessy M. and Condon K.C. Burns. 1996
[135] Lalau J.D., Bresson R., Charpentier P., Coliche V. *et al.* Diabetes Metab. 2002
[136] Lim T.C. and Tan W.T. Br.J.Plast.Surg. 1992
[137] Agren, M. S. (1996). Four alginate dressings in the treatment of partial thickness wounds: A comparative experimental study. British Journal of Plastic Surgery, 49(2), 129–134.

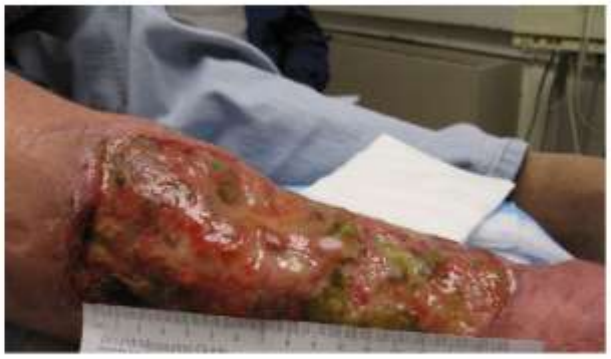

FIG. 1. Chronic leg ulcer with significant exudate before use of alginate dressing.

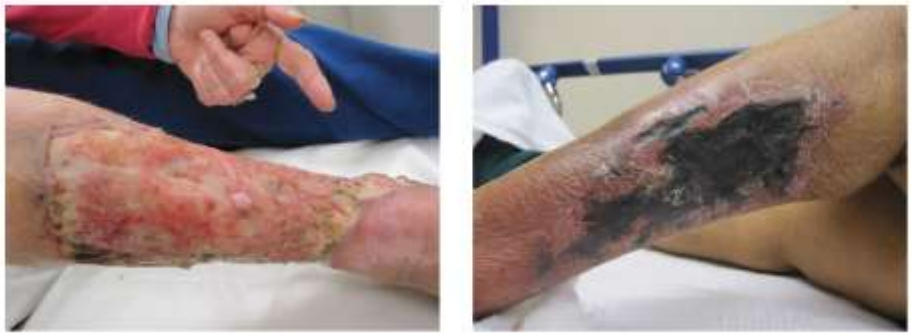

Figure 16. Use of different alginate dressing in wound healing

3.3.1. Role of calcium and zinc in wound healing

Alginate dressings, as well as derived gels, have a pharmacological function in all the wound healing phases, due to the action of the calcium ions present inside the dressing.[138] Those ions, when released into the wound, play a physiological role aiding in the clotting mechanism (hemostat) during the first stage of wound healing.[139,140,141] Furthermore calcium alginate shows also a direct action on cellular mechanism of wound healing. Schmidt and Turner[142] and Doyle

[138] Lansdown AB. 2002. Calcium: A potential central regulator in wound healing in the skin. Wound Repair Regen 10:271–285.
[139] Jarvis PM, Galvin DAJ, Blair SD. 1987. How does calcium alginate achieve haemostasis in surgery? Proceedings of the 11th Congress on Thrombosis and Haemostasis, 58: 50.
[140] Collins F, Hampton S, White R. 2002. A-Z Dictionary of Wound Care. Dinton, Wiltshire: Mark Allen Publishing Ltd.
[141] Blair SD, Jarvis P, Salmon M, McCollum C. 1990. Clinical trial of calcium alginate haemostatic swabs. Br J Surg 77:568–570.
[142] Schmidt RJ, Turner TD. 1986. Calcium alginate dressings. Pharm J 236:578.

et al.[143] showed that calcium alginate increased proliferation of mouse fibroblasts but not their motility in vitro and Thomas *et al.*[144] have reported that some alginate dressings activate human macrophages to produce tumor necrosis factor-α (TNFα) which initiates inflammatory signals, as part of the wound healing process. Zinc-containing alginate dressings were also shown to possess greater potentiating effect on prothrombotic coagulation and platelet activation than calcium.[145] This may be related to the role of zinc ions as a cofactor for the coagulation factor XII and the intrinsic pathway of coagulation. Further support was found in the study by Kowalska *et al.*[146], reporting enhanced zinc ion aggregation to adenosine diphosphate (ADP) during wound healing. These evidences suggest that the effects of the dressing may have been mediated by ions released from the alginate and therefore calcium or zinc alginate may improve some cellular aspects of wound healing.

Calcium alginate dressing have been claimed to have antimicrobial properties in wound management, but clinical studies showed no evidence to support this use.[147] Divalent cations such as Cu^{2+} and Zn^{2+} were found to exhibit higher anti-microbial activities than Ca^{2+} against the skin pathogens *S. aureus* and *P. aeruginosa* and were generally compatible with common topical anti-microbial agents[148].

3.3.2. Clinical use of alginate dressings

Since the first clinical use by Major George Blaine in the 1940s,[149] many useful applications of alginates are included in wound care management. Alginates can be made into various forms such as sponges, films and extrudates (woven or nonwoven dressing) depending on the process of cross-linking and several commercial products of alginate-based dressings are available. Alginate dressings are useful for moderate to heavily exuding wounds because they can absorb large volumes of exudates while providing an adequately moist environment for

[143] Doyle JW, Roth T, Smith M. 1996. Effects of calcium alginate on cellular wound healing processes modelled in vitro. J Biomed Mater Res 32:561–568.
[144] Thomas A, Harding KG, Moore K. 2000. Alginates from wound dressings activate human macrophages to secrete tumour necrosis factor-a. Biomaterials 21:1797–1802.
[145] Segal H.C., Hunt B.J. and Gilding K. J.Biomater.Appl. 1998
[146] Kowalska M.A., Juliano D., Trybulec M., Lu W. *et al.* J.Lab Clin.Med. 1994
[147] Lansdown AB. 2002. Calcium: A potential..
[148] Goh C.H., Heng P.W.S., Huang E.P.E., Li B.K.H. et al. Journal of Antimicrobial Chemotherapy. 2008
[149] BLAINE G. Ann.Surg. 1947

wound healing.[150] The absorbent ability of the dressing can be affected by the constitution of the fibers, relative proportions of mannuronic and guluronic acid residues, and content of calcium and sodium ions.[151] Dressings with high mannuronate content are capable of greater uptake of moisture but the fibers are characteristically weaker.[152] Since alginate dressings require moisture to function effectively, they cannot be used for dry wounds and those covered with hard necrotic tissue. This is because it could dehydrate the wound, delaying healing, which represents a major disadvantage.[1]

One important consideration of a wound dressing will be its mechanical integrity. The dressing should be sufficiently strong during the intended use. It was reported that the composition of the alginates (mannuronate to guluronate or M/G ratio) influenced the physical attributes of the fibers formed[153]. However, alginate dressings in the form of fibers when trapped in a wound are readily biodegraded and can be rinsed away with saline irrigation. Subsequent removal therefore, does not destroy granulation tissue, making dressing change virtually painless.

The present interest in alginates for wound care management involves an understanding of the advantages of alginates and the strategies are largely targeted at improving their functionality as a wound healing promoter. Moreover with the advent of tissue engineering, extensive work has been done to integrate wound healing components/cells with biocompatible polymers to provide an environment capable of sustaining viable cells for tissue repair[154].

[150] Thomas, A., Harding, K. G., & Moore, K. (2000). Alginates from wound dressings activate human macrophages to secrete tumor necrosis factor-. Biomaterials, 21(17), 1797–1802.
[151] Walker M., Hobot J.A., Newman G.R. and Bowler P.G. Biomaterials. 2003
[152] Miraftab M., Qiao Q., Kennedy J.F., Anand S.C. *et al.* Carbohydrate Polymers. 2003
[153] Miraftab, (2003)
[154] Goh 2012

Table 8. Alginate dressing currently available on the market in Italy

Commercial name	Composition	Therapeutic indication	Contraindications	Interactions
ALGISITE M	Calcium alginate fiber dressing	Partial and total thickness lesions with moderate to high exudate, and tending to minor bleeding, such as: sores, ulcers of the lower limbs, surgical wounds, diabetic ulcers, cancer lesions , burns.	Known allergy to alginate dressings. Hemostasis in profusely bleeding wounds	Unknown interactions
ALGOSTERIL	Calcium alginate fiber dressing	Wounds with medium / high exudate: chronic wounds (leg ulcers, pressure ulcers, diabetic foot), deep wounds, bleeding wounds, infected and desquamating wounds.	Unknown controindications	Unknown interactions
CURASORB	Calcium alginate dressing	Venous ans arterial stasis ulcers, sores, diabetc ulcers, lacerations, abrasions, surgical incisions, second degree burns and other external wounds with moderate exudate. Can be used on infected wounds only if replaced at least once a day.	Do not use on third degree burns and heavily bleeding injuries.	Unknown interactions
KALTOSTAT	Calcium sodium alginate dressing	Moderately to highly exuding chronic and acute wounds, and for wounds with minor bleeding. Exuding wounds such as diabetic ulcers, venous stasis ulcers, bedsores, skin donor sites, superficial wounds and abrasions, superficial burns, cancer wounds	Known allergy to alginate dressings or one of their component. Third degree burns, heavily bleeding injuries. Small exudating injuries	Unknown interactions
MELGISORB	Calcium sodium alginate dressing	Moderately to highly exuding chronic and acute wounds. Exuding wounds such as diabetic ulcers, venous stasis ulcers, bedsores, Superficial wounds and abrasions, skin donor sites, superficial burnsand other acute skin lesions	Do not use on dry wound and Third degree burns	Unknown interactions
SEASORB	Calcium sodium alginate and carboxymethylcell ulose dressing	Indicated for the management of moderate to heavily exuding wounds such as pressure ulcers, leg ulcers, diabetic ulcers, second-degree burns, grafts, skin donor sites or traumatic wounds. Local hemostatic effect	Do not use on dry wound with no exudate. Inaffective for bleeding management of surgical wound	Unknown interactions
SUPRASORB A	Calcium sodium alginate dressing	Indicated for the management of moderate to heavily exuding wounds.	Unknown controindications	Unknown interactions
TEGADERM ALGINATE	Calcium alginate fiber dressing	Partial and total thickness lesions with moderate to high exudate, and tending to minor bleeding.	Do not use on dry wound, surgical implants and Third degree burns	Unknown interactions
ASKINA SORB	Calcium alginate and hydrocolloyd	Indicated for the management of moderate to heavily exuding	Do not use on surgical implants and Third	Topical treatment with antimicrobial

	(carboxymethilcell ulose) dressing	wounds, also in presence of infection. Exuding wounds such as diabetic ulcers, venous stasis ulcers, bedsores, skin donor sites, superficial wounds and abrasions, superficial burns, cancer wounds	degree burns	or antiseptics. In case of anaerobial bacteria it shouldn't use with an occlusive dressing
CURASORB ZN	Calcium-zinc alginate dressing	Use as secondary dressing in case of external wounds such as diabetic ulcers, venous stasis ulcers, bedsores, or similar wounds.	Do not use on third degree burns and heavily bleeding injuries.	Unknown interactions
FARMACTIVE ALGINATO	Calcium alginate and carboxymethilcellu lose dressing	Not available	Not available	Not available
HYALOGRAN	Calcium alginate and Hyaff (hyaluronic acid ester)	Necrotic lower limb Necrotic ulcers, surgical wounds and bedsores. Useful in wound with diffused necrotic tissue	Known allergy to alginate dressings. In presence of infection the dressing should be daily changed	Unknown interactions
SORBALGON SORBALGON T	Calcium alginate and polysorbate	Indicated for the management of moderate to heavily exuding wounds or bleeding wounds, such as pressure ulcers, leg ulcers, diabetic ulcers, severe burns, grafts, extended surgical wounds	Unknown controindications	Unknown interactions
SUPRASORB A+AG	Calcium alginate and silver	Indicated for infected and heavily exuding wounds, such as pressure ulcers, leg ulcers, diabetic ulcers, severe burns, extended surgical wounds.	Known allergy to alginate dressings.	Avoid contact with conductive gel or electrodes during ECG, EEG measurements Not use in patients subject to Magnetic Resonance.
URGOSORB	Calcium alginate and hydrocolloyd (carboxymethilcell ulose) dressing	Indicated for the management of moderate to heavily exuding wounds, during the wound cleaning and the granulation phase	Used as nasal swabs. Use with caution on poorly exuding wounds, third-degree burns and in leg ulcers	Unknown interactions

CHAPTER II

ALGINATE – HYALURONIC ACID HYBRID HYDROGELS AD SPONGES FOR WOUND HEALING

Introduction

Wound repair of is one of the most complex biological processes that occur during human life. After an injury occurs, various intracellular and intercellular pathways need to be activated and coordinated if tissue integrity and homeostasis are to be restored.[155] Dressings have been applied to open wounds for centuries to prevent wound from further injury and bacteria invasion, but nowadays their design has evolved toward multifunctionality, that is to better control potential infection and to aid healing process.[156] A key property of modern dressings is their ability to retain and create a moist environment around the wound which is expected to facilitate healing. Amid modern dressings, hydrogels cover a large area due to their well-recognized compatibility.[1] Above their glass transition temperature, hydrogels are typically soft and elastic due to their thermodynamic compatibility with water and useful in many different biomedical applications.[157,158] Hydrogels have shown excellent potential in a variety of biomedical applications, including scaffolds for tissue engineering or carriers for drug delivery systems.[159]

Alginates include a whole family of water-soluble polysaccharides extracted from brown seaweed. ALGs are linear copolymers containing blocks of (1,4)-linked β-d-mannuronate (M) and α-l-guluronate (G) residues. These blocks are composed of consecutive G residues (GGGGGG), consecutive M residues (MMMMMM), and alternating M and G residues (GMGMGM). The proportional and sequential arrangements of the M and G residues depend upon the type of algae tissue from which the polysaccharide is extracted, as well as on the season in which the algae is collected and more than 200 different ALGs are currently being manufactured.[160] Due to its biocompatibility and bioresorption properties, ALGs has been

[155] Gurtner G.C., Werner S., Barrandon Y. and Longaker M.T. Nature. 2008
[156] Queen D., Orsted H., Sanada H. and Sussman G. Int Wound J. 2004
[157] Peppas N.A., Huang Y., Torres-Lugo M., Ward J.H. *et al.* Annu.Rev.Biomed.Eng. 2000
[158] Slaughter B.V., Khurshid S.S., Fisher O.Z., Khademhosseini A. *et al.* Advanced Materials. 2009
[159] Lee K.Y. and Mooney D.J. Prog.Polym Sci. 2012
[160] Tonnesen H.H. and Karlsen J. Drug Dev.Ind.Pharm. 2002

widely used in regenerative medicine.[161] ALG dressings offer a series of advantages and for this reasons they have been successfully applied to cleanse a wide variety of secreting lesions. The high water absorption occurs via strong hydrophilic gel formation, which limits wound secretions and minimizes bacterial contamination.[162] Furthermore, ALG dressings maintain a physiologically moist microenvironment that promotes healing and formation of granulation tissue. ALGs can be rinsed away from wound bed with saline irrigation, so removal of the dressing does not interfere with healing granulation tissue. This makes dressing changes virtually painless. Another important aspect of the wound healing process is related to wound exudate management, particularly with respect to controlling maceration of peripheral tissue. ALG dressings can be designed to absorb large volumes of exudate, whilst continuing to provide a moist wound healing environment and thus they well adapt to management of moderate to heavily exudating wounds.[163] ALG-based absorbent wound dressing may be used on multiple wound types, including but not limited to diabetic wounds, venous wounds, pressure ulcers, cavity wounds, and some bleeding wounds.

Ionically cross-linked ALG hydrogels can be formed in the presence of several divalent cations such as Ca^{2+}, Ba^{2+}, Sr^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, and Co^{2+}.[164] Divalent cations allow interchain interactions between G-rich regions of one polymer chain with the G-rich regions of adjacent polymer chains in what is termed the 'egg-box' model of cross-linking resulting in hydrogel formation.[165] Soluble calcium salts are widely employed to cross-link ALGs (in solution or solid). These methods typically leads to rapid and inadequately controlled gelation that results in the formation of hydrogels with scarce structural homogeneity and poor mechanical properties.[166,167] To overcome this drawback, internal gelation of ALG through $CaCO_3$-GDL (D-glucono-δ-lactone) system has been attempted and found very useful to obtain homogenous hydrogel useful as scaffolding materials for wound healing applications. The kinetics of the gelation process has been studied extensively[168] and it has been found to be affected by alginate concentration, composition, and particle size of the calcium salts.

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[163] Motta G.J. Ostomy Wound Manage. 1989
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[168] Draget K.I., Ostgaard K. and Smidsrod O. Carbohydrate Polymers. 1990

Hyaluronan (HA) is a nonsulfated, linear glycosaminoglycan (GAG), consisting of repeating units of (b, 1–4) glucuronic acid-(b, 1-3)-N-acetyl glucosamine. HA is present in most living tissues as a high molecular mass polymer (> 100 kDa) and in significant amounts in the skin (dermis and epidermis), brain, and central nervous system. Toole B.P. Nat.Rev.Cancer. 2004 HA has a crucial role in tissue repair, including wound healing.[169] Several studies have shown that exogenous HA exerts beneficial effects on the wound-healing process. Topically applied HA has been shown to accelerate skin wound healing in rats[170] and hamsters[171]. HA may also play a role in the control of angiogenesis during tissue repair.[172] In addition, it has been proposed that HA may protect granulation tissue from oxygen free radical damage by its ability to scavenge reactive oxygen species (ROS),and that it could reduce the deleterious effect of oxygen free radical on wound healing.[173] HA and its fragments may play crucial roles in the skin wound-healing process, by modulating the expression of fibroblast genes involved in remodeling and repair of extracellular matrix (ECM).[174] However, the detailed mechanisms of how HA exerts its biological effects is far to be elucidated.

The main objective of this study was to develop a ALG/HA hydrogel with functional properties with potential use as wound dressing. Internal gelation technique was used for cross-linking ALG/HA solutions. Hydrogels were shaped as discs and characterized as hydrated system or dry system. Finally, biocompatibility and wound healing properties were evaluate on human adipocyte-derived Mesenchimal Stem Cells.

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[173] Trabucchi E., Pallotta S., Morini M., Corsi F. *et al.* Int J.Tissue React. 2002
[174] David-Raoudi M., Tranchepain F., Deschrevel B., Vincent J.C. *et al.* Wound Repair Regen. 2008

Materials and methods

2.1. Materials

Sodium alginate (ALG) (viscosity 360cps) was purchased from Farmalabor (Italy). Analysis-grade ethanol was purchased from Carlo Erba (Italy). Hyaluronic acid (HA) sodium salt from *Streptococcus equi* (1.5-1.8 * 10^6 Da), tranexamic acid (TA), calcium carbonate ($CaCO_3$), calcium chloride dihydrate ($CaCl_2$ * $2H_2O$), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), D-glucono-δ-lactone (GDL) and all reagents used for sulforhodamine assay were obtained from Sigma-Aldrich (St. Louis, MO). Media, sera, and antibiotics for cell cultures were from Lonza (Lonza Group Ltd, Basel, Switzerland). The water used throughout this study was depurated and filtered (Millvi Q filter).

2.2. Determination of the chemical composition of Alginate by ^{1}H NMR spectroscopy

The chemical composition of ALG, the M/G ratio and the sequence of the blocks along the polymeric chain determine its functional properties, including the solubility, viscosity and gelification.[175,176] The chemical composition of ALG was assessed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The ^{1}H NMR spectra of ALG in solution were recorded on a NMR spectra were recorded on a Varian INOVA 700 MHz spectrometer (Varian inc., USA) equipped with a z-gradient 5mm triple-resonance probe head. All the spectra were recorded at a temperature of 25 and 45 °C. The spectra were calibrated relative to TSP (0.00 ppm) as internal standard. Prior to analysis, the molecular weight and the viscosity of the ALG were reduced by a mild partial acid hydrolysis, using 1 M HCl solution, according to the ASTM Standard F 2259.[177]

2.3. ALG/HA hydrogel preparation

Structurally homogeneous calcium Alginate gels were made as previously described by Kuo and Ma.[178] Briefly ALG (1% for hydrogels and 2% for sponges w/v) was dissolved in

[175] Pereira R., Carvalho A., Vaz D.C., Gil M.H. *et al.* Int J.Biol.Macromol. 2013
[176] Salomonsen T., Jensen H.M., Larsen F.H., Steuernagel S. *et al.* Food Hydrocolloids. 2009
[177] American Society for Testing and Materials. 2003
[178] Kuo C.K. and Ma P.X. Biomaterials. 2001

deionized water and mixed with calcium carbonate ($CaCO_3$) 30mM to form a suspension. A fresh aqueous 64 mM GDL solution was then added to the suspension and vortexed to initiate gelation. A $CaCO_3$ to GDL molar ratio of 0.5 was maintained to achieve a neutral pH post-crosslinking. The HA was dissolved directly in the alginate solution to obtain the concentrations of 10% and 20% w/w. The solutions were cast in 96 well plate (50 µl, well size: 5 mm diameter, 20 mm height) to form circular discs 2 mm in thickness and 5 mm in diameter or in a 24-well plate (1 ml, well size: 15mm diameter, 20mm height) to form circular discs 5 mm in thickness and 15 mm in diameter, depending on use. The well plate were capped, sealed with Parafilm®, and gelled on a leveled plate at room temperature for 24 h. After gelation the ALG disc were washed with water and stored at 4°C.

2.4. ALG/HA sponge preparation

To obtain a structurally homogenous ALG/HA sponge with useful properties for drug delivery, was prepared a 2% w/v ALG solution and gelled with the previously described method in a 24-well plate. This hydrogels was frozen overnight at −40°C, and then lyophilized at 0.01 atm and -60°C in a Modulyo apparatus (Edwards, UK). After lyophilization the ALG sponge were stored at room temperature under vacuum.

To obtain TA loaded Alg/HA sponges, 10 mg of TA were dissolved in ALG solution for each well.

2.5. Hydrogel characterization

The viscosity of ALG/HA colloidal dispersions was evaluated using a Brookfield viscometer model DV8+pro using the impeller 61. The rotation speed used for this experiment was 3 RPM.

Homogeneity of the cylindrical ALG gels was characterized with dry to wet weight ratios. Gels were cut perpendicular to the cylinder axis into 4 slices with approximately the same thickness. The slices were labeled 1-4 from top to bottom. After measuring their wet weights, the slices were dried at 45° C for 48 h. The specimens were weighed again after drying, and their dry/wet weight ratios were calculated. The averages and the standard deviations of triplets were reported.

Gelation time was assessed using a method adapted from Kuo and Ma.[179] Gelation time was defined as the time between the addition of GDL and the formation of the gel. Briefly the sodium ALG solution was added to $CaCO_3$ suspension in H_2O, mixed and vortexed, and transferred into vials for each sample. The suspensions were allowed to equilibrate to the room temperature for 45 min. A fresh aqueous GDL solution were subsequently added to each sample. The final suspension was vortexed for 20 s. The gel was said to be formed when the sample no longer flowed when tilted at an angle of 45° for longer than 30 s The averages and the standard deviations of triplets were reported.

Cross-linking degree of the ALG hydrogels was evaluated by soaking the hydrogel gel disc was in 15 mL of 1, 3 and 5 mM solution of $CaCl_2*2H_2O$ to varying calcium ion concentrations. The swelling experiments were carried out at 37° C in high humidity by keeping the samples in a thermostatic bath under gentle shaking. The medium was changed every day. At the scheduled times, the specimens were retrieved and hydrogel surfaces were quickly blotted twice on a filter paper. The specimens were weighed on an analytical balance accurate to 10^{-4} g. The initial wet weight (W_0) was obtained after 24 h gelation. Wet weight (W) during immersion experiment was recorded at designated times. The swelling ratio was defined as W/W_0. Samples were tested in triplets. Averages and standard deviations are reported.

2.6. Sponge characterization

The bulk morphology of the ALG/HA sponge was analyzed through scanning electron microscopy (SEM). The samples were stuck on a metal stub and coated with gold under vacuum evaporator for 90–120 s. Images were obtained using Quanta 200 FEG (FEI, USA).

The average porosity of ALG/HA sponge was determined by a fluid replacement method.[180] Ethanol was chosen as the displacement liquid because it penetrates easily into the pores and did not induce shrinkage or swelling. The geometrical volume (V_s) of the sponge samples was calculated by measuring diameter and height, and the pore volume (V_p) was measured by ethanol displacement method. The dry sponge was weighed (W_0) and immersed in absolute ethanol at room temperature, and then placed in a degasser for 5 min to remove air bubbles from the sponge pores. After wiping gently with a filter paper to remove surface

[179] Kuo C.K. and Ma P.X. Biomaterials. 2001
[180] Aziz Z., Abu S.F. and Chong N.J. Burns. 2012

ethanol, samples were weighed immediately (We). The porosity of the sponge was calculated according to the following equation:

$$P = \frac{V_p}{V_s} X\ 100 = \frac{W_e - W_0}{\rho_e V_s} X\ 100$$

where ρ_e represents the density of ethanol (0.789 g/cm^3). An average value of five replicates for each sample was taken.

The density (ρ) of the sponge was calculated according to the following equation:

$$\rho = \frac{W_0}{V_s}$$

Hydration properties of sponges have been evaluated by means of modified Enslin apparatus employing Phosphate Buffer Saline (PBS, NaCl 120 mM, KCl 2.7 mM, Na_2HPO_4 10 mM) at pH 7.4[181] as hydration medium. PBS was used as hydration medium to simulate biological fluids in tissue injuries. Three replicates were performed for each sample.

The *in vitro* release profile of tranexamic acid (TA) from ALG and ALG/HA sponge matrix was evaluated in Phosphate Buffer Saline (PBS, NaCl 120 mM, KCl 2.7 mM, Na_2HPO_4 10 mM) at pH 7.4. TA loaded sponge were incubated in 10 ml of PBS and placed in a thermostatic bath at 37 C. At scheduled time intervals, the release medium was withdrawn and replaced with the same volume of fresh filtered medium. The supernatant was analyzed for TA content by an indirect method using a UV-vis spectrophotometer after derivatization with fluorescamine.[182] Briefly, TA samples were diluted 1:2 by volume in a fluorescamine solution at 0.5% (w/v) in ethanol and incubated in the dark at room temperature for 1 hour before analysis. The absorbance (ABS) of TA samples was measured at 390 nm using a Shimadzu 1800 spectrophotometer (Shimadzu, Japan) fitted out with a 1 cm quartz cell (Hellma, Germany). The linearity of the response was verified over the concentration range 5–100 μg/ml ($r^2 \geq 0.999$).

To measure the water retention rate, sponge sample soaked in water for 30 min then carefully removed and placed in a centrifuge tube. The sponge was centrifuged at 3500 rpm for

[181] Rossi S., Marciello M., Sandri G., Ferrari F. *et al.* Pharm.Dev.Technol. 2007
[182] El-Aroud K.A., Abushoffa A.M. and Abdellatef H.E. Chem Pharm.Bull.(Tokyo). 2007

3 min to eliminate the excess of water, and the wet weight was recorded. Water retention rate (WR) was calculated with the following formula:

$$WR = \frac{M_h - M_D}{M_d} X\ 100$$

Where M_h (g) is the weight of the sponge after centrifugation, and M_d (g) is the initial dry weight. An average value of five replicates for each sample was taken.

2.7. Water uptake of hydrogels and sponges

Water uptake was determined by placing the cross-linked freeze-dried sponges in water. Three circular samples of 15 mm diameter were obtained from a 24 well plate. The initial weight of each sample was recorded using an analytical balance. The samples were placed in 5 mL of water at a temperature of 37 °C maintained by a water bath. Samples were taken out and excess water was removed using tissue paper and after having been weighed were re-immersed in water. The samples weight was recorded at intervals of 1h up to 6h and every 24 h from there onwards until equilibrium was established. Water was replaced after every weight. The swelling ratio per cent (*SR* %) at each time point was calculated by the equation

$$SR\% = \frac{W - W_0}{W_0} \times 100$$

where *W* is the mass of the swollen sample and W_0 is the mass of the initial dry sample. The equilibrium water content (EWC) per cent was calculated by the equation:

$$\text{EWC}\ (\%) = \frac{W_e - W_d}{W_e} \times 100$$

where W_e is the mass of the swollen sample at equilibrium and W_d is the mass of the dry sample at equilibrium.

2.8. In vitro toxicity of hydrogels: Human adipose tissue-derived mesenchymal stem cell culture and viability.

Human adipose tissue biopsies were digested with collagenase and Mesenchymal Stem Cell (Ad-MSC) were isolated as previously reported.[183] Ad-MSC were cultured at 37°C with Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1) with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cultures were maintained in humidified atmosphere of 95% air and 5% CO_2 at 37° C.

Ad-MSC viability was assessed using 0.4-μm pore polycarbonate membranes (Costar, Cambridge, MA). Cells were loaded at 50,000 cells per insert in the lower chamber. The following day, the hydrogels were added to the upper chamber in DMEM F12 (1:1) 10% FBS. Upon 24h, sulforhodamine assay was used for determination of cell viability.[184] Briefly, the upper chamber was removed and the cells were fixed with 50% trichloroacetic acid for at least 2 h at 4 C. Then, cells were washed 5 times with distilled and de-ionized water. After air drying, cells were stained for 30 min with 100 μl 0.4% sulforhodamine dissolved in 1% acetic acid. Unbound dye was removed by five washes with 1% acetic acid. After air drying, 10 mM Tris solution (pH 7.5) was added to dissolve the protein-bound dye. Cell survival was assessed by optical density (OD) determination at 510 nm using a microplate reader. Three replicate wells were used for each data point, and the experiment was performed three times.

2.9. Cell motility - Wound healing Assay

Ad-MSC were seeded into six-well microplates and grown in a complete medium to a confluent monolayer. Then, the cells were wounded by manually scratching with a p20 pipette tip. Reference points near the "scratch" were marked to guarantee the same area of image acquisition. The wells were washed twice with phosphate buffered saline (PBS) and incubated at 37 C with hydrogels. Images of wound gap were taken at 0 and 24 h by a digital camera coupled to the microscope and percentage of closure was calculated with NIH IMAGE J. These experiments were repeated at least three times.

[183] D'Esposito V., Passaretti F., Hammarstedt A., Liguoro D. *et al.* Diabetologia. 2012
[184] Chiba K., Kawakami K. and Tohyama K. Toxicol.In Vitro. 1998

Results and discussion

3.1. Characterization of Alginate

The chemical composition of ALG, which can vary with the material batch and producer, can greatly influence its properties. Indeed, the affinity of certain ions with the G units, the M/G ratio and the number of repeated sequential G and M units strongly affect the gelation process and, in turn, hydrogel physical structure (strength and porosity).. The analysis of the chemical composition of the raw ALG powder used, allowed to quantify the M/G ratio and the sequence of the blocks along the polymeric chain, which influence the properties of the resulting hydrogels. M/G ratio and the block structure were determined by ^{1}H NMR spectroscopy following the method outlined by Torres *et al.*[185] It was found that the M and G fractions were 0.61 and 0.39,respectively with a M/G ratio of 1.54, a value corresponding reasonably well with those previously reported for ALG produced by *Macrocystis pyrifera*.[186] Although it has been shown that ALG with a high G content demonstrate a much higher *in vivo* stability[187], ALGs with high mannuronic content were reported to show greater biocompatibility and recommended as suitable biomaterials for implants.[188] To improve the mechanical properties of ALG, an internal gelation technique was selected since it allows formation of strong and homogeneous hydrogels despite a low G fraction. Draget *et al.*[189] have developed as first a method to obtain uniform ALG hydrogel controlling the release of Ca^{2+} directly in the ALG solution. This slow gelation process leaded to a very homogeneous hydrogels with uniform pore size and distribution and consequent well-controlled material properties. Furthermore, the ALG-calcium mixture can be poured into molds before gelation is complete and suitably shaped.

3.2. Preparation and characterization of ALG and ALG/HA hydrogels

Control over the material properties of a hydrogel is crucial for many biomedical applications. Structural uniformity is necessary not only for uniform drugs distribution, but also for consistently well-controlled material properties. Covalent crosslinking is an elective way to

[185] Torres M.R., Sousa A.P., Silva Filho E.A., Melo D.F. *et al.* Carbohydr Res. 2007
[186] Moe S.T., Draget K.I., Skjåk-Bræk G., and Smidsrød O. Alginate in Food Polysaccharides and Their Applications. 1995
[187] Kong H J, Alsberg E, Kaigler D, Lee K Y *et al.* Adv.Mater. 2004
[188] Orive G., Ponce S., Hernandez R.M., Gascon A.R. *et al.* Biomaterials. 2002
[189] Draget K.I., Ostgaard K. and Smidsrod O. Carbohydrate Polymers. 1990

stabilize three dimensional polymer networks for a variety of applications and has also been used in hydrogel formation with permanent 3-D structures. However, covalent crosslinking reagents are usually toxic and not suitable in biomedical application. In this work we have used $CaCO_3$-GDL system to produce strong, uniform, transparent, and three-dimensionally defined ALG gels (Figure 1a). This method is based on the use of an inactivate form of calcium ($CaCO_3$) that is mixed with alginate solution. The controlled release throughout the alginate solution of the crosslinking ion is usually obtained by means of a decrease of pH caused by the addiction of a slowly hydrolyzing molecule such as GDL. The use of $CaCO_3$ and GDL in a molar ratio of 2 guarantees only slightly acidic pH in the final hydrogel.[190] By adding a supplementary freeze-drying step, ALG-based sponges were obtained (Figure 1b). The presence of HA did not affect the macroscopic appearance of ALG/HA sponge.

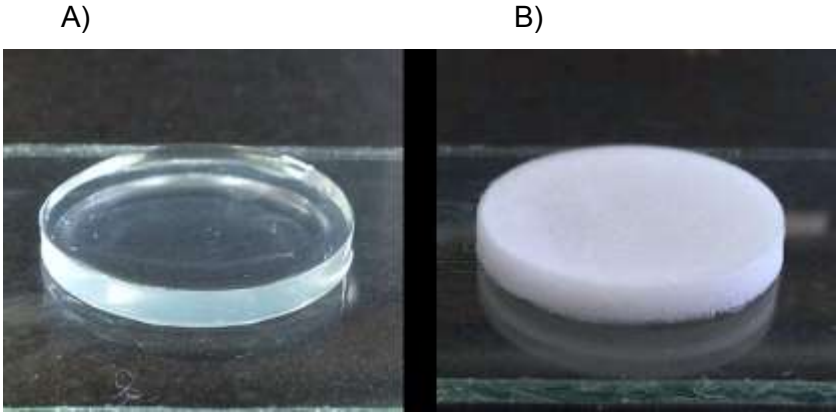

Figure 17. ALG/HA 10 hydrogel (a) obtained by internal gelation and ALG/HA 10 sponge (b) obtained by internal gelation/freeze-drying.

One of the aims of the present study was to formulate hydrogels which would remain intact after hydration and with sufficient strength to be removed from, wound bed. The hydrogels showed sufficient strength to be handled, cut with scissors or packed without difficulties.

Gelation time is an important variable in hydrogel formulation, as previous studies have shown that a fast gelation time can lead to a poorly homogeneous gel structure.[191] Gelation time

[190] Draget K.I., Ostgaard K. and Smidsrod O. Applied Microbiology and Biotechnology. 1989
[191] Kuo C.K. and Ma P.X. Biomaterials. 2001

may depend on the availability of ALG chains for crosslinking with calcium ions, which can be impaired by the presence of HA in the medium.

Gelation time was found to be dependent on the presence of HA in solution (figure 2) In fact gelation time increased with increasing HA content, which also progressively increased the viscosity of the medium.

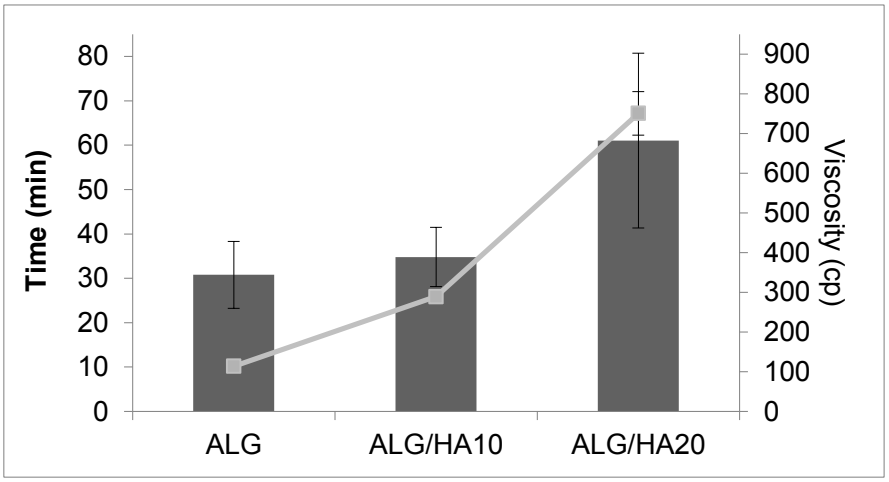

Figure 18. Gelation time and viscosity of ALG solution with or without HA.

Viscosity increase could be directly related to the gelation time as the ALG chains having less free movement, due to the increased viscosity of the solution, interact with each other more slowly to form "egg box" structures.

3.2.1. Hydrogel homogeneity

Amid the large number of factors that can influence ALG gelation time[192], concentration of calcium carbonate in the polymer solution is of utmost importance since it controls availability of calcium ions for crosslinking. On the other hand, calcium carbonate is dispersed in polymer medium and can progressively accumulate down of the recipient thus giving non-homogeneous cross-linking. It is worth of note that structural uniformity is crucial for biomedical applications not only from a drug delivery viewpoint to achieve uniform distribution of the drug in the matrix, but especially to get good material properties. To evaluate the homogeneity of hydrogels made with $CaCO_3$-GDL, cylindrical gels with varying compositions

[192] Alexander B.R., Murphy K.E., Gallagher J., Farrell G.F. *et al.* J.Biomed.Mater Res.B Appl Biomater. 2011

were prepared and sliced along the vertical axis. Comparable homogeneity profiles of ALG gels at different HA concentrations indicated that the presence of HA did not significantly affect gel homogeneity in the concentration range studied (Figure 3). The slightly higher dry/wet weight ratio of the bottom slice was likely due to either adherence of the $CaCO_3$ powder to the bottom during mixing or some sedimentation during gelation.

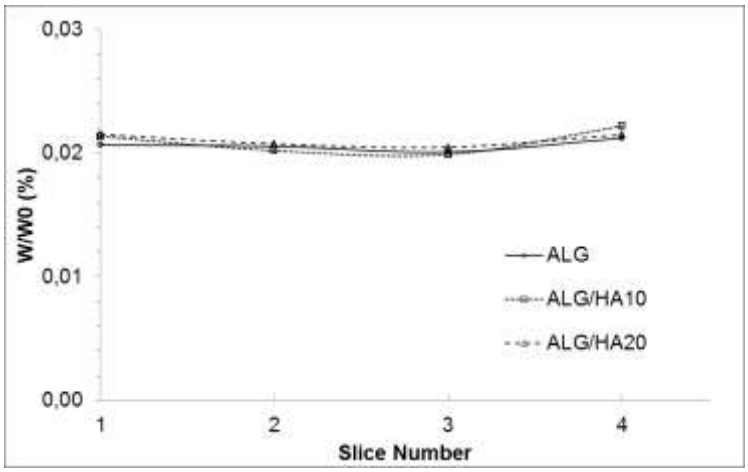

Figure 19. Dry/wet weight ratios of ALG hydrogel slices with varying ALG/HA concentrations numbered 1-4, from top to bottom.

3.2.2. Cross-linking degree

The swelling behavior of ALG hydrogels is a function of crosslink density, ALG concentration, and ALG chemical composition.[193] In particular, structural integrity and mechanical properties are highly dependent on crosslinking of G-blocks, that in presence of Ca^{2+} form more highly ordered regions.[194] During the internal gelation, Ca^{2+} is released directly inside the ALG solution, and for this reason its diffusion in the solution is essential to achieve a homogenous gelation. To determine whether hydrogels compositional variables should be considered for controlling swelling behavior as well, the effects of HA on swelling behavior were investigated. Swelling profiles were obtained for ALG and Alg/HA hydrogels immersed in medium with different Ca^{2+} concentrations for 7 days (figure 4).

[193] {Kuo, 2008 587 /id}
[194] Smidsrod O. and Skjak-Braek G. Trends Biotechnol. 1990

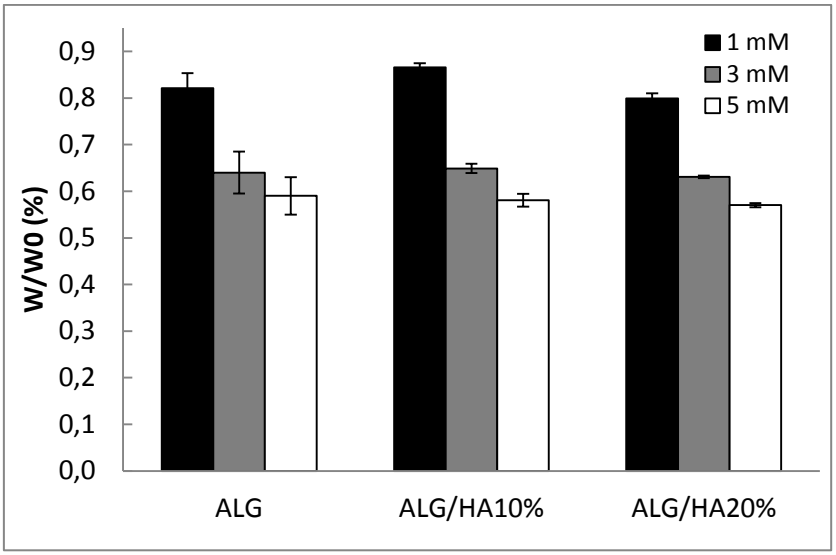

Figure 20. Swelling behavior of ALG hydrogels immersed in medium of different Ca2+ for 7 days.

It was found that all gels with or without HA gave a similar swelling behavior with a shrinking that directly depends from the calcium concentration in the medium. The W/W_0 ratio was similar for all the formulation suggesting that the presence of HA had a minimum influence on the crosslinking degree. These results demonstrated also that cross-linking of G-rich ALG sequences was not complete.

3.3. Preparation and characterization of ALG and Alg/HA sponges

3.3.1. Morphology and water retention

ALG sponges, with or without HA, exhibit a macroporous and interconnected networks comprising numerous pores and channels. This highly porous structure is of utmost importance to ensure water absorption and mechanical strength, especially if an application as wound dressing is envisaged.

The freeze drying method, which can prevent the destruction of the porous structure during the removal of water, was adopted in the preparation of crosslinked sponges starting from hydrogels obtained by internal gelation. To have sponges with good handling properties 2% w/w alginate hydrogels were employed. The freezing method is useful to obtain regular pore sizes since the sponge porosity is directly correlated with the size of the ice crystal formed

during the freezing process.[195] In particular Kang *et al.*[196] showed that the sponge prepared at freezing temperatures near -20 °C, showed a three-dimensional structure with interconnected pore without the use of any additives and organic solvents.

The Alg/HA sponges were imaged by SEM. The surface of ALG sponge exhibited a random highly porous structure from interconnected pores with size in the range of 100−300 μm. Comparison of the ALG sponge (Fig. 5 A) and the ALG/HA sponges (Fig. 5 B-C) revealed that HA filled most of the sponge voids.

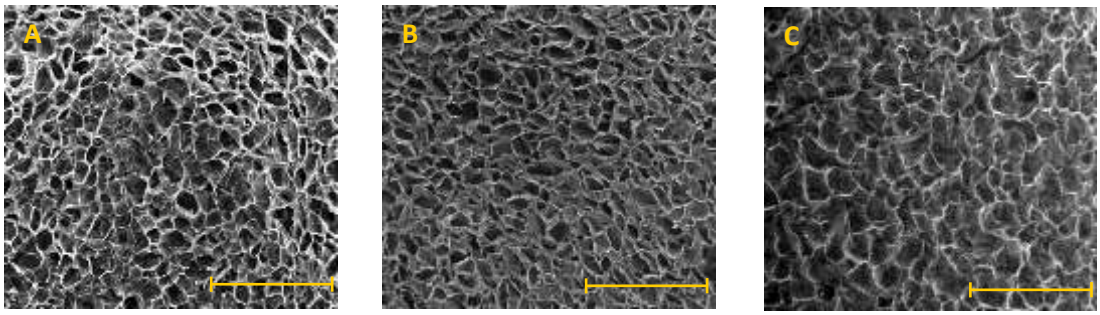

Figure 21. SEM images of ALG (a) ALG/HA10 (b) and ALG/HA20 (c). Scale bar=1 mm.

Water absorption (WA) and water retention (WR) are very important properties for sponge materials, which are highly dependent on their inherent structure and morphology. Main properties of Porosity (P), WA and WR values for the various ALG/HA sponges are summarized in Table 1.

Table 7. Characteristics of ALG sponges.

	Porosity (%)	Density (mg/cm^3)	Water absorption (%)	Water Retention (%)	Equilibrium Water Content EWC (%)
ALG	56.61 ± 1.03	30.76 ± 1.00	5103.95 ± 39.66	67.3 ± 1.8	98.14 ± 0.02
ALG/HA10	72.27 ± 3.21	34.74 ± 1.05	4851.34 ± 63.77	78.8 ± 4.1	98.08 ± 0.09
ALG/HA20	47.70 ± 3.11	34.59 ± 1.85	5070.04 ± 114.86	77.1 ± 2.4	98.09 ± 0.07

[195] Shapiro L. and Cohen S. Biomaterials. 1997
[196] Kang H.W., Tabata Y. and Ikada Y. Biomaterials. 1999

In comparison with ALG/HA, ALG crosslinked sponges, held more open and ordered pores as well as solid internal structure and exhibited the highest water absorption (>5100 g water per 100 g of solid sponge). Furthermore, the presence of HA influenced the sponge density, making them less porous. In Alg/HA sponges HA partially fills the gaps between the ALG chains, and this explain the reduction in porosity despite the constant pore diameters. The uniform and solid porous structure of crosslinked sponges should "lock" the water molecules and prevent the easy runoff of water. Comparing the water retention between neat ALG and ALG/HA sponges after the centrifugation treatment, WR of Alg/HA sponges was higher as compared to ALG sponge, this difference being limited. This may be attributed to the high hydrophilic character of hyaluronate[197] that promote strong interactions with water.

3.3.2. Water uptake and degradation behavior of ALG/HA sponge

The ability to absorb fluids is essential to envisage an use of these systems as advanced dressings. In fact high water absorption capacity is considered to be beneficial in application on highly suppurating wounds.[1] Moreover, when the sponge incorporates a drug to deliver, drug release process is strictly related to water absorption kinetics, which are in turn affected from sponge physical structure. In order to clarify this point, water absorption kinetics of the various sponges were evaluated. Figure 6 shows water absorption capacity of sponges with different composition.

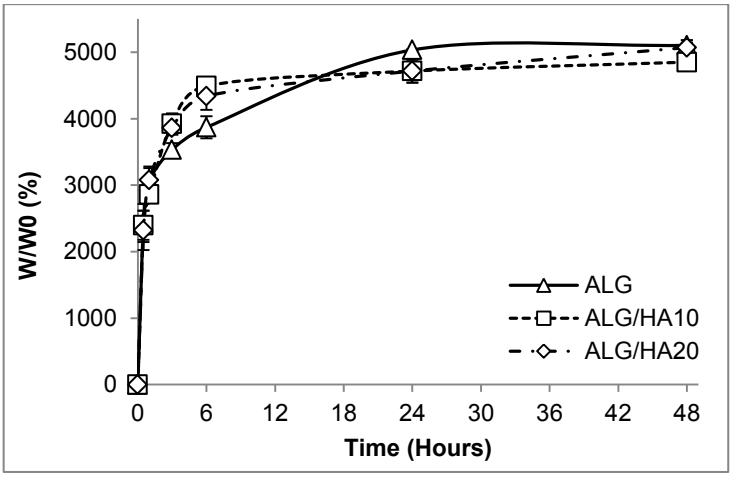

Figure 22. Kinetics of water uptake of ALG and ALG/HA sponges. Results are reported as mean ± SD (n= 3).

[197] Toole B.P. Nat.Rev.Cancer. 2004

Contrariwise to equilibrium water content, water absorption kinetics were influenced by the presence of HA in the sponge. The absorption curves of the ALG/HA hydrogels showed a higher rate of water uptake within the first 6h of immersion (equilibrium swelling) as compared with ALG sponges, while after 24 h all the formulations displayed the same water uptake. Differences in sponges porosity does not seem the main property to explain water uptake kinetics. Nevertheless, the high hydrophilic and polyanionic character of hyaluronate[198] may lead to a such increase of the hydrostatic pressure within the sponge and promote its hydration rate.

To get an insight into sponge behavior in a moist environment, the stability of ALG sponges in contact with a physiological fluid was investigated. The degradation time, and consequently the expected *in vivo* residence time, has been evaluated using a modified Enslin apparatus in which phosphate buffered saline (PBS) at pH 7.4 was employed as wetting medium. In the current study water uptake and stability were evaluated in an ionic solution which mimics the composition of blood plasma and synovial fluid with respect to inorganic ions. Since the calcium ion concentration in these solutions is too low to stabilize the hydrogels, slow degradation as a result of cross-linker ion loss is expected to occur. Sponges show completely different behaviors depending on the composition. in case of ALG sponge, its porous structure was maintained for three days while complete hydration was reached after 6 days (Figure 23). A complete loss of structural integrity was reached in about 14 days.

[198] Toole B.P. Nat.Rev.Cancer. 2004

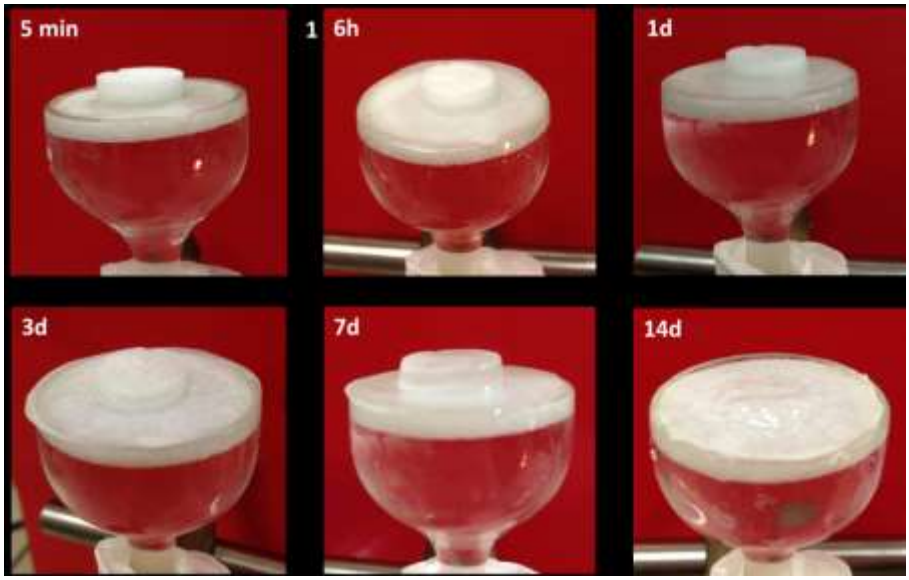

Figure 23. Degradation kinetics of ALG sponge.

Sponges containing HA, at both concentrations studied (Figure 24), show a different behavior. Indeed, Alg/HA sponges degrade more quickly than ALG sponge, reaching complete hydration in about 3 days, while the loss of structural integrity occurred after 7 days.

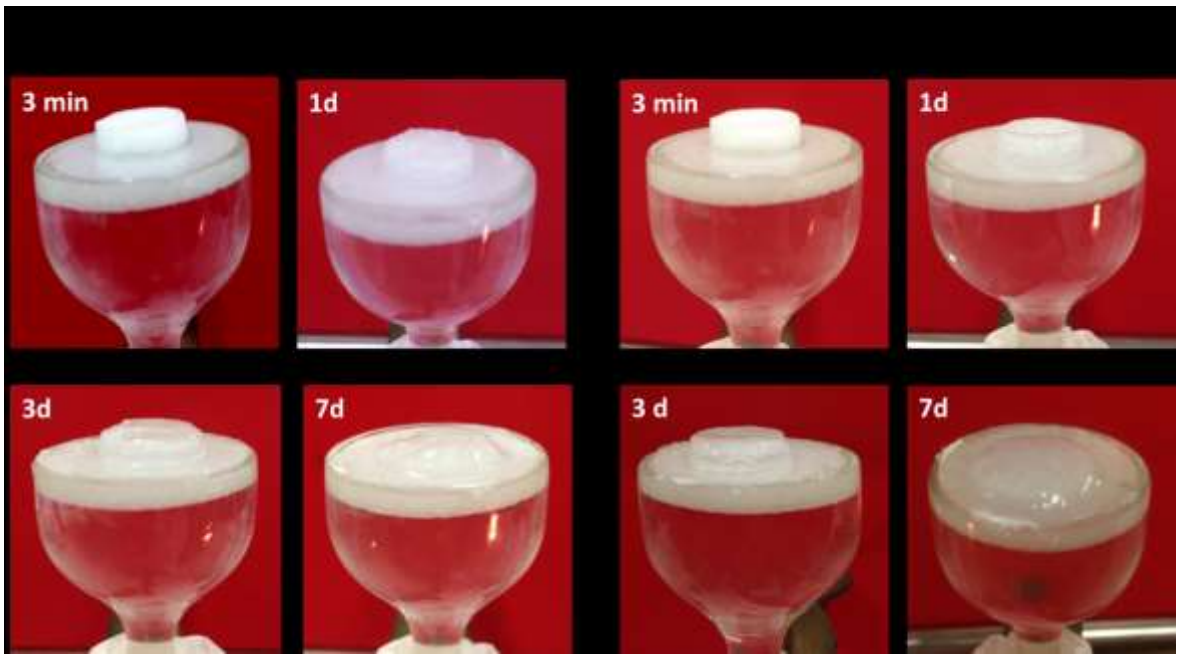

Figure 24. Degradation kinetics of Alg/HA10 and Alg/HA20 sponge

The hydrophilic nature of HA is likely to affect also sponge degradation *in vitro* because it acts modifying both WA and WR. The consequent faster increase in ion exchange inside the sponge leads to disassembly of the egg box structure with a loss in structural properties. Furthermore, the Ca^{2+} diffusion throughout seems to be accelerate in presence of HA.[199] However, contribution of enzymatic degradation of HA operated by hyaluronidasis should be take into account.

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3.3.3. Drug release from sponges

The impact of matrix composition on drug release from loaded sponges was investigated. Sponges were loaded with TA, a drug with hemostatic properties, and its release followed to evaluate the ability of the system to reduce bleeding upon wound application. The ALG sponge showed the slowest release rate, whereas the presence of HA accelerated it (figure 25). The release of hydrophilic molecules from swellable sponges depends mainly from swelling kinetics, thus pointing to the presence of HA as a key parameter in affecting the release rate. Comparison between the release profiles of TA and water uptake curves highlight that the water uptake reached over 70% of its final value in 3 hours while in the same period almost 95% of TA is released. Thus, the swelling process reaches ends up in 6 hours, when all TA has been released. The incorporation of HA in the composite sponge significantly affected TA release profile because it causes a faster water absorption with a consequent larger burst effect.

[199] Oerther S., Le G.H., Payan E., Lapicque F. *et al.* Biotechnol.Bioeng. 1999

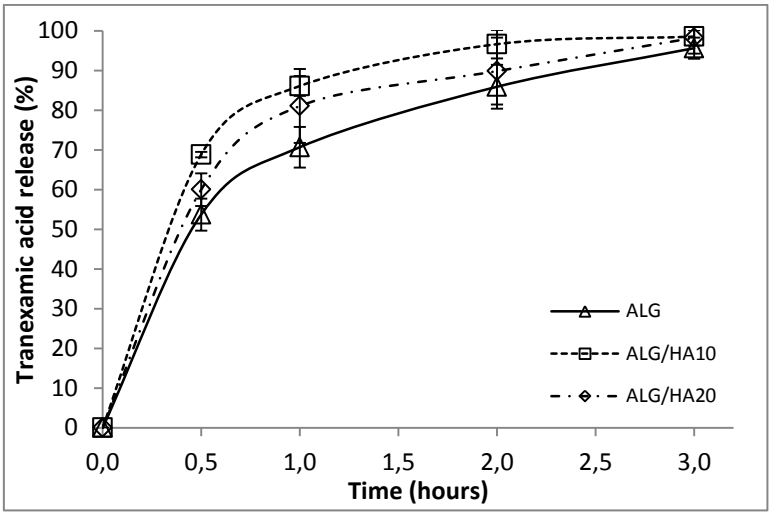

Figure 25. Tranexamic acid release profile in PBS pH 7.4

3.4. In vitro toxicity: Effect of Alg/HA hydrogels on Ad-MSCs viability and motility.

In order to evaluate the in vitro toxicity of hydrogels, human Mesenchymal Stem Cells (Ad-MSCs) previously isolated from subcutaneous adipose tissue biopsies were used.[200] Cell viability was then investigated using a transwell system. The upper chamber was filled up with the hydrogel, while the lower chamber was loaded with a cell suspension in DMEM F12 (1:1), containing a 10% FBS. As shown in figure 26 sulforhodamine assay revealed that Ad-MSC viability in the presence of ALG and ALG/HA hydrogels was comparable to that achieved with DMEM F12 (1:1) 10% FBS.

[200] {D'Esposito, 2012 504 /id}

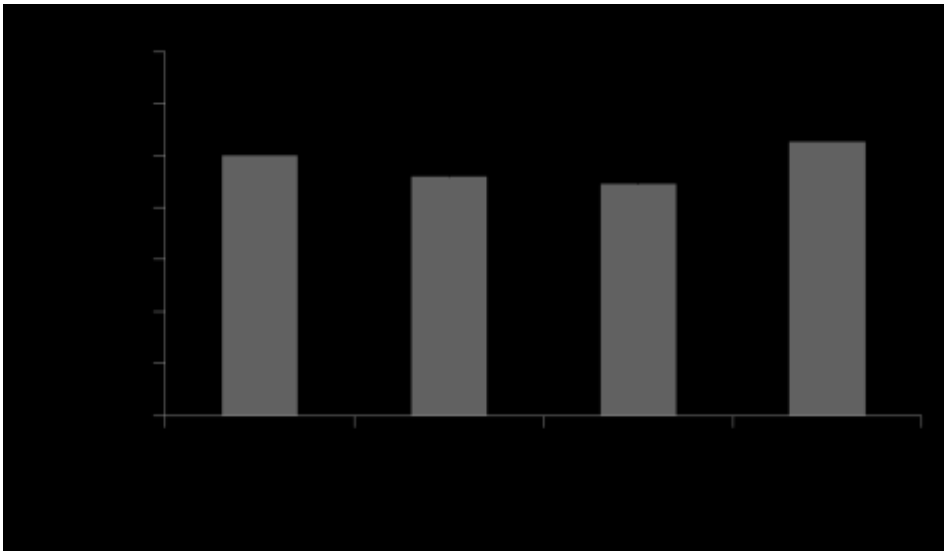

Figure 26. Effect of ALG and ALG/HA hydrogels on Ad-MSCs viability. Ad-MSCs have been loaded at 50,000 cells per insert in the lower chamber on a 0.4 μm pore polycarbonate membrane. The following day, ALG hydrogels or ALG-HA hydrogels (ALG/HA10% - ALG/HA20%) have been added in the upper chamber of the transwell in DMEM F12 (1:1) 10% FBS. Upon 24 hours, cell viability has been determined, as optical density, by sulforhodamine assay. The results have been reported as percentage of viable cells compared with cells incubated in DMEM F12 (1:1) 10 % FBS in absence of hydrogels (considered as 100% viable cells). Bars represent the mean ± standard deviation of triplicate determination in three independent experiments.

Next, to investigate whether Alg/HA hydrogels could affect cell motility a wound healing assay was performed. Confluent monolayers of Ad-MSCs were scratched and incubated with ALG and ALG/HA hydrogels in medium without serum supplementation (DMEM F 12 -0.25% BSA). Images were taken at 0 and 24 h after wounding. As shown in figure 27, serum induced an almost complete wound closure. Even in absence of serum, ALG and Alg/HA hydrogels significantly promoted wound closure as compared to serum-free medium.

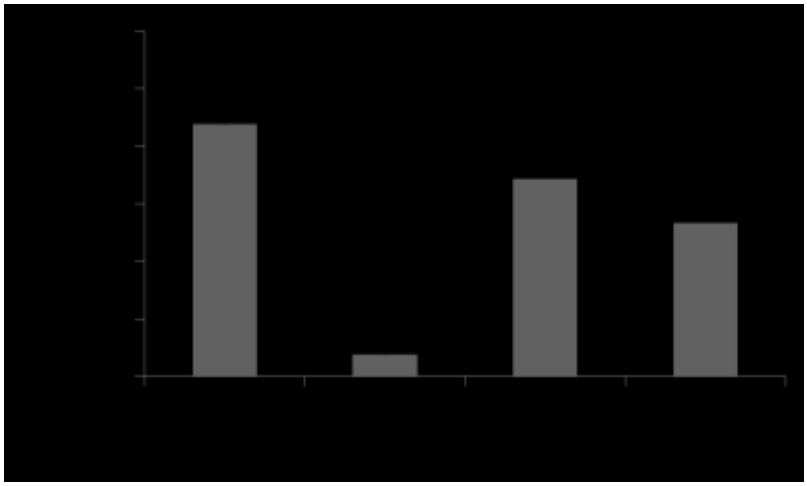

Figure 27. Effect of ALG and ALG/HA hydrogels on Ad-MSCs motility. Confluent monolayers of Ad-MSCs, grown in DMEM F12 (1:1) 10% FBS, have been incubated with serum free medium (DMEM F12 1:1 0.25% BSA) over night. The following day, cells have been subjected to scratch assays, washed twice with phosphate buffered saline (PBS) and incubated at 37°C in presence of ALG or ALG/HA (ALG/HA20%) hydrogels in DMEM F12 (1:1) 0.25% BSA. Images of wound gap have been taken at 0 and 24 h by a digital camera coupled to the microscope and percentage of closure was calculated with NIH IMAGE J. The results have been reported as percentage of wound distance at 24 h compared with the starting point (time 0). Bars represent the mean ± standard deviation of triplicate determination in three independent experiments. Asterisks denote statistically significant differences respect to cells in DMEM F12 (1:1) 0.25% BSA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Very interestingly, the stimulatory activity of HA on Ad-MSCs motility is evidenced also when it is included in the hydrogel. In fact, degradation of the hydrogels in the medium occurs and correspondent release of calcium ions and HA is realized. Thus, stimulatory effect on cellular proliferation exerted by HA and proteoglycan synthesis (particularly aggrecan) according to various mechanisms is likely to occur. It has been proven that HA induces protein kinase activity through CD44 receptor and activates a cytoplasmic signal transduction pathway.[201] This pathway may include mitogen-activated protein activation, resulting in nuclear translocation of extracellular signal-regulated kinase 1 to stimulate cell proliferation.[202]

[201] Slevin M., Kumar S. and Gaffney J. J.Biol.Chem. 2002
[202] Knudson C.B. and Knudson W. Clin.Orthop.Relat Res. 2004

Conclusions

Hydrogels useful for wound treatment in form of hydrated and spongy-like sheets have been successfully produced. The internal gelation technique allow as to obtain strong tridimensional hydrogels with good handling characteristics. Presence of hyaluronan, whose seems to have a crucial role in wound healing, affected the alginate gelation time, but had a minimum influence on the crosslinking degree. In the case of sponge, hyaluronan increased hydrophilicity and porosity of the matrix with the consequent increased water uptake. These effects were demonstrated to modulated release rate of a hydrophilic drug incorporated in the sponge. Cellular studies highlight that hyaluronan incorporation in the alginate hydrogels promotes wound healing without any effect on cell viability. Taken together, these results demonstrate how integration of hyaluronan in a physically cross-linked alginate matrix can be a useful strategy to promote wound healing.

CHAPTER III

ALGINATE - HYALURONIC ACID HYBRID HYDROGELS FOR NANOSILVER DELIVERY TO INFECTED WOUNDS

Introduction

Throughout the centuries a large number of traditional wound dressings were developed for wound healing, but only in 1962 the real revolution in wound care occurred when George D. Winter demonstrated that a moist environment can ensure a more rapid re-epithelialization as compared with the healing of the lesion left to dry.[203] Nevertheless, moisture promotes the bacterial growth with consequent wound infections, which represents one of the main adverse effects that limit recovery of the diseased tissue.

Silver has a long history as an antimicrobial agent[204,205]and has gained renewed interest in the wound-management industry as an effective antimicrobial agent due to increased antibiotic resistance. Several silver-containing wound dressings have been developed. Semi-solid preparations such as silver sulphadiazine cream[206] and silver nitrate ointment[207] are already used to treat bacterial infection since they remain on the surface of the wound for a longer period of time as compared with solutions. However for highly exuding wounds, semi-solid preparations are not very effective as they rapidly absorb fluid, lose their rheological characteristics and become mobile[208]. For this reason the use of solid wound dressings is preferred in case of exudative wounds as they are capable of a correct exudate management and persistence at wound site where they exert healing action.

Nanosilver (NS) consists in clusters of silver molecules that range from 1 to 100 nm which have proven to be a highly effective form of silver with antimicrobial activity against bacteria, viruses and other eukaryotic micro-organisms.[209] It's simple synthesis and highly effective antibacterial activity make them a very attractive way to administer silver.[210,211]In

[203] {Winter, 1962 37 /id}
[204] {Klasen, 2000 582 /id}
[205] {Klasen, 2000 583 /id}
[206] {Hudspith, 2004 581 /id}
[207] {Liao, 2006 28 /id}
[208] boateng
[209] {Rai, 2009 601 /id}
[210] {Chernousova, 2013 569 /id}

order to show any antimicrobial properties, silver has to be in its ionized form.[212,213] In fact, silver in its non-ionized form is inert[214], but in contact with moisture silver ions (Ag^{+}) can be generated.[215] In addition to its well-recognized antibacterial properties, NS can be used for modulate local and systemic inflammation and accelerate wound healing process[216,217] due the capacity to reducing cytokine release[218], decreasing lymphocyte and mast cell infiltration[219] and inducing apoptosis in inflammatory cells.[220,221]

Advanced dressings comprising both conventional barrier functions and bioactivity are designed to provide a fast and correct wound healing process. Novel wound dressings create a moist environment around the wound to facilitate wound healing and to maintain site hydration along time.[222] Through the years a number of modern dressings have been developed as an advance upon the traditional wound healing agents, and their use on different wound types found to be highly dependent on the material employed.[223] Among the materials already used in commercial wound dressing, sodium alginate (a water-soluble polysaccharide extracted from brown seaweed) is considered one of the most versatile. Alginates (Alg) can be easily cross-linked through divalent ions such as Ca^{2+} and Zn^{2+} forming hydrogels with improved mechanical properties. Since Alg are highly absorbent, Alg-based dressings can fit application on highly exuding wounds where microbial infections are common. Furthermore Ca^{2+} cross-linked Alg dressings show biological function due to the presence of calcium ions that orchestrate some cellular events of wound healing.[224,225] Nevertheless release of calcium from cross-linked Alg dressings plays a physiological role aiding the clotting mechanism (hemostasis) during the first stage of wound healing.[226,227]By incorporating silver ions into Alg

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[211] {Rizzello, 2014 603 /id}
[212] {Lok, 2007 590 /id}
[213] {Rai, 2009 601 /id}
[214] {Guggenbichler, 1999 579 /id}
[215] {Kumar, 2005 585 /id}
[216] {Tian, 2007 614 /id}
[217] {Chaloupka, 2010 499 /id}
[218] {Castillo, 2008 567 /id}
[219] {Boucher, 2008 565 /id}
[220] {Wright, 2002 629 /id}
[221] {Nadworny, 2008 533 /id}
[222] Boateng, 2008
[223] {Queen, 2004 600 /id}
[224] {Doyle, 1996 17 /id}
[225] {Thomas, 2000 610 /id}
[226] {Blair, 1990 564 /id}
[227] {Blair, 1988 563 /id}

fibers, it is expected that a highly absorbent wound dressing with good antimicrobial properties can be obtained.

Hyaluronan or hyaluronic acid (HA) is a long-chain polysaccharide that is found naturally in the body and acts both as a structural component of the extracellular matrix (ECM) and as a mediator of various cellular functions. Several studies indicates that HA is involved in each phase of wound healing[228] and one of his function is the modulation of inflammatory cell and dermal fibroblast activities.[229,230,231] HA functions during wound formation of a porous networks during inflammation and as a space filler in granulation tissue. In contrast, short HA fragments have stimulatory and attracting properties toward fibroblasts and stimulate collagen production.[232] Furthermore, different HA lengths stimulate the production of specific collagen types, which can promote or inhibit scar formation.[233] Since HA is found in the ECM throughout the body, biomaterials based on HA derivatives show promising properties for clinical application and can be taken into consideration to promote tissue repair and wound healing.[234]

The aim of this work is to develop a NS-cloaded Alg dressing able to accelerate tissue repair and to control bacterial infection. To this purpose, we propose a Ca^{++} cross-linked Alg dressing containing dispersed HA of medium molecular weight to help repair and releasing silver ions in the wound bed. Biological behavior of the hydrogels were studied on human Adipose Mesenchymal Stem Cells and in a rat model of wound healing to evaluate their toxicity and wound repair potential. Finally, NS-loaded hydrogels were tested on clinical isolates from infected wounds to evaluate antimicrobial activity.

[228] {Frenkel, 2012 577 /id}
[229] {Evanko, 2001 575 /id}
[230] {Papakonstantinou, 1995 599 /id}
[231] {Bourguignon, 2006 566 /id}
[232] {Moseley, 2002 594 /id}
[233] David-Raoudi, 2008
[234] {Voigt, 2012 620 /id}

Materials and methods

6.1. Materials

Pharmaceutical grade sodium alginate (Alg) extracted from *Laminaria hyperborea* (viscosity 360 cps) was purchased from Farmalabor (Italy). Analysis-grade ethanol was purchased from Carlo Erba (Italy). Calcium carbonate ($CaCO_3$), D-glucono-δ-lactone (GDL), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4) and hyaluronic acid sodium salt from *Streptococcus equi* (1.5-1.8 * 10^6 Da) were obtained from Sigma-Aldrich (USA). Colloidal silver solution (NS) was provided from Cluster nanotech s.r.l. Media, sera, and antibiotics for cell cultures were from Lonza (Lonza Group Ltd, Basel, Switzerland). Depurated and filtered (Milli Q filter) water was used throughout this study.

Male Wistar albino rats weighing 300-350 g were purchased from Harlan-Italia (San Pietro al Natisone, UD). All the rats were housed singly, to prevent fighting and attack on the wounds, for one week in Plexiglas cage at temperature of 22 ± 1 °C, with alternate cycle of 12 hours of light and 12 hours of dark. All animals received food and water ad libitum throughout the experimental period. All manipulations were performed using aseptic techniques. This study was carried out in strict accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 of Ministero della Salute and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC).

6.2. NS characterization

NS was characterized to evaluate size and shape. The size was obtained from dynamic light scattering using a Zetasizer Nano ZS (Malvern instruments, UK). Particle size and polydispersity were registered at a scattering angle of 90◦and at a temperature of 25°C.

NS morphology was visualized by Transmission Electron Microscopy (TEM) Sample for TEM analysis was prepared by placing one drop of NS onto a copper grid. After approximately 1 hour images were captured by a transmission electron microscope. TEM analysis was performed with a FEI Tecnai G12 (LAB6 source) equipped with a FEI Eagle 4 K CCD camera (Eindhoven, The Netherlands) operating with an acceleration voltage of 120 kV.

6.3. Hydrogel preparation

Structurally homogeneous calcium Alg hydrogels were made as previously described by Kuo and Ma.[235] Briefly Alg was dissolved in deionized water (1% w/v) and mixed together with calcium carbonate ($CaCO_3$) 30mM to form a suspension. A fresh aqueous 64 mM GDL solution was then added to the suspension and vortex mixed to initiate gelation. A $CaCO_3$ to GDL molar ratio of 0.5 was maintained to achieve a neutral pH post-crosslinking. The HA was dissolved directly in the Alg solution to obtain the concentrations of 10% and 20% w/w. The different solution were cast in 96 well plate (50 µl, well size: 5 mm diameter, 20 mm height) to form circular discs 2 mm in thickness and 5 mm in diameter or in a 24-well plate (1 ml, well size: 15 mm diameter, 20 mm height) to form circular discs 5 mm in thickness and 15 mm in diameter, depending on use. The well plate were capped, sealed with Parafilm®, and gelled on a leveled plate at room temperature for 24 h. Different concentrations of nanosilver were added to Alg solution before gelation to obtain nanosilver-loaded gels. After gelation the Alg hydrogels were washed with water and stored at 4°C until use.

Table 8. Hydrogel compositions tested in the study.

	Alginate (% w/v)	**HA (% w/w)**	**NS**
ALG	1 %	-	-
ALG/NS10	1%	-	10 ppm
ALG/NS50	1%	-	50 ppm
ALG/NS10/HA10	1 %	10 %	10 ppm
ALG/NS50/HA10	1 %	10 %	50 ppm
ALG/NS10/HA20	1 %	20 %	10 ppm
ALG/NS50/HA20	1 %	20 %	50 ppm

6.4. Hydrogel characterization

Water uptake was determined by placing the cross-linked hydrogels in water. The initial weight of three circular samples (15 mm diameter) was measured by using an analytical balance. The samples were then placed in 5 mL of water at 37 °C in a thermostatic bath. Samples were taken out and excess water was removed by gentle blotting with tissue paper.

[235] Kuo C.K. and Ma P.X. Biomaterials. 2001, 2001

Samples were weighed and then placed again in 5 mL of water at 37 °C. The weight of the samples was recorded at regular intervals until no weight change was recorded was established. Results are expressed as swelling ratio percent (SR %) at each time point according the equation:

$$SR\ (\%) = \frac{W\text{-}W_0}{W_0} \times 100$$

where *W* is the mass of the swollen sample and *W0* is the mass of the initial dry sample. The equilibrium water content (EWC) per cent was calculated by the equation:

$$EWC\ (\%) = \frac{W_e - W_d}{W_e} \times 100$$

where W_e is the mass of the swollen sample at equilibrium and W_d is the mass of the dry sample at equilibrium.

Changes of hydrogel aspect along time upon contact with a medium simulating biological fluids in tissue injuries were registered.[236] Hydrogels were placed in contact with a Phosphate Buffer Saline (PBS, NaCl 120 mM, KCl 2.7 mM, Na_2HPO_4 10 mM) at pH 7.4 (Ph.Eur 8) by means of a modified Enslin apparatus. Pictures of the hydrogels are taken over time. Three replicates were performed for each sample.

6.5. Cell culture studies

6.5.1. Cell lines

Adipose Mesenchymal Stem Cell (Ad-MSC) were isolated as previously reported[237] from human adipose tissue biopsies through digestion with collagenase. Ad-MSC were cultured at 37°C with Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1) with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cultures were maintained in humidified atmosphere of 95% air and 5% CO_2 at 37° C.

[236] {Rossi, 2007 20 /id}
[237] {D'Esposito, 2012 504 /id}

6.5.2. Cell viability

Ad-MSC viability was assessed by sulforhodamine assay.[238] Briefly, Ad-MSCs (5,000 cells/well) were seeded in 96-well culture plates in a complete medium. The following day the cells were incubated with raising concentrations of silver nanoparticles (NS). Upon for 24h, the cells were fixed with 50% trichloroacetic acid for 2 h at 4 C. Then, cells were washed 5 times with distilled and de-ionized water. After air drying, cells were stained for 30 min with 100 μl 0.4% sulforhodamine dissolved in 1% acetic acid. Unbound dye was removed by five washes with 1% acetic acid. After air drying, 10 mM Tris solution (pH 7.5) was added to dissolve the protein-bound dye. Cell survival was assessed by optical density (OD) determination at 510 nm using a microplate reader.

To investigate the effect of Alg hydrogels on Ad-MSC viability, 50,000 cells were seeded in the lower chamber of a 24-transwell culture system (0.4-μm pore polycarbonate membranes; Costar, Cambridge, MA). The following day, the hydrogels were added to the upper chamber in complete medium. Upon 24h, sulforhodamine assay was performed. Three replicate wells were used for each data point, and the experiment was performed three times.

6.5.3. Cell motility- Wound healing Assay

Ad-MSCs were seeded into six-well microplates and grown in a complete medium to a confluent monolayer. Then, the cells were wounded by manually scratching with a p20 pipette tip. Reference points near the "scratch" were marked to guarantee the same area of image acquisition. The wells were washed twice with phosphate buffered saline (PBS) and incubated at 37 C with hydrogels. Images of wound gap were taken at 0 and 24 h by a digital camera coupled to the microscope and percentage of closure was calculated with NIH IMAGE J. These experiments were repeated at least three times.

6.6. Antibacterial activity on clinical isolates

The antibacterial activity of the ALG/NS or ALG/NS/HA hydrogels was evaluated by the Luria-Bertani agar plate method against clinical isolates of both Gram-positive (methicillin-resistant *Staphylococcus aureus, Staphylococcus epidermidis*) and Gram-negative (*multidrug* resistant *Pseudomonas aeruginosa, Escherichia coli*, *Acinetobacter baumanni* , *Klebsiella*

[238] {Chiba, 1998 502 /id}

pneumoniae) bacterial strains, obtained from the Diagnostic Unit of Bacteriology and Micology of the University Federico II of Naples. The bacterial isolate were grown under aerobic conditions in Brain-heart infusion broth (Becton Dickinson) at 37°C until to mid-log phase. The inoculum of each bacterial strain was adjusted to 10^5 colony-forming units (CFU)/ml and 1 ml incubated in the presence of the hydrogels or Alg solution for 24 h or 48 h at 37°C. Three concentration (2, 10 and 50 ppm) of silver solution and two concentrations (10 and 50 ppm) of Alg/NS or Alg/NS/HA hydrogels were used. Each assay was performed in triplicate. After incubation period, a viability count was performed by dilution and plating on LB agar and incubation at 37°C for 24 h. The viable cells were counted by quantization of CFUs and expressed as mean and standard deviation of three assays.

6.7. In vivo wound healing studies

A full-thickness excision wound model was used to monitor wound closure. All the animals were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and xylazine (5 mg/Kg) by intraperitoneal administration, and the dorsal hair was shaved using a shaving machine. The surgical area was disinfected with Betadine R 10%. A full-thickness wound with a diameter of 2,5 cm was excised from the back of the rats using sterile scissors at the depth of loose subcutaneous tissues and the wounds were left open. Hemostasis was obtained by direct pressure using a sterile gauze. Before hydrogel application the wound was moistened with 300 µL of saline solution. Animals (n=3) were divided into four groups and wounds were treated with a single application of unloaded hydrogels (Alg, Alg/HA20) or NS-loaded hydrogels (Alg/NS, Alg/NS50/HA20) containing 50 ppm NS.. On top of the wounds, a piece of Tegaderm™ (3M, USA) was applied to prevent the rats from removing the treatments. For wound closure study, changes in wound area were measured on day 1 and 5 and wound closure was calculated according to Equation:

$$\%\ \text{Wound closure} = \frac{A_0 - A_t}{A_0} X\ 100$$

Where A_0 is the initial wound area and A_t is the open area of wound at day 1 and 5 after biopsy.

6.8. Statistical analyses

Statistical analyses were undertaken using GraphPad Prism®, version 4.00 (GraphPad Software, San Diego, USA). Data were compared using a Student's t-test, a one-way ANOVA, with a Bonferroni post-test (parametric methods).

Results and discussion

7.1. Nanosilver containing hydrogels

NS employed in our studies have a mean diameters of 0.8 nm. A low PI (0.110) indicates good size homogenity of NS solution (figure 28).

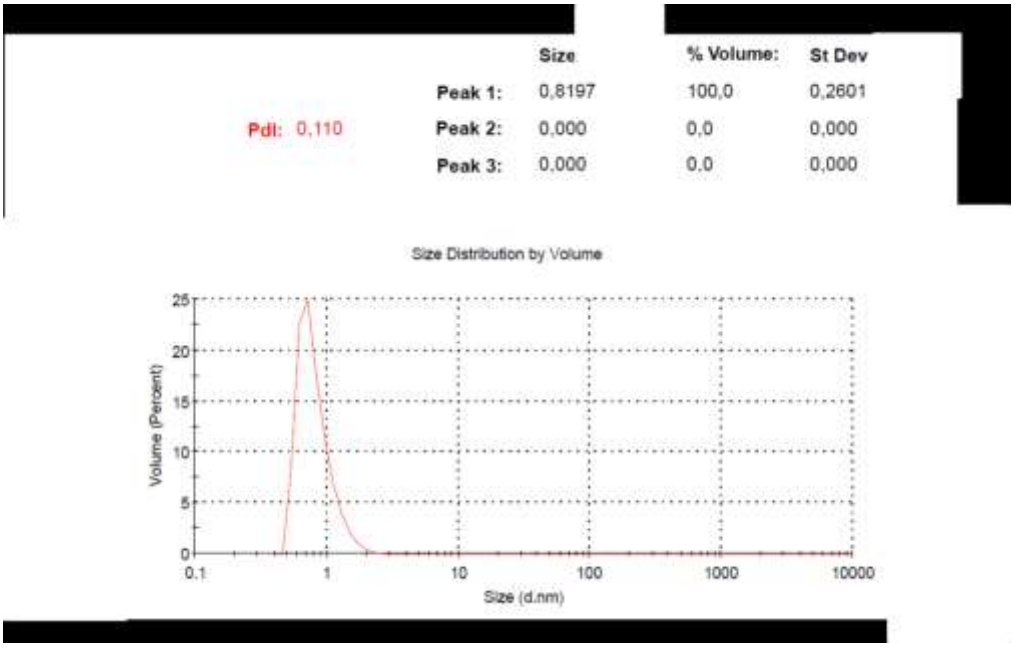


Figure 28: Size analysis of NS

TEM images show that Ag NPs are less than 1 nm, in agreement with DLS, and characterized by spherical shape (data not shown).

The bactericidal effects of NS depends directly from the particle size, since smaller particles induce a stronger inhibition of microorganism growth with respect to larger ones. Morones et al.[239] found that NS in the range of 1-10 nm are attached the cell membrane with more affinity as compared with larger nanoparticles, drastically perturbing the membrane function with consequent cellular death.

Solid transparent ionically cross-linked Alg/HA hydrogels or Alg/NS/HA sheets with different compositions were prepared (Table x) using the internal gelation technique. This

[239] {Morones, 2005 593 /id}

method, which is characterized by a slow release of Ca^{2+} ions directly from the inside the Alg solution, allow as to obtain homogeneous hydrogels which have sufficient strength to be handled, cut with scissors or packed without difficulties.[240],[241],[242] Visually, hydrogels Alg and Alg/HA have a homogeneous aspect and the presence of NS does not have any effects on hydrogels physical properties. The physical characterization of this system gave results close to those reported in chapter X.

The ability to absorb fluids is essential for using hydrogels as advanced dressings and water absorption capacity is considered to be beneficial in application on suppurating wounds {Boateng, 2008 635 /id}. A silver-containing hydrogel acts by absorbing exudates and releasing the silver onto the wound bed, killing microorganisms both in the wound and within dressings. Xiu et al.[243] demonstrated that the antibacterial activities of NS is entirely due to the release of Ag^{+} ions in the medium rather than to NS themselves. To evaluate the absorption rate, ionically cross-linked ALG and ALG/HA hydrogels were placed in water and the swelling ratio (SR%) was measured in terms of mass increase at regular time intervals (figure 29).

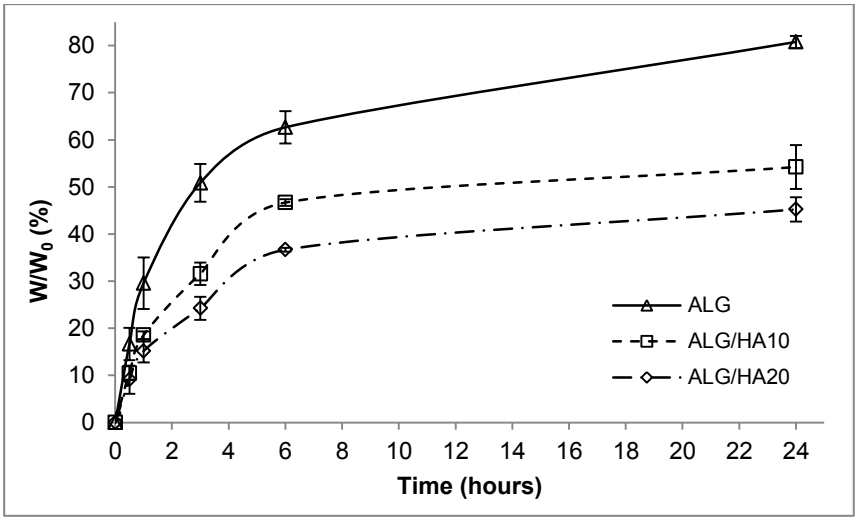


Figure 29. Water uptake of Alg hydrogels.

[240] {Draget, 1997 124 /id}
[241] Kuo C.K. and Ma P.X. Biomaterials. 2001
[242] {Kuo, 2008 587 /id}
[243] {Xiu, 2012 630 /id}

When comparing the weight change behavior of each hydrogel, it is clear that the hydrogel of Alg has a higher swelling ratio than Alg/HA hydrogels. The absorption curves of the Alg/HA hydrogels showed a similar water absorption rate within the first 3 h of immersion, while after this point the higher swelling of Alg hydrogel allows to absorb a greater amount of water. The fastest water uptake is probably due to the high hydrophilic and polyanionic character of hyaluronan[244] that may lead to an increase of the hydrostatic pressure within the hydrogels and promote its hydration rate.

Hydrogel stability along times upon contact with a simulated physiological fluid was examined. The degradation time, and consequently the *in vivo* residence time, has been evaluated using a modified Enslin apparatus in which the wetting liquid, a phosphate buffered saline (PBS) at pH 7.4, is in contact with one side of the hydrogel. The extent of degradation changed depending on the composition of the hydrogel, being in the order Alg/HA20 > Alg/HA10 > Alg (Figure 30 and 31). The degradation mechanism for the crosslinked hydrogels, and the consequent loss of structural integrity in time, is correlated to hydrolysis of the glycosidic bonds and the Na^+ ions (present in PBS) interchange with cross-linking Ca^{2+} ions.[245]

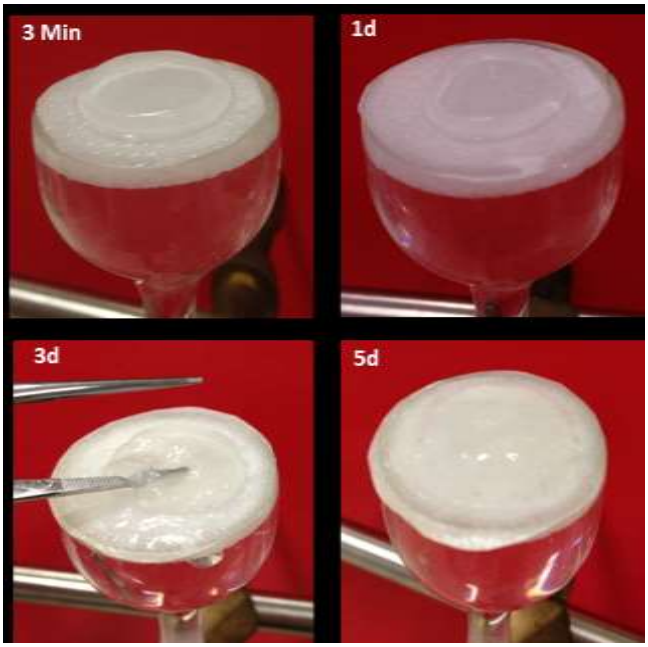


Figure 30. Degradation kinetics of ALG hydrogel.

[244] {Toole, 2004 40 /id}
[245] {Lee, 2012 660 /id}

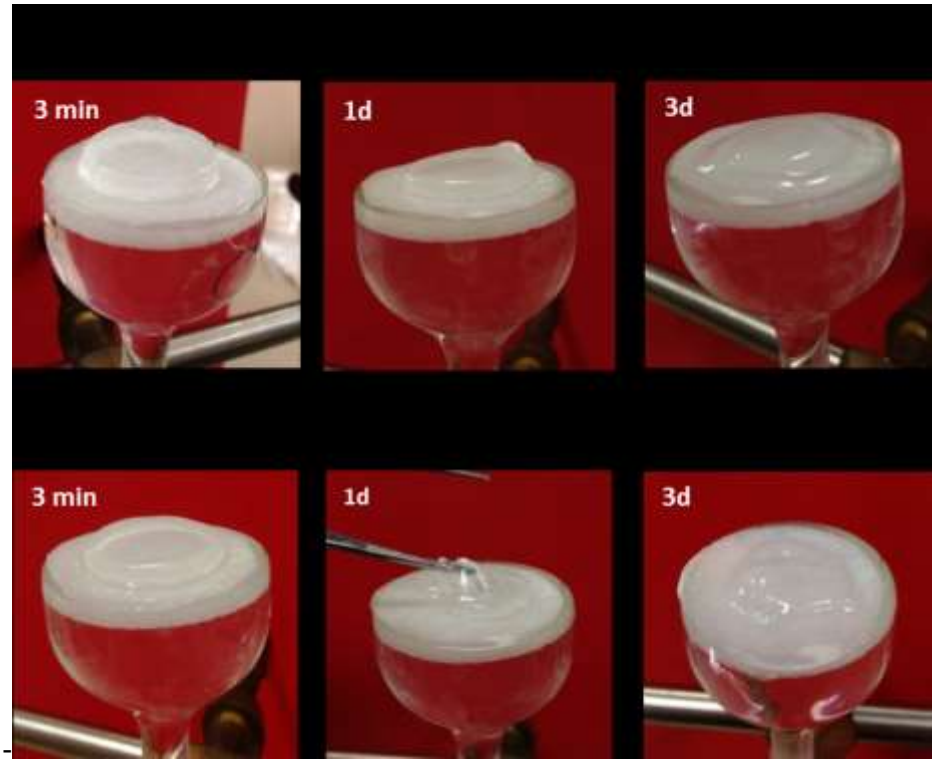


Figure 31. Degradation kinetics of Alg/HA10 and Alg/HA20 hydrogels.

The difference in degradation rate between the hydrogel ALG/HA and Alg is greater at 3 days than at 1 days. This phenomenon can be explained by the fact that the ALG/HA polymeric matrix is less cross-linked due to the presence of HA chains that hampers ionic interaction between Alg and Ca^{2+} ions.[246] The hydrophilic nature of HA[247] increased the degradability of the hydrogels likely due to a faster water absorption and a consequent faster exchange of Ca^{2+} ions with Na^{+} and K^{+} ions.

7.2. Effect of nanosilver (NS) and Alg/NS hydrogels on Ad-MSC viability

In order to evaluate NS and Alg/NS hydrogels toxicity on a cellular model, we employed human Mesenchymal Stem Cells (Ad-MSCs) previously isolated from subcutaneous adipose

[246] {Oerther, 1999 493 /id;}
[247] {Toole, 2004 40 /id}

tissue biopsies.[248] As assessed by sulforhodamine assay, 10 and 50 ppm NS did not interfere with cell viability (fig.x). At variance, higher concentrations of NS (70, 100, 150 ppm) significantly reduced Ad-MSC viability (figure 32).

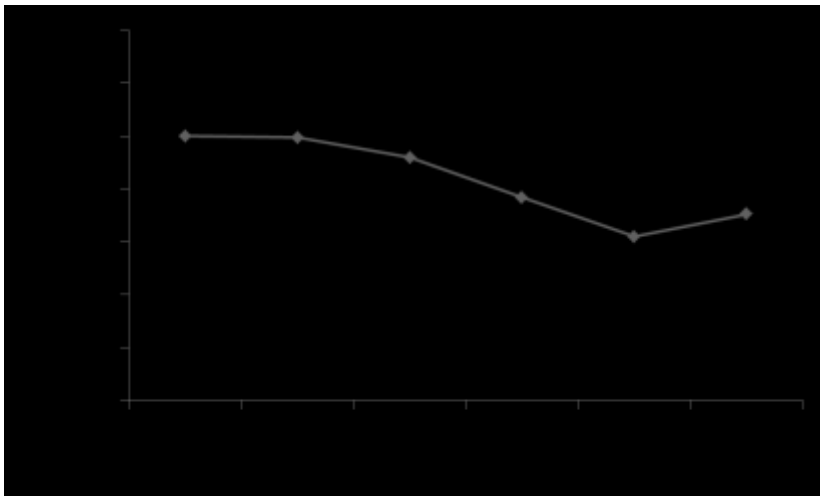


Figure 32. Effect of NS on Ad-MSCs viability. Ad-MSCs (5,000 cells/well) have been seeded in 96-well culture plates in DMEM F12 (1:1) 10% FBS. The following day the cells have been incubated with 10, 50, 70, 100 or 150 ppm silver nanoparticles (NS). Upon 24 hours, cell viability has been determined, as optical density, by sulforhodamine assay. The results have been reported as percentage of viable cells compared with cells incubated in DMEM F12 (1:1) 10 % FBS in the absence of NS, considered as 100% viable cells. Bars represent the mean ± standard deviation of triplicate determination in three independent experiments.

Then, we investigated the effect of Alg/NS hydrogels on cell viability using a transwell system. The upper chamber was filled up with Alg hydrogels, while the lower chamber was loaded with cell suspension in complete medium. As shown in figure 33, sulforhodamine assay revealed that Ad-MSC viability in the presence of Alg and Alg/HA hydrogels (at both HA concentration used) was comparable to that achieved with DMEM F12 (1:1) 10% FBS. The same test was performed on hydrogels loaded with 10 ppm of NS and also in this case the results indicated that the NS-loaded systems produced did not impair cell viability (fig. y).

[248] {D'Esposito, 2012 504 /id}

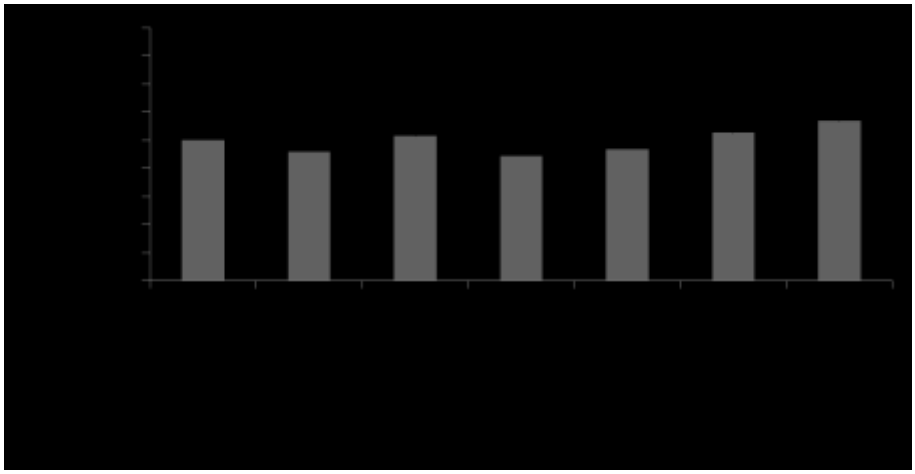


Figure 33. Effect of NS loaded Alg hydrogels on Ad-MSCs viability**.** Ad-MSCs have been loaded at 50,000 cells per insert in the lower chamber of a 24-transwell culture system (0.4μm pore polycarbonate membrane). The following day, Alg hydrogels (ALG), ALG/NS hydrogels, ALG/HA hydrogels (ALG/10% HA- ALG/ 20% HA) or ALG/NS/HA 10%-20% hydrogels, have been added in the upper chamber of the transwell in DMEM F12 (1:1) 10% FBS. Upon 24 hours, cell viability has been determined, as optical density, by sulforhodamine assay. The results have been reported as percentage of viable cells compared with cells incubated in DMEM F12 (1:1) 10 % FBS in absence of hydrogels, considered as 100% viable cells. Bars represent the mean ± standard deviation of triplicate determination in three independent experiments.

7.3. Effect of nanosilver (NS) and Alg/NS hydrogels on Ad-MSC motility

Next, to investigate whether NS and Alg/NS hydrogels could affect cell motility, a wound healing assay was performed. Confluent monolayers of Ad-MSCs were scratched and incubated with 10 ppm of NS and NS-loaded hydrogels in medium without serum supplementation (DMEM F 12 -0.25% BSA). Images were taken at 0 and 24 h after wounding. As shown in fig. z, serum induced an almost complete wound closure. Even in the absence of serum, NS, Alg and Alg/NS significantly promoted wound closure compared to serum free medium (figure 34). Very Interestingly Alg/HA20 induced a higher extent of wound closure which was only partly reduced by NS incorporation (figure 34). Thus, NS and ALG/NS hydrogels did not interfere with cell viability, but improved cell motility depending on the presence of NS and HA.

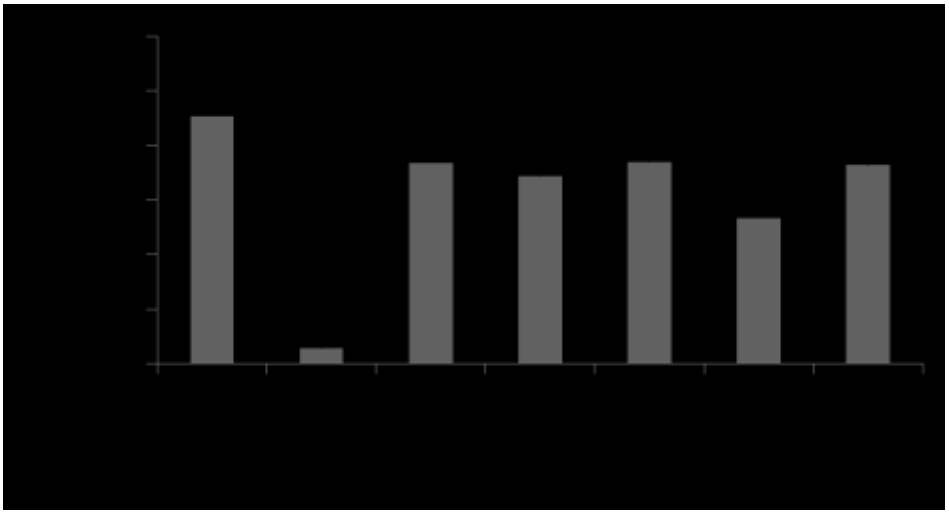


Figure 34. Effect of NS and Alg/NS hydrogels on Ad-MSCs motility**.** Confluent monolayers of Ad-MSCs, grown in DMEM F12 (1:1) 10% FBS, have been incubated with serum free medium (DMEM F12 1:1 0.25% BSA) over night. The following day, the cells have been subjected to scratch assays, washed twice with phosphate buffered saline (PBS) and incubated at 37°C in presence of 10 ppm NS, Alg hydrogels (ALG), ALG/NS hydrogels, ALG/NS/HA20 hydrogels in DMEM F12 (1:1) 0.25% BSA. Images of wound gap have been taken at 0 and 24 h by a digital camera coupled to the microscope and percentage of closure was calculated with NIH IMAGE J. The results have been reported as percentage of wound distance at 24h compared with the starting point (time 0). Bars represent the mean ± standard deviation of triplicate determination in three independent experiments. Asterisks denote statistically significant differences respect to cells in DMEM F12 (1:1) 0.25% BSA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

7.4. Antimicrobial activity

The antibacterial activity of NS at 2, 10 and 50 ppm indicated as NS2, NS10 and NS50, respectively was tested to search for the minimal bactericidal concentration [MBC] to be used in subsequent tests with NS-loaded hydrogels. At all NS concentrations strong antibacterial effects against *P. aeruginosa*, *E. coli*, *S. aureus* and *S. epidermidis* at 24 h were observed, whereas only NS10 and NS50 resulted active against the same bacterial strains at 48 h (figure 35). Therefore, the higher concentrations were used in all subsequent experiments with hydrogels.

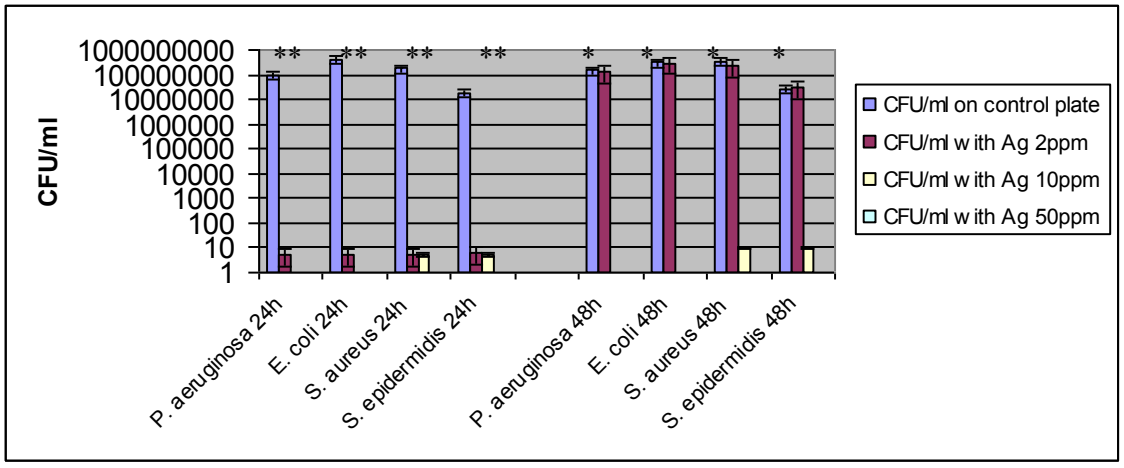
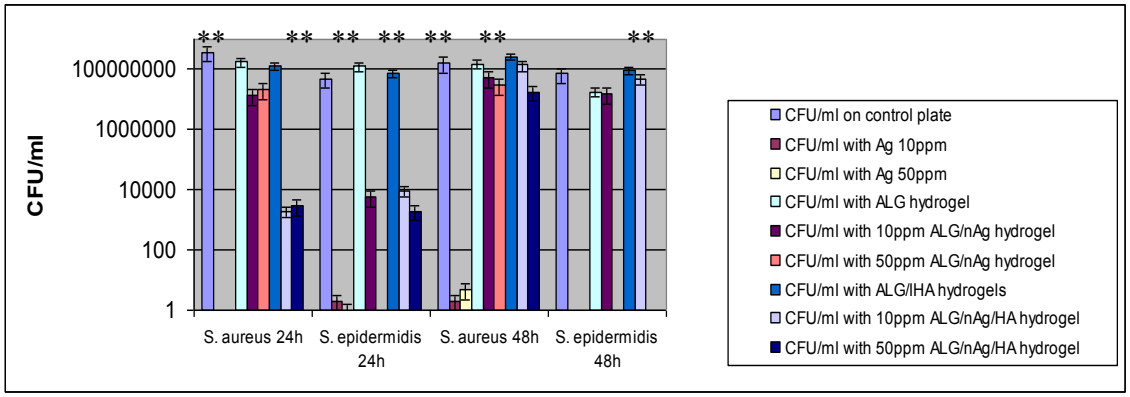


Figure 35. Colony forming units (CFU)/ml of viable bacteria after 24h and 48h incubation with different ppm of silver solution. Significant differences (P values < 0.05) in CFU/ml in comparison to control are indicated with asterisks. Data are shown as means of three independent experiments with SD indicated by the error bar.

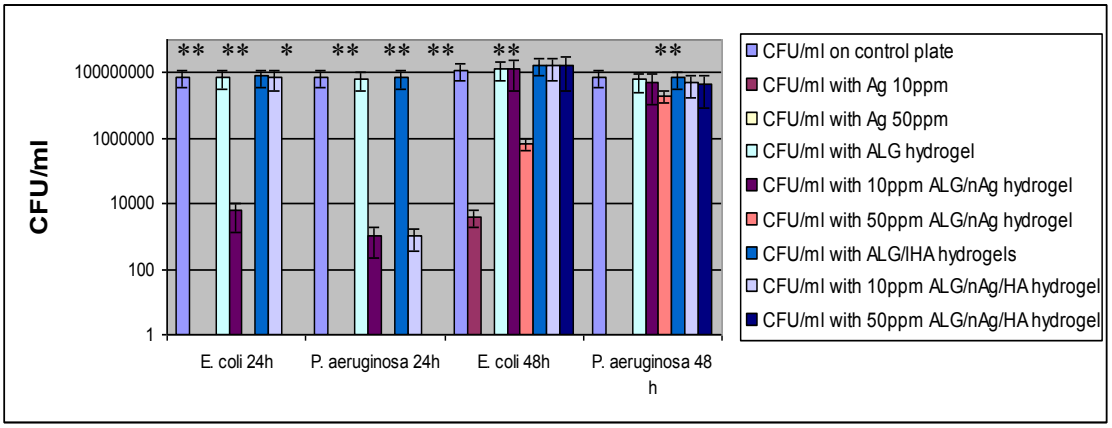
Figure 36 (A) shows the antibacterial effects of Alg/NS and Alg/NS/HA20 hydrogels on Gram-positive bacteria. Both 10 and 50 ppm ALG/NS/HA20 hydrogels showed bactericidal activity against *S. aureus* after 24 h incubation, whereas hydrogels were ineffective at 48 h. Alg/NS and Alg/NS/HA20 hydrogels significantly reduced the viable counts of *S. epidermidis* after 24 h of treatment with all the concentrations tested, but only Alg/NS50 and ALG/NS50/HA20 hydrogels were bactericidal at 48 h.

Figure 36 (B and C) shows the antimicrobial activity of Alg/NS and Alg/NS/HA20 hydrogels on Gram-negative strains. At both 10 and 50 ppm, NS-loaded Alg and Alg/HA20 hydrogels showed bactericidal effects on *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* after 24 h treatment; the total number of *E. coli* cells was significantly reduced after 24 h incubation at both NS concentrations, but only ALG/NS50/HA20 hydrogel showed antibacterial activity in comparison to the control. After 48h treatment, ALG/NS and ALG/NS/HA hydrogels were ineffective; only the *A. baumannii* viable cells were significantly reduced by 50 ppm NS-loaded ALG hydrogel.

A)



B)



C)

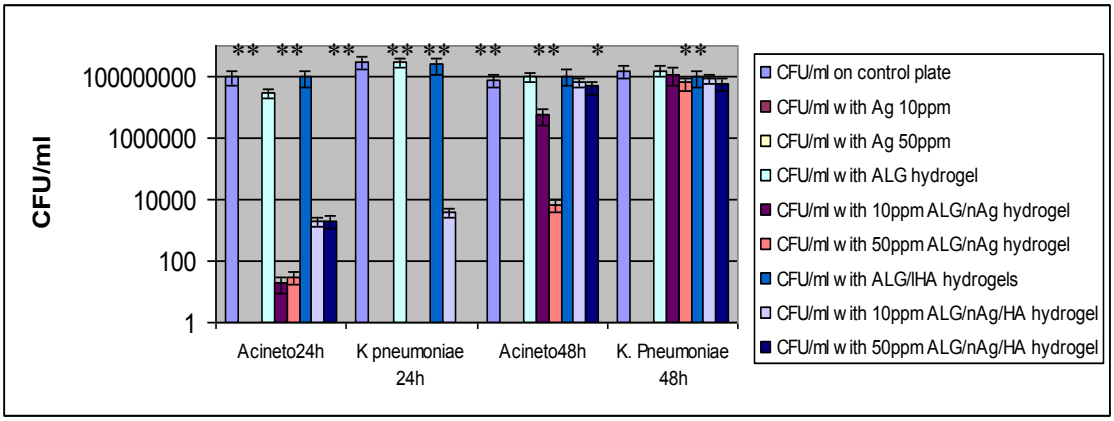


Figure 36. Colony forming units (CFU)/ml of viable bacteria (A: *S. aureus* and *S. epidermidis*; B: *E. coli* and *P. aeruginosa; C: A. baumannii and K. pneumoniae*) after 24h and 48h incubation with different ppm of silver solution and ALG/NS/HA and ALG/NS hydrogels. Significant differences (P values < 0.05) in CFU/ml in comparison to control are indicated with asterisks. Data are shown as means of three independent experiments with SD indicated by the error bar.

There are two possible explanations to why gram-positive bacteria are less susceptible to Ag^+ than gram-negative bacteria. The first involves the charge of peptidoglycan molecules in the bacterial cell wall. Indeed, gram-positive bacteria have more peptidoglycan than gram-negative bacteria because of their thicker cell wall, and because peptidoglycan is negatively charged and silver ions are positively charged, more silver may get trapped by peptidoglycan in gram-positive bacteria than in gram-negative bacteria. [249]The decreased susceptibility of gram-positive bacteria can also simply be explained by the fact that the cell wall of gram-positive bacteria is thicker than that of gram-negative bacteria.

7.5. Wound healing effect of Alg/NS hybrid hydrogels

Evaluation of wound healing process induced by hydrogels was evaluated by the excision wound model in rats (figure 37).

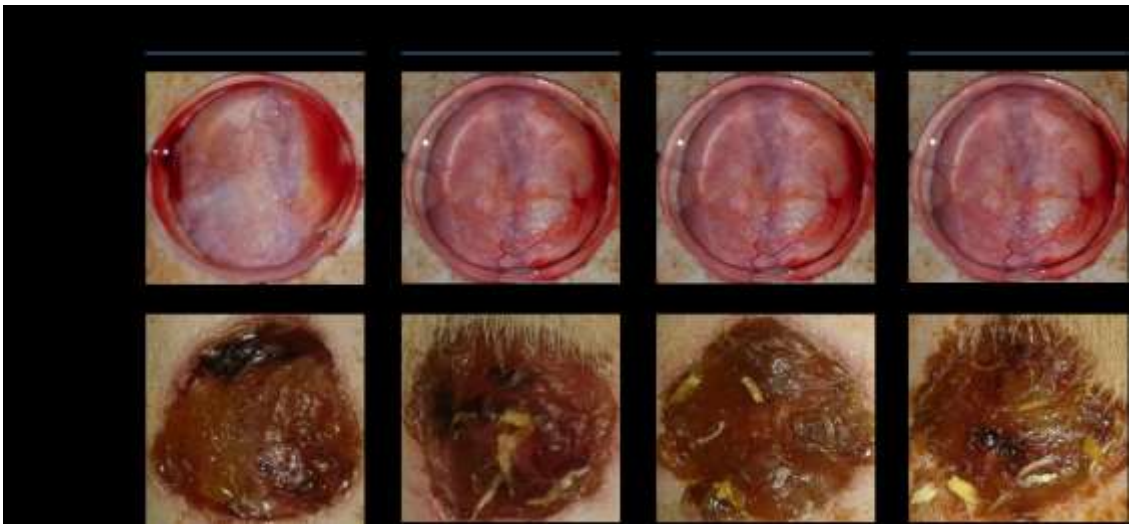


Figure 37. Macroscopic appearances of skin wounds treated with different Hydrogels at day 0 and 14 in excision wound model.

Wound closure is reported for each group as percentage of the reduction in wounded area at day 0, 1, and 5 (Figure 38).

[249] {Kawahara, 2000 672 /id}

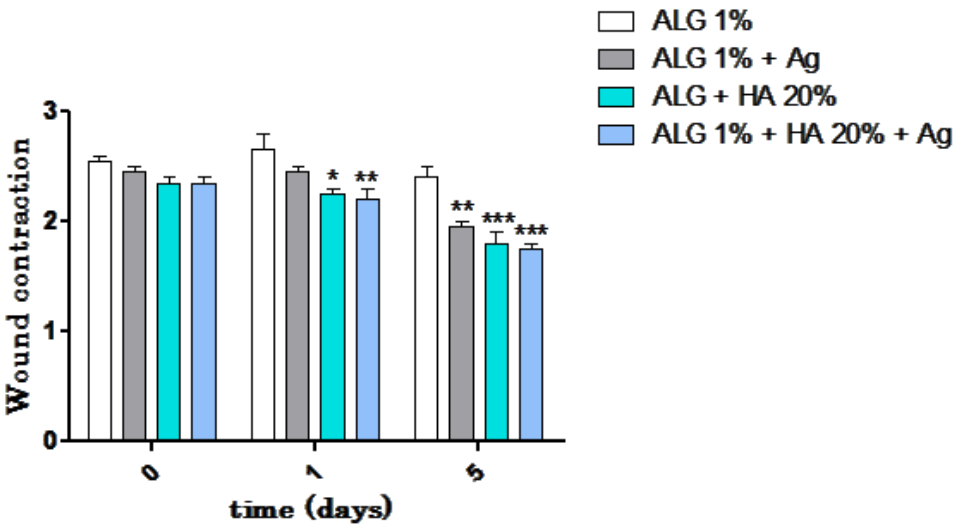


Figure 38. Effects of Alg hydrogels on wound healing process in a rat model. *P<0.05; **P<0.01; ***P<0.001 vs ALG.

All experimental groups began to show reductions in open wound area from day 1 (Figure 38). By day 5, the wound area reductions observed for Alg/NS50/HA20 hydrogel were significantly greater than with the untreated control group. As compared to the Alg treated group, Alg/NS50 had no significant effect on wound area reduction at day 1, while the formulation containing HA significantly reduced wound area. After 5 days all the tested formulations have a significant action on wound healing, with Alg/HA20 and Alg/NS50/HA20 hydrogels showing the morst pronounced effect.

Conclusions

Silver is used from many centuries as antimicrobial agent in wound healing, but recently new studies highlighted his influence on wound healing. The bactericidal mechanisms of silver nanoparticles (NSs) were intensively studied and experimental evidence show that a very small particle size is important for the antimicrobial effects. In this work NS was dispersed in an alginate hydrogel to obtain an biomedical device with a direct action on wound healing. The internal gelation technique allow as to obtain strong tridimensional hydrogels with good handling characteristics. Moreover, the adding of hyaluronic acid, a natural polysaccharide whose seems to have a crucial role in wound repair, was investigated to enhance the wound healing effects. The presence of hyaluronic acid is directly correlated to hydrogels physical properties while NS don't interfere with them. Alg/NS/HA hydrogels seem to have a strong antimicrobial activities against gram+ and gram- bacteria without any effect on cell viability. *In vitro* tests showed that Alg/NS/HA composite hydrogels can improve cell motility depending on the presence of NS and HA. In the *in vivo* experiments, Alg/NS/HA composite exhibited good wound healing activity in full-thickness excision wound model in rats. Overall, our results suggested that combination of bioactivity of hyaluronic acid and the antimicrobic activities of NS promoted tissue reconstruction processes, indicating that Alg/NS/HA hydrogels are a potential wound dressing for cutaneous wound healing.

CHAPTER IV

IN SITU FORMING ALGINATE HYDROGELS CONTAINING A TEA TREE OIL NANOEMULSION FOR WOUND HEALING

1 Introduction

From centuries wound dressings are used to protect the surface of the wound from microbial contamination. Even though wound dressings such as plasters and bandages can protect the wound, they may also cause sufferings and pains to the patient during the dressing and removal processes.

Alginates (Alg) are generally referred to as a family of polyanionic copolymers derived from marine kelp, mainly the brown sea algae[250], and are of growing importance in the healthcare and pharmaceutical industry.[251] The various applications of Alg gels have exploited the wide range of gelling approaches, physical properties, cell adhesion, and degradation behaviour of this family of materials.[252] The ability of Alg to form crosslinks in presence of divalent ions has permitted the development of biocompatible hydrogels that helps to maintain the lesion at an optimum moisture content and healing temperature.[253] Sodium Alg, in combination with other biopolymers or active agents, is widely used as film dressings to improve wound healing and prevention of burns infection in human.[254,255]

Hydrogels are a network of polymers filled with water that may be applied to absorb wound exudates and protect wounds from secondary infection. Furthermore, hydrogels can be readily removed from the wound site by washing with water. An *in situ*–forming hydrogel (ISG) is initially fluid at room temperature but becomes a fixed gel *in situ* due to specific conditions, including ionic cross-linking, pH, and temperature change.[256] ISGs have some merits over traditional dressings, including conformability without wrinkling or fluting of the wound bed, ease of application, good patient compliance, and comfort. With these characteristics, ISGs are

[250] {Laurienzo, 2010 659 /id}
[251] Boateng
[252] {Lee, 2012 36 /id}.
[253] (Peng et al., 2012)
[254] {Brachkova, 2011 637 /id}
[255] {Dantas, 2011 641 /id}
[256] {Ruel-Gariepy, 2004 667 /id}

the optimal choice to prepare a multifunctional wound dressing. Polysaccharide ISGs obtained by simultaneous and alternate spraying of polyanions and polycations (layer-by-layer, LbL) are gaining in interest because they allow to quickly obtain highly uniform thin film over a large surface area.[257,258] The alternate polysaccharide deposition on a solid substrate represents an appealing option to applicate ISGs, because it is much faster and easier to adapt at an industrial level.[259] This type of systems can deliver preferentially hydrophilic molecules inside wound bed.

Tea tree oil (TTO) is a natural essential oil steam-distilled from the Australian native plant *Melaleuca alternifolia.* TTO natural composition is well-characterized by approximately 100 constituents, the majority being monoterpene and sesquiterpene hydrocarbons and their alcohols.[260] TTO is a very promising agent for the treatment of dermatologic disease due his antimicrobial effects against a wide spectrum of microorganisms[261,262] and minimal impact on developing resistance.[263] TTO also has potent activity against many fungi,[264,265] protozoa,[266,267] and certain viruses, including herpes simplex and influenza viruses.[268,269] Besides the well-known antimicrobial activities, TTO has been shown to possess a number of other therapeutic properties, including anti-inflammatory activities [270,271,272] and anti-tumor properties,[273] especially in the skin cancer.[274,275] Hydrogels containing TTO were already tested in burn wound model and seams increasing the rate of wound healing.[276] The physical characteristics of TTO present certain difficulties for the formulation of essential oils based products, thus limiting applicability. In particular their lipophilicity leads to miscibility problems in water-

[257] {Cado, 2012 638 /id}
[258] {Schlenoff, 2000 669 /id}
[259] {Schaaf, 2012 668 /id}
[260] {Brophy, 1989 115 /id}
[261] {Pazyar, 2013 664 /id}
[262] {Carson, 2006 639 /id}
[263] {Hammer, 2012 653 /id}
[264] {Hammer, 2003 651 /id}
[265] {Hammer, 2004 652 /id}
[266] Carson, 2006
[267] {Mikus, 2000 661 /id}
[268] {Garozzo, 2011 649 /id}
[269] {Carson, 2001 44 /id}
[270] {Hart, 2000 43 /id}
[271] {Koh, 2002 657 /id}
[272] {Pearce, 2005 665 /id}
[273] {Bozzuto, 2011 636 /id}
[274] {Greay, 2010 650 /id}.
[275] {Ireland, 2012 654 /id}
[276] {Jandera, 2000 655 /id}

based products, while its volatility means that the encapsulation method must provide an adequate barrier to volatilization.

Nanoemulsions (NE) are of great interest in drug delivery, as they represent a convenient way to encapsulate hydrophobic drugs, and have emerged as novel vehicles for sustained or controlled release for many administration routes of drugs.[277] NEs appears as an attractive and competitive system due to many benefits such as easy manufacturing, small droplet size (20−200 nm), high thermodynamic stability, and enhanced solubilisation for hydrophobic ingredients. In recent years, nanoemulsions have been investigated as potential drug delivery vehicles for transdermal and dermal delivery of many compounds especially hydrophobic compounds in order to avoid clinical adverse effects associated with oral delivery of the same compound.[278] Furthermore, nanoemulsion droplets can be easily incorporated into Alg hydrogels to create composite hydrogels with a controlled release profile.[279] Nanoencapsulation can represent a viable and efficient approach to increasing physical stability of essential oil, protecting them from undesired interaction and increasing their bioactivity. In particular Donsi et al.[280,281] demonstrated that NEs can enhances the antimicrobial activity of encapsulated essential oil increasing their water solubility and the consequent capacity to interact with cell membranes.

The objective of the study was to develop antimicrobial *in situ*-forming Alg wound dressings incorporating TTO. Alg hydrogels were prepared by a layer-by-layer sparay deposition method with the aim to minimize the discomforts especially during dressing application. After characterization of TTO NE and TTO NE-loaded Alg hydrogels, their antimicrobial effect was tested.

[277] {Fanun, 2012 648 /id}
[278] {Shakeel, 2012 670 /id}
[279] {Josef, 2010 656 /id}
[280] {Donsi, 2011 642 /id}
[281] {Donsi, 2012 643 /id}

2 Experimental

2.1 Materials

Pharmaceutical grade sodium Alg acid sodium salt (Alg) extracted from *Laminaria hyperborea* (viscosity 360 cps) and Tea Tree Oil (TTO) were supplied by Farmalabor (Italy). Polysorbate 80 (Tween® 80), calcium chloride dihydrate ($CaCl_2$ * $2H_2O$), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), and nile red (NR) were obtained from Sigma-Aldrich (USA). Analysis-grade ethanol was provided by Carlo Erba reagenti (Italy). The spray pump used for the spray deposition (Classic line equipped with SL pump) was kindly gifted by Aptar Pharma (France). The water used throughout this study was depurated and filtered (Milli Q filter).

2.2 Pseudoternary Phase Diagrams

The boundaries of the NE domains were determined, with the aid of pseudo ternary phase diagrams for the polysorbate 80:ethanol, TTO and water systems, at different ethanol:polysorbate 80 volume ratios. A titration method was employed for the construction of phase diagrams. Different mixtures of polysorbate 80 and ethanol (1:1, 2:1 and 3:1) were weighed in a dark-brown, screw-cap glass vial, mixed using a magnetic bar on a stirring plate for 1 h and subsequently stored overnight at room temperature. TTO was then added at ratios ranging from 9:1 to 1:9 to different vials. Finally, aqueous phase was slowly added (under vigorous stirring) with a graduated syringe up to clouding of homogenous mixture of oil and surfactants:co-surfactants. Approximately 10 data points were obtained to determine each pseudoternary phase diagram. No attempts were made to completely identify the other regions of the phase diagrams in detail, and these have been described in terms of their visual and external appearance. To graphically show the phase variations of polysorbate:ethanol–water–TTO system a pseudo-ternary phase diagram was built using Microsoft excel software.

2.3 Preparation of nanoemulsion

Once the nanoemulsion region was identified, a surfactant/co-surfactant mixture was prepared and dissolved in TTO using a magnetic stirring plate. An oil-in-water NE was prepared by slowly adding water to the oil phase (oil plus surfactants) under continue magnetic stirring. To prepare the NR-loaded NE, the probe was dissolved directly into TTO at the concentration of 20 μg/ml. All the formulations were prepared at room temperature and tested after 24 h, the

time necessary for allowing equilibrium. Resulting mixtures were visually inspected and turbidity was measured using a spectrophotometer.

2.4 Characterization of nanoemulsion

Droplet size and polydispersity index (PDI) of NE was measured by a zetasizer NanoZs (Malvern instruments, UK). NEs were diluted 1:10 with deionised water prior of the experiment to do away with the effect of viscosity caused on account of emulsion ingredients and also to trim down multiple scattering effect. Particle size and PDI measurements were performed at a scattering angle of 90◦and at a temperature of 25°C.

NEs droplet morphology was visualized by Transmission Electron Microscopy (TEM) Sample for TEM analysis was prepared by placing one drop of NEs onto a copper grid. After approximately 1 hour images were captured by a transmission electron microscope. TEM analysis was performed with a FEI Tecnai G12 (LAB6 source) equipped with a FEI Eagle 4 K CCD camera (Eindhoven, The Netherlands) operating with an acceleration voltage of 120 kV.

Turbidity of the formulated NEs was evaluated on a UV/VIS spectrophotometer (UV 1800, Shimadzu, Japan) at 502 nm fitted out with a 1 cm quartz cell (Hellma, Germany). The turbidity was calculated as turbidity × path length = 2.303 × absorbance {Fletcher, 1995 35 /id}.

The pH of each formulations were evaluate using a crison basic 20 pHmeter equipped with an elecrode 50 10T (crison, Spain).

2.5 Stability studies

2.5.1 Centrifugation study

The formulated NEs were studied for their resistance to centrifugation. All the formulations were subjected to centrifugation (mikro 20, Hettich, Germany) at 13,000 rpm for 30 min and observed for phase separation, creaming and cracking (if any).

2.5.2 Heating–cooling cycle

This study was performed to check the effect of temperature variations on the stability of NEs formulations. Samples were stored between 4 and 40 °C for a period of 48 h at each temperature. The heating–cooling cycle was repeated four times. The NEs formulations that did

not show any instability such as cracking, creaming and phase separation were chosen and subjected to freeze–thaw stress.

2.5.3 Freeze–thaw cycle

In this study, NEs formulations were subjected to freeze–thaw stress between −21 and +25°C, with storage at each temperature for a minimum period of 48 h. Three freeze–thaw cycles were performed and the formulations that resulted to be stable to this stress were further characterized and preferred for application study.

2.5.4 Kinetic stability

NEs formulations were preserved at room temperature for checking out the intrinsic stability. The emulsion formulations were observed for phase separation, creaming and cracking with respect to prolonged storage time period. Kinetic stability was investigated by measuring droplet size of the NEs at different intervals of time.

2.5.5 Dilution test

The dilutability of the NEs studied was assessed to know whether these systems could be diluted with the external phase of the system without phase separation. In an effort to imitate the dilution process that occur when the NEs loaded hydrogel were prepared, the selected NEs were diluted with water in proportions of 1:1 and 1:10. Dilution was performed with vigorous vortexing for 5 min at ambient temperature and assessed visually for transparency and size for a period of at least 48 h. Diluted systems were considered as NEs if they maintained their physical integrity.

2.6 Hydrogels preparation and characterization

Hydrogels were obtained by spraying-deposition method using a pharmaceutical grade spray pump. The selected pump is calibrated to dispense exactly 140 μL of an Alg solution at 1% w/v in each actuation. The spraying-deposition procedure was standardized as follows: a 1% (w/v) Alg water solution was sprayed twice on a lightly wet glass petri dish (36 mm diameter), followed by a single spray of a 2% w/v $CaCl_2$ aqueous gelling solution. A thin layer of hydrogel formed immediately. Three layers were deposited one above the others for each preparation.

The hydrogels containing NeTTO were prepared by pre-dispersing 20 mL of NEs in 100 mL of Alg solution under magnetic stirring.

Hydrogel homogeneity was determined by the dry to wet weight ratio method.[282,283] Hydrogels were cut in four slices (labeled A-D as shown in Figure 39) and weighed on analytical balance. The specimen were dried to constant weight (60° C for 24h under vacuum) and weighed again. The calculated dry to wet weight ratio provides an indication of gel homogeneity. The reported data are the average of three samples ± standard deviations.

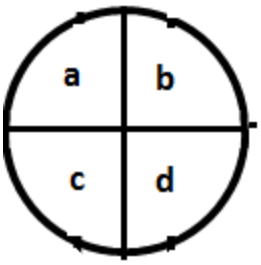


Figure 39. Schematic representation of hydrogels division for homogeneity evaluation.

Water content was determined gravimetrically on hydrogels as obtained by spray deposition. The initial weight of each sample was recorded using an analytical balance. Successively, the hydrogels were frozen overnight at −40°C, and then lyophilized at 0.01 atm and -60°C in a Modulyo apparatus (Edwards, UK). After lyophilization the samples were reweighed and the water content (WC%) was calculated using the equation:

$$WC\% = \frac{W - W_0}{W_0} \times 100$$

where W is the mass of the freeze dried sample and W_0 is the mass of the initial wet sample. The weight loss, expressed as %, corresponds to water content.

Degradation of hydrogels containing NEs was followed through an modified Enslin apparatus (see chapter II) designed for mimic the wound bed. Hydrogels were placed on a porous glass filter and maintained in constant contact with an hydration medium to simulate biological fluids in tissue injuries. As simulated fluid we employing Phosphate Buffer Saline (PBS, NaCl 120 mM, KCl 2.7 mM, Na_2HPO_4 10 mM) at pH 7.4, lactate solution at pH 4.8, or a

[282] Kuo, 2001
[283] {Alexander, 2011 494 /id}

saline solution. Three replicates were performed for each sample and photos were taken occasionally during the experiment.

3 Results and discussion

3.1 Phase diagrams

Polysorbate 80 is a nonionic surfactant (HLB=15) giving NEs without the aid of a co-surfactant but over only a very limited range of concentrations. In preliminary experiments we found that the maximum amount of TTO that could be incorporated in the NEs was 10% (w/w) with a total surfactant content of 80–85% (w/w) (Data not shown). The use of a non-ionic surfactant, as the Polysorbate 80, alone makes difficult the spontaneous formation of the zero mean curvature lipid layers, necessary for NEs formation. For this reason to achieve the ultra-low interfacial tensions necessary for the formation of the small droplets an high amounts of surfactant is required. To extend NEs area, a co-surfactant such as a short chain alcohol has been traditionally employed because alcohols are able to increase the hydrophilic–lipophilic balance of Polysorbate 80 by decreasing the hydrophilicity of the polar solvent (external aqueous phase).[284] The presence of a short chain co-surfactant allows the interfacial film sufficient flexibility to adopt the different curvatures required to form NEs.

A pseudo-ternary phase diagram, that describes the experimental conditions in which the components must be combined to form clear preparations is of relevance to characterize NE systems. Pseudo-ternary phase diagrams composed of polysorbate 80 (S), ethanol (used as co-surfactant, CoS), TTO (termed the oily phase) and water (W) were constructed at room temperature to show the relationship between composition and phase behavior of the samples (Figure 40). The construction of phase diagrams makes easy to find the concentration and ratio of the components allowing the existence of a range of NEs.

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[284] {Moreno, 2003 662 /id}

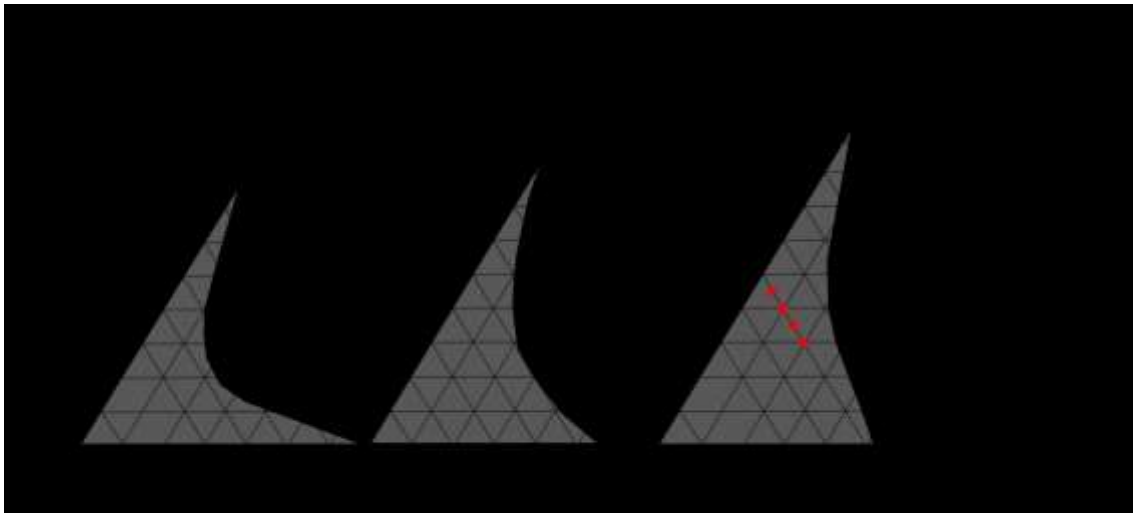

Figure 40. Phase diagrams obtained at different S:CoS volume ratios. W = water phase, O= oil phase, S/CoS = Surfactant/co-surfactant

For the selected components, the shaded areas of the pseudo-ternary diagrams in Figure 2 show the regions allowing stable (and transparent) NEs formation. In all the cases, the areas of stable NE formation extended over a more or less limited area in the S/CoS-rich part of the phase diagram. Using different S/CoS ratio gave a smaller zone of stable NE formation caused by the effect of ethanol on interface fluidity and consequently hydrophilic–lipophilic balance (HLB). The HLB describes the simultaneous attraction of the surfactant mixture for the oil and aqueous phases, so when it is similar to the required HLB of the NE oil phase, the system provides the minimum energy condition for NE formation. The polysorbate 80/ethanol 1:1 (v/v) ratio was used to produce stable NEs in order to obtain the largest NE formation area. In this particular region four formulations were selected in order to obtain oil-in-water systems. The remaining region of the phase diagram allows to form conventional emulsions..

The red dots shown in figure 2 represent the specific composition of the formulations giving different TTO concentrations. The data of the pseudo-ternary phase diagram shows that it is possible to add a large volume of aqueous phase and a limited volume of the oily phase maintaining the thermodynamic stability of the O/W systems.

3.2 Characterization and stability of NEs

Based on the phase diagrams, four NEs were selected for further investigation. This formulation differed in the amount of oil included in the internal phase (Table 1).

Light scattering is a routine technique to determine the diameter of the internal phase of NEs. The light scattering measures were made for liquid NEs, which were diluted with aqueous buffer in order to avoid experimental errors due to short interparticle space between globules.[285] The mean diameters and the PDI of selected NEs are shown in table 1.

Table 9. characteristic of the spray deposited hydrogels

Formulation	W/S-CoS composition (%)	TTO (%)	Diameter (nm ± D.S.)	Polydispersity index	Turbidity (%)	pH
NeTTO20	30/50	20	21.95 ± 0.20	0.232±0.012	1.74	5.6 ±0.1
NeTTO15	35/50	15	18.31 ± 0.67	0.209±0.038	3.39	5.7±0.1
NeTTO10	40/50	10	14.06 ± 0.33	0.206±0.060	2.82	5.8±0.1
NeTTO5	45/50	5	11.92± 0.04	0.211±0.054	3.56	5.9±0.1

TTO-loaded NEs showed a very small particle size and narrow size distribution, as determined by light scattering analysis. The final TTO concentration influenced the average size which increased as TTO amount increased. Low polydispersity (~0.2) was indicative of homogeneous monodispersed particles. Morphology of NEs was visualized by TEM (figure 41). Emulsion droplets were spherical in shape and the droplet size was confirmed in nanometric range.

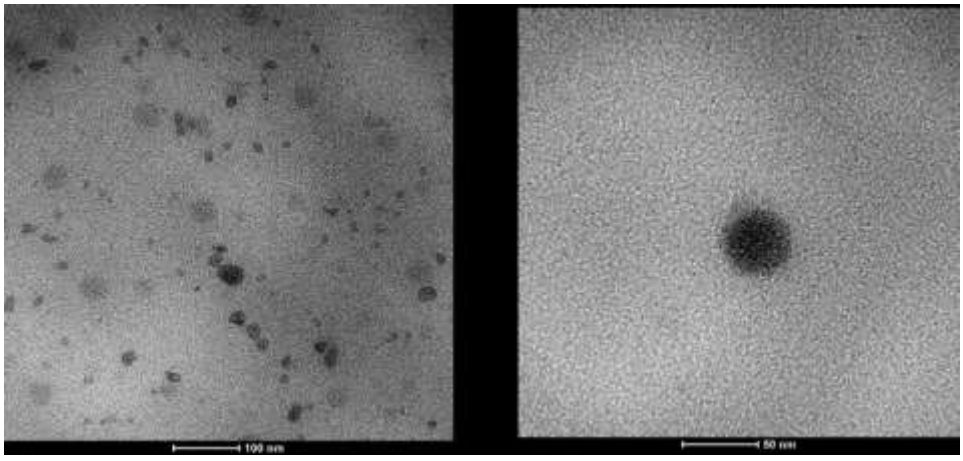

Figure 41. Droplet size of NeTTO20.

[285] {Orthaber, 2000 663 /id}

The pH value for all formulations are within the physiological range.

NEs are thermodynamically stable by definition but even so accelerated testing methods are required for evaluation of long term stability. Thermodynamic stability of NEs formulations prepared with different oil concentrations were investigated by visual inspection and size determination after centrifugation, freeze–thaw and heating–cooling cycles. The centrifugation test allowed the evaluation of the absence of phase separation after mechanical stress. After centrifugation all NEs were found to be stable, as no change in any of their properties was observed. Another mean to test the NEs stability is to subject them to rapid temperature changes. All the systems underwent freeze–thaw and heating–cooling cycles, remained stable since no significant changes in droplet size were observed after the thermal treatments (data not shown). Kinetic stability of NEs was studied by storing the formulation for a prolonged period time at room temperature. After 3 months the size remains constant and no sign of phase separation was found (figure 42).

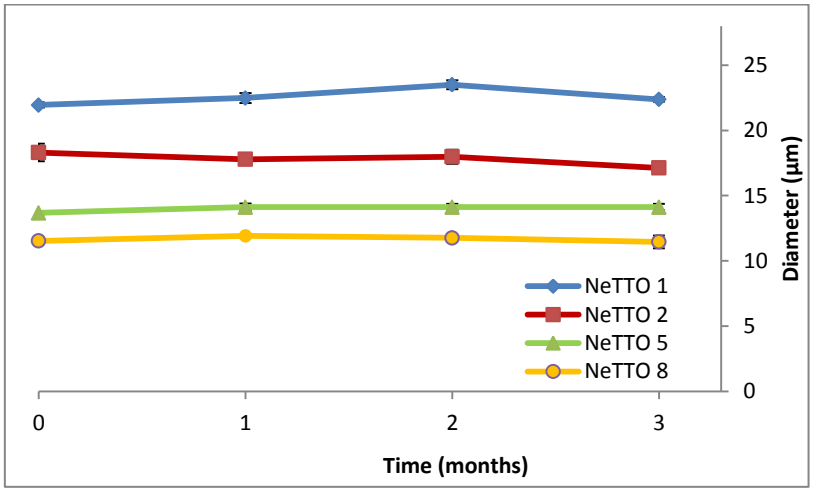

Figure 42. Kinetc stability of tea tree oil nanoemulsion.

NE stability to dilution was assessed to verify their capacity to be dispersed in the Alg solution employed to produce hydrogels. The dilution test performed in water showed that all the NEs studied in this work remained stable for at least 2 days at room temperature after being diluted, because no physical changes apparently occurred during this period of time.

3.3 Hydrogels preparation and characterization

Hydrogels were obtained by alternate spraying of Alg and $CaCl_2$ solutions. The spray deposition technique allows to realize thin coatings as well as thick hydrogels by cumulative

spray. Hydrogels are transparent (Figure 43), with an irregular top surface but a quite regular thickness and diameter (Table 9).

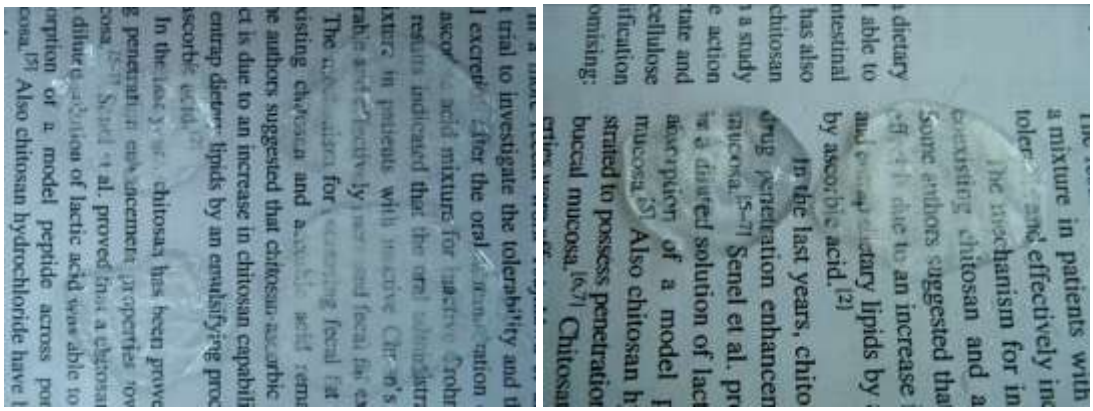

Figure 43. Left: Alg hydrogel, Right: Alg/NeTTO20 hydrogel.

The spray procedure to obtain Alg *in situ* forming hydrogels was standardized as a function of two parameters: Alg and $CaCl_2$ solution concentration and number of relative Alg/$CaCl_2$ sprays. Alg concentrations higher than 1% w/v were e not suitable for the spray deposition technique, due to their high viscosity. Different $CaCl_2$ concentrations were tested (1, 2, 4, 6, 8%), being 2% the most suitable to give adequate gelation time and homogeneous gels. As a matter of fact, 1% $CaCl_2$ was not enough to give a solid gel, while at concentration > 2% a too fast gelation occurs causing surface shrinking. Excessively rapid gelation causes the formation of an irregular surface upon which successive spray layers give rise to a stratified, not homogeneous final gel. The optimal conditions were set as follows: two sprays of 1% Alg followed by one spray of 2% $CaCl_2$. Thicker and homogeneous gels were obtained by cumulative spray. The number of consecutive layers was set to three in the following. The gels were let to complete gelation for 30 min before further characterization.

Weight, thickness and diameter of freshly-prepared hydrogels are reported table 10. The values are averaged on five specimens and averages and standard deviations are reported.

Table 10. Properties of Alg and Alg/NeTTO20 hydrogels.

Hydrogel	Weight (g)	Thickness (mm)	Diameter (cm)	Water content (%)
Alg	1.250 ± 0.025	1.41 ± 0.43	2.53 ± 0.05	72.56 ± 2.35
Alg/NeTTO20	1.154 ± 0.05	0.96 ± 0.12	2.73 ± 0.06	61.14 ±1.29

Structural uniformity of spray hydrogels is crucial for biomedical applications not only from a drug delivery viewpoint to achieve uniform distribution of the drug in the matrix, but especially to get good material properties. The homogeneity of Alg and Alg/NeTTO20 hydrogels was estimated by the dry/wet weight ratio method. A constant weight ratio across the constituent slices is indicative of homogeneous gel. Homogeneity profiles of gels showed no relevant variations between the slices (figure 44), indicating that the spraying-deposition process here described can be applied to produce homogeneous Alg hydrogels suitable for wound dressing. Moreover, the presence of NEs did not significantly affect gel homogeneity in the concentration range studied

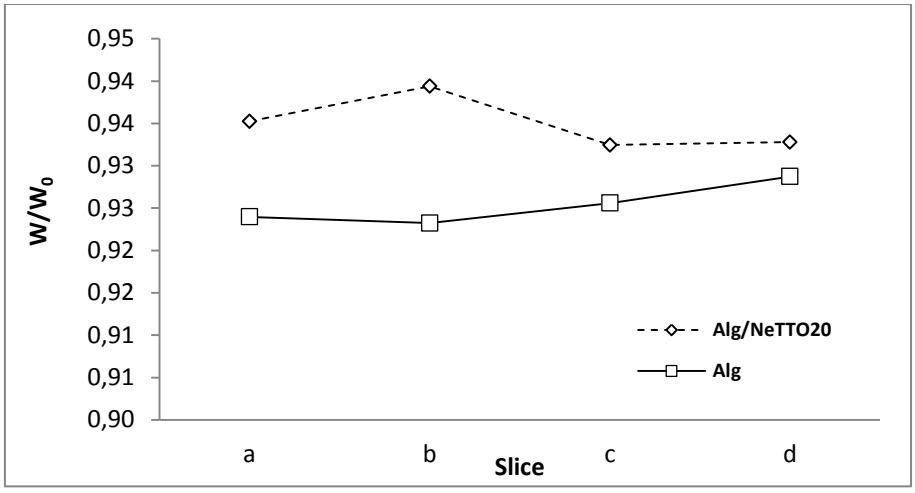

Figure 44. Dry/wet weight ratios of Alg and Alg/NeTTO20 hydrogel slices.

Mean water content, determined on five different preparations of Alg and Alg/NETTO20 hydrogels are reported in Table 2. The decrease of water content in NE-loaded hydrogel is consistent with the reduced water content of the initial Alg solution.

In order to ensure a prolonged and durable protection of the wounds, hydrogels are expected to be stable when in contact with the wound bed for a reasonable time. Degradation has been qualitatively assessed by simulating the wound bed through the experimental system described in section 2.6.3. As the exudate pH in chronic wounds is reported to fall in the 4,8 - 9,8 pH range,[286] solutions with different pH, namely 4,8 and 7,2, were tested. Nevertheless, no

[286] (Britland et al, 2011)

differences were appreciated. Hydrogels swelled in the first 24 h while becoming thinner after 48 h, indicating that some degradation occurred due to exchange of Ca^{2+} ions with those present in the medium.

3.4 Alg/NeTTO composite hydrogels

Alg has been extensively studied as wound dressing material, but as a hydrophilic polymer, it hardly incorporates as such lipophilic liquids such as TTO. Using an o/w NEs we can uniformly disperse TTO into Alg hydrogels and increase its dispersability. The methodology of incorporating NEs droplets in an Alg hydrogel was already investigated by Josef *et al.*[287] who verified the existence of intact NEs droplets in a crosslinked Alg matrix.

To obtain Alg/NeTTO composite hydrogels different amounts of NEs were dispersed in the Alg solution and then cross-linked the solution by spray deposition. Mixing NE with Alg resulted in a clear solution, with no observable phase separation. The addition of a polymer to a NEs system can change its structure, and possibly break the spherical droplets. To verify the presence of intact NEs, their size inside the Alg solution was checked and, as shown in figure 45, only a slight difference in size was detected (the mean size changes from 20 to 26 nm). This data are in agreement with those reported by Josef *et al.*[288] and suggest that the structure of the NEs is unchanged upon mixing with Alg.

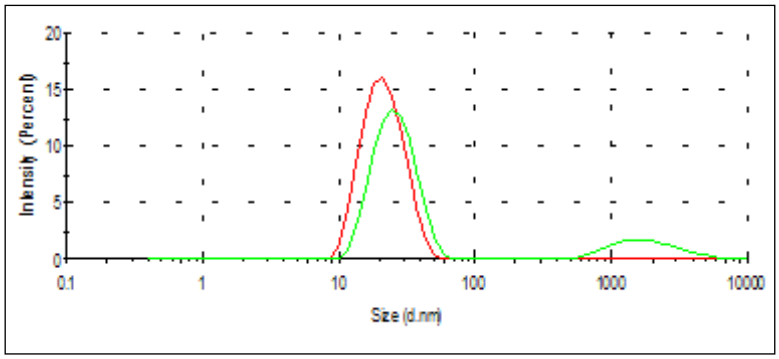

Figure 45. NEs size inside the Alg solution. Red line: NeTTO20; Green line: Alg/NeTTO20

[287] {Josef, 2010 656 /id}
[288] Josef, 2010

4 Conclusions

Tea tree oil is an natural compound with well recognized wound healing properties and for this already used in topical treatments. The methodology of incorporating nanoemulsion droplets into alginate hydrogels is already used to create composite hydrogels and could be applied to improve the TTO solubility and activities. Stable, NEs was formulated using tea tree oil, Tween 80 and water with a very low droplet diameter, directly dependent from the oil ratio. Selected NEs formulations were dispersed in alginate solution and cross-linked to obtain solid hydrogels. The spray deposition technique used to have *in situ* forming hydrogel allow as to have an homogenous composite system with good phisical properties and no phase separation. The existence of intact MEs droplets in the gel was verified and no morphologic change due to NEs dispersion were detected. This finding confirmed that the inclusion of TTO in composite hydrogels hold great potential in spray deposited hydrogel for wound management

CHAPTER V

MELT-SPUN BIOACTIVE SUTURES CONTAINING NANOHYBRIDS FOR LOCAL DELIVERY OF ANTINFLAMMATORY DRUGS

Introduction

Sutures are biomedical devices of natural or synthetic origin used to held together tissues that have been separated due to surgery or traumatic injury. Despite the presence of different devices for wound closure (staples, tapes and glues) available on the market, sutures are the most widely diffused in the medical practice and have a market of around 1.3 billion dollars a year.[289] A suture should fulfil a number of requirements and, unfortunately, no ideal product is available and the surgeon generally operates a selection on the basis of availability and familiarity.[290,291] Nevertheless, an appropriate suture should take into account aspects such as mechanical properties, resorption rate, risk of infection, and inflammatory reactions that may occur during wound healing process. Over the years, new surgical suture materials have been developed to better respond to particular surgical needs. Recently, the research has switched toward a novel concept of medicated suture that includes a bioactive compound which can be released in a defined time frame and help tissue repair.

Research in this area, although being very attractive, has leaded to very few products successfully entering the market.[292,293,294] The first commercial antimicrobial suture, a Polyglactin 910 suture loaded with triclosan, a broad-spectrum antibacterial agent, (Vicryl Plus®) was approved for clinical uses by the US Food and Drug Administration (US FDA) since 2002.[295] The basic concept in these sutures consist in coating a preformed polymeric filament with a second biodegradable polymer layer embedding triclosan with the aim to create a zone of inhibition to the spread of bacteria and to exert a preventive action against the possible infection

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of the surgical site.[296] Nowadays antimicrobial suture are successfully used in a number of surgical procedures[297,298,299,300,301,302] with a reduction of wound site infection and consequent cost saving.[303] For all this reasons this treatment strategy was found to be very promising and sutures coated with other drugs such as antithrombotic, analgesics, antineoplastic and antiproliferative agents are under investigation.[304]

Recently *Lee et al.*[305] have proposed a new method to obtain medicated suture where a commercial suture is covered with a polymeric sheet loaded with a pain relief drug. These sutures have been proven to have suitable mechanical properties and a drug release only for 6 days. Nevertheless, control of drug release rate is a critical factor to design a bioactive suture in view of an optimized biological effect. For this reason, more suitable strategies are needed to attain both efficient control over drug release rate and adequate mechanical properties. As an alternative to coated fibers, electrospun aligned fibers have been developed where different active agents are dispersed in a polymer to give a matrix-like structure.[306],[307] Unfortunately, fibers show weak mechanical properties and electrospinning is difficult to scale-up, making these systems difficult to be applied.

At this regard, a promising alternative is represented by melt-spinning technology. In this case, a polymer melt is forced through a spinneret capillary to obtain fibers with properties strongly related to the applied drawing extent. The application of this process, even if scalable up to industrial level, is limited in the biomedical field where the usual thermolability of bioactive additives as drugs and/or the relatively poor elongational properties of biocompatible polymer melts, furtherly worsened by incorporation of additives, may prevent a satisfactory drawing of fibers compromising their ultimate mechanical properties.

Among the strategies useful to control drug release from a polymer matrix, the inclusion of lamellar structures opens new opportunities to develop smart systems. Recently, magnesium

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and aluminium hydroxycarbonates referred to as hydrotalcite-like compounds (HT) intercalating bioactive molecules have been proposed.[308,309,310,311] These systems consist of a layer of inorganic clays which, under specific conditions, self-organize to form a bilayer. HTs have already been proven to be biocompatible and some of them are already used in clinical practice as antiacids because of their antipepsin activity.[312] In specific conditions, HT can intercalate different anions or biologically active molecules such as anionic non-steroidal anti-inflammatory drugs (NSAID),[313] antibiotics,[314] up to around 50% by weight and form nanohybrids. Depending on drug features (solubility, molecular weight, affinity to HT), fast dissolution or sustained release of the drug can be accomplished as a consequence of a de-intercalation process.[315,316] Furthermore, a body of interest is growing on the development of novel composite systems based on inorganic layered materials and organic polymers. Recent studies report on the possibility to introduce organically-modified HT in different polymers as fillers opening a new way to integration of bioactive HT in polymeric films, membranes or fibers with different potential applications in industrial and biomedical field.[317,318,319] In this context, on the basis of an European patent owned by some participants to this research,[320] *Sammartino et al.*[321] incorporated nanohybrids containing the NSAID Diclofenac (DIC) into poly(ε-caprolactone) (PCL) films and demonstrated effective control of drug release as compared to free drug directly dispersed into the polymer.

Prompted by these considerations, in this paper we offer a proof of principle on the possibility to obtain a sustained-release biodegradable suture through the incorporation of a drug-HT nanohybrid in a thermoplastic polymer. To this purpose DIC-intercalated HT was incorporated in a fiber of poly(ε-caprolactone) (figure 1). The fiber was produced by melt spinning and characterized in terms of morphology, size, mechanical properties, drug release and *in vivo* performance.

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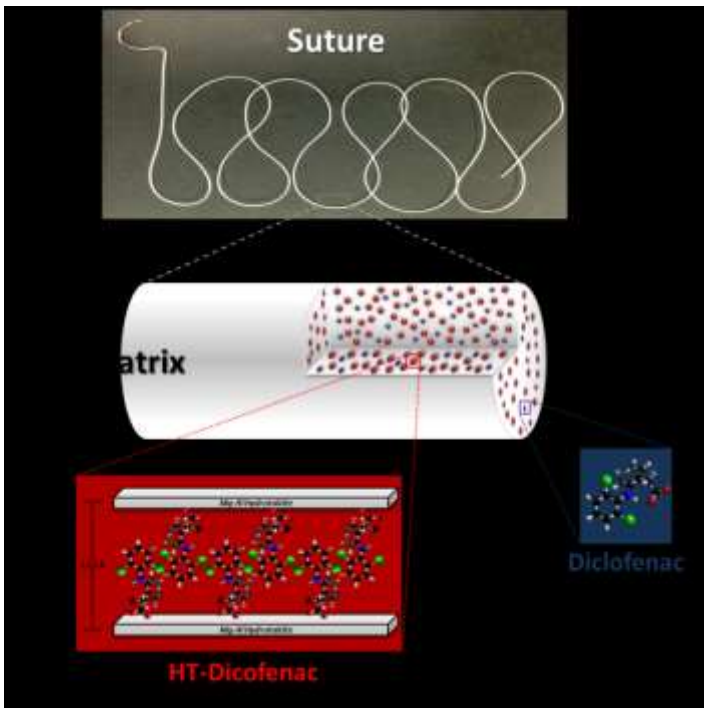


Figure 46. Graphical representation of loaded monofilament.

Materials and methods

2.1. Materials

A nanohybrid containing synthetic hydrotalcite and Diclofenac (HT-Dic, $[Mg_2Al(OH)_6](C_{14}H_{10}Cl_2NO_2)$ * $2H_2O$, Mg/Al Ratio 1.8, distance between layers = 23.6 Å, moisture 7.0 %, Diclofenac loading =59.6 %) was obtained from Prolabin & Tefarm (Italy). Diclofenac sodium (Dic) was purchased from Farmalabor (Italy). Poly(ε-caprolactone), PCL, used for this study (CAPA® 6800) was from Perstorp corporation (UK). HPLC-grade tetrahydrofuran (THF), acetonitrile and methanol, analysis-grade acetone and dichloromethane were from Carlo Erba (Italy). Synthetic hydrotalcite (HT, $Mg_6Al_2(CO_3)(OH_{16})$ * $4H_2O$), sodium chloride, potassium chloride, HPLC-grade trifluoroacetic acid (TFA), sodium phosphate dibasic and Potassium phosphate monobasic (HPLC grade) were obtained from Sigma-Aldrich (USA). Distilled filtered (0.22 μm) water was employed.

2.2. Fiber production

Fibers were prepared through extrusion, drawing and subsequent cold drawing to the final diameter of approximately 300 μm. Prior to the extrusion process, the components were separately sieved to obtain a fine powder (97 % of the powder passed through a #140 sieve with a mesh size of 106 μm according to Ph. Eur. 7th edition). The mean diameter and size distribution of powders was determined by laser light scattering (Coulter LS 100Q, USA). Particle size is expressed as volume mean diameter (μm) ± SD of values collected from three different batches. For Zeta potential measurements, HT-Dic was dispersed in water and analyzed on a Zetasizer Nano Z (Malvern Instruments, UK).

The base materials were mixed in a HAAKE twin screw extruder using a screw speed of 20 rpm and applying a temperature profile going from 60 °C, at feed zone, to 100 °C at the die. The filament was cooled in stagnant air (at 23°C) and collected with a take-up speed of 4 m/min. The as-spun fibers (with a diameter of about 900 μm) were drawn, using a (DSM Xplore Conditioning Unit) with a temperature of 50°C, to the final diameter of about 300 μm (corresponding to a draw ratio of 9).

Different compositions of the fibers, reported in Table 1, were selected in order to investigate the effect of Dic intercalated in HT (Dic/HT) as compared to free Dic (PCL/HT-Dic *vs.* PCL/Dic); ii) the influence of HT in systems containing free Dic (PCL/HT/Dic *vs.* PCL/Dic) and iii) the possible effect of free Dic in fibers involving intercalated Dic (PCL/HT-Dic *vs.*

PCL/HT-Dic/Dic). We fixed Dic loadings in the fiber at 9 and 18% by wt in order to test the system at high loading where control of drug release is generally poor.

2.3. Mechanical properties

Tensile properties of straight and simple knotted (according to Ph. Eur. 7th edition) fibers were measured using a universal testing machine (Alpha Technologies mod. 2020) equipped with a 10 N load cell. All tensile experiments were carried out at a strain rate of 300 mm/min, using a gage length of 150 mm, at 23°C. For each sample, at least five specimens were analyzed (if the fiber broke in a clamp or within 1 cm, the result was discarded and the test was repeated) and average results are reported with their standard deviation. The mean diameter was obtained after three measurements at points evenly spaced along the suture using a 0.001 mm accuracy digital micrometer. Mechanical properties of fibers are expressed in terms of elastic modulus, breaking load, breaking stress and percent elongation at break.

2.4. Scanning electron microscopy/Energy Dispersive X-Ray Microanalysis

Sutures shape and morphology were analyzed by scanning electron microscopy (SEM) (Quanta 200 FEG; FEI, USA). To analyze internal structure of fiber wire sections were prepared. The sample was included into Tissue Tek® OCT (Sakura, Japan) and then cut into 40 μm slice using a Tissue-Tek® Cryo3® Microtome/Cryostat (Sakura, Japan). The samples were stuck on a metal stub and coated with gold under vacuum evaporator for 90–120 s. Surface composition of the samples was investigated by Energy Dispersive X-ray spectroscopy (EDS) microanalysis through a X-EDS detector (Oxford Inca Energy System 250 equipped with INCAx-act LN2-free detector) .

2.5. HPLC analysis of Diclofenac sodium

Dic analysis was carried out by RP-HPLC on a system consisting of a FCV-10ALvp mixer, a LC-10ADvp pump equipped with a SIL-10ADvp autoinjector, a SPD-10Avp UV–vis detector and a C-R6 integrator from Shimadzu (Japan). The analysis was performed on a Luna 5μ C18 (250 × 4.6 mm) (Phenomenex, USA) at a flow rate of 1.0 mL/min. The injection volume was 20 μL in all the experiments and the detection wavelength fixed at 238 nm.

For Dic quantitative analysis the mobile phase was a acetonitrile/acid water both modified with 0.1% TFA mixture in the ratio 70:30 v/v. A Dic standard solution in water was

prepared and stored at 4° C until use. Calibration curve was constructed by injecting solutions with concentrations in the range 0.3—30 μg/mL LOD was 0.08 μg/mL whereas QOD was 0.26 μg/mL.

2.6. In vitro release studies

In vitro release of Dic from the HT-Dic nanohybrid was evaluated by dispersing 2 mg of HT-Dic in 5 mL of release medium at 37°C. Three different release media were prepared: NaCl/KCl solution (NaCl 120 mM, KCl 2.7 mM), Phosphate Buffer Saline (PBS, NaCl 120 mM, KCl 2.7 mM, Na_2HPO_4 10 mM) and Phosphate Buffer Saline at double chloride concentrations (PBS2, NaCl 240 mM, KCl 5.4 mM, Na_2HPO_4 10 mM) both at pH 7,4. At predetermined time intervals 1 mL of supernatant was collected after centrifugation (5000 rpm 15 min 4°C) and analyzed by the analytical method previously described for Dic quantification.

The *in vitro* release of Dic was evaluated by immersing three fiber portion (3 cm) taken from different part of extruded suture in 5 mL of Phosphate Buffer Saline (PBS, NaCl 120 mM, KCl 2.7 mM e Na_2HPO_4 10 mM) at pH 7,4 and 37°C under gentle shaking. Periodically 1 mL of release medium was collected and replaced with the same volume of fresh medium. The samples were analyzed by the analytical methods previously described and results are reported in terms of released Dic percent.

Fiber morphology was evaluated also at the end of the release experiment. To this purpose, fiber was washed with water three times, freeze-dried and then analyzed by SEM.

2.7. In vivo experiments

Male CD1 mice (30-35 g) (Harlan, Italy) were purchased from Harlan Italy (San Pietro al Natisone, UD, Italy) and housed in stainless steel cages in a room kept at 22 ±1 °C with a 12:12 h light/dark cycle. The animals were acclimated to their environment for 1 week and had ad libitum access to standard rodent chow pellets. All procedures met the guidelines of the Italian Ministry of Health (D.L. no.116 of January 27, 1992) and guidelines in the European Communities Council (Directive of November 24, 1986, 86/609/ECC).

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), the back was shaved and scrubbed with betadine, and an incision (4 cm in length, and 0.2 cm in deep) was made in the middle using a number 12 blade. Five different sutures were used: 1) PCL suture; 2) PCL/HT/Dic, 3) PCL/Dic; 4) PCL/HT-Dic/Dic; 5) PCL/HT-Dic. The wounds were closed with three sutures for sub-cutaneous and four sutures for cutaneous tissues. After 3 days from surgery, mice were killed and cutaneous and sub-cutaneous tissues were removed. The

samples were fixed in 10% neutral buffered formalin, then processed and embedded in paraffin, according to standardized protocol. Sections of 4 micron were stained with hematoxylin and eosin.

Two histological parameters were evaluated in each sample: inflammatory infiltrate and granulation tissue. They were graded according to semi quantitative score as:

+ = weak inflammatory infiltrate/ low amount of granulation tissue

++ = moderate inflammatory infiltrate/ moderate amount of granulation tissue

+++ = intense inflammatory infiltrate / intense amount of granulation tissue

Results and discussion

3.1. Fiber extrusion and morphology

The aim of this work was to obtain a drug-eluting synthetic suture with antiinflammatory activity and to test its effects in vivo. Fine tuning of drug delivery rate was considered as a key parameter to achieve regional prolonged release and to promote healing process. We focused on melt-spinning technique to produce polymeric fibers with regular circular cross section since it couples good versatility, fast industrial scale up and does not require organic solvents. Melt-spinning perfectly fits loading of very hydrophilic drugs in water-insoluble polymers such as PCL ensuring high entrapment efficiency. After characterization, the HT-Dic nanohybrid was included in PCL fibers, which were then drawn to obtain suitable diameter and fully investigated.

Properties of HT-Dic nanohybrid are reported in Figure 2. The size distribution curve showed a monomodal trend and a HT-Dic mean particle diameter of 2.4 ± 0.2 μm. Surface charge of the nanohybrid was slightly negative. SEM analysis confirmed the size and the HT lamellar structure. The release profile of Dic from the HT-Dic nanohybrid was evaluated in physiologically-simulated conditions. HT consists of layers of magnesium hydroxide, with aluminium isomorphically substituted to give a net positive charge inside the layers. This charge is balanced by interlayer hydrated anions, resulting in a multilayer of alternating host layers and gallery ions.[322] To achieve drug release, the presence of an anion capable of exchange with that located inside the nanohybrid is needed. Since in a physiological environment the most available anions are chlorines and phosphates, Dic release from HT-Dic was tested in media at different chloride and phosphate concentration (Figure 47c). As expected for this type of system, release rate was strongly influenced by the ionic composition of the medium. PBS ensured a complete release of Dic from the intercalation product and was selected for further release experiments on fibers.

[322] Rives V. Layered double hydroxides: present and future. in . 2001

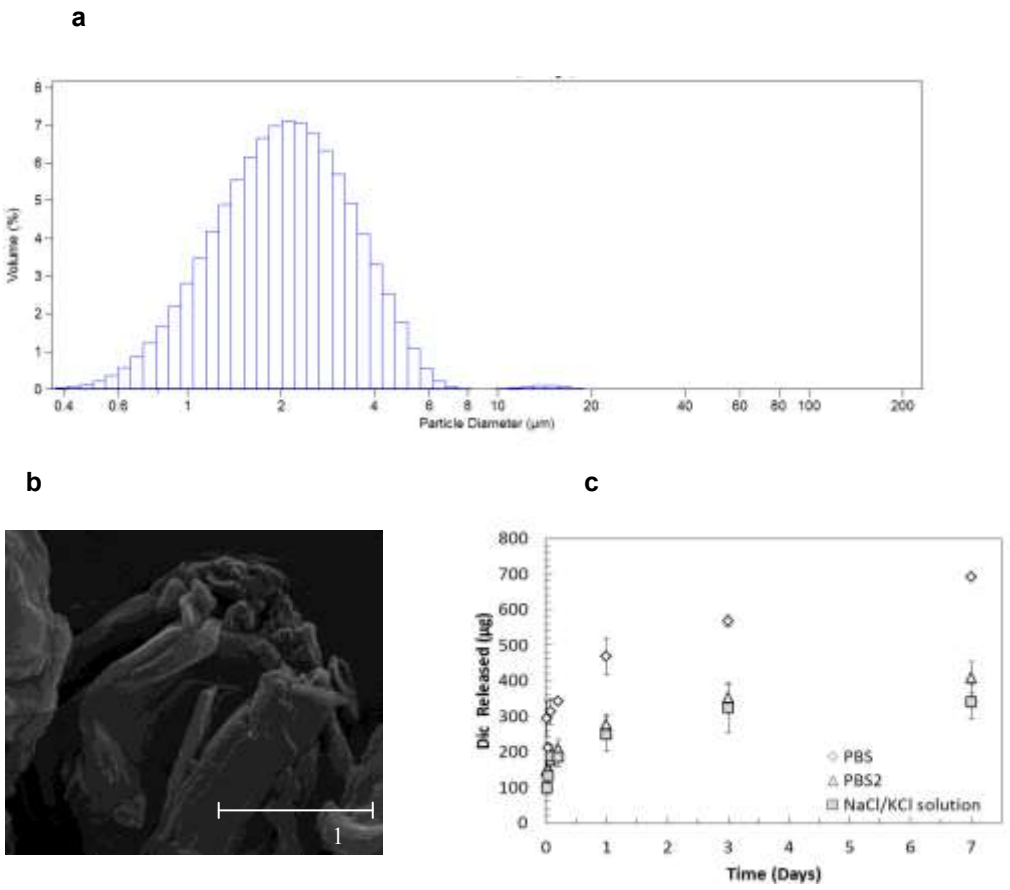


Figure 47. Properties of HT-Dic. a) size distribution; b) SEM micrograph; c) release profile of Dic in PBS (NaCl 120 mM, KCl 2.7 mM, Na2HPO4 10 mM), PBS2 (NaCl 240 mM, KCl 5.4 mM, Na2HPO4 10 mM) and NaCl/KCl (NaCl 120 mM, KCl 2.7 mM) solution at pH 7.4 and 37 °C. Results are reported as mean ± SD of three measurements.

Biodegradable PCL fibers with different compositions were processed by a two-step melt-spinning method (melt extrusion and hot draw) (Table 11). Melt spinning has proven to be a robust method for the production of several biomedical devices and it has been found to be useful in the pharmaceutical industry as well.[323] Since many years this technique has been already used for the industrial production of sutures because it is an economical process with reduced production time, few processing steps, and offering the possibilities to work in continuous. Furthermore, the capacity of melt extrusion to disperse in a matrix different active pharmaceutical ingredients at molecular level (forming solid solutions) has been seen as a possible strategy to increase solubility and bioavailability of water-insoluble compounds.[324,325]

[323] maniruzziman
[324] Maniruzzaman M., Rana M.M., Boateng J.S., Mitchell J.C. *et al.* Drug Dev.Ind.Pharm. 2013

One of the disadvantages of this production method is the necessity to operate at high temperature in order to melt the polymer while most drugs may degrade at high temperature. In the present case, RP-HPLC qualitative analysis of Dic extracted from fiber after extrusion confirms that the production method does not affect drug stability. Nevertheless, this loading strategy can be of benefit for drugs poorly stable upon heating as well as drugs undergoing polymorphism in order to maintain their chemical-physical integrity.

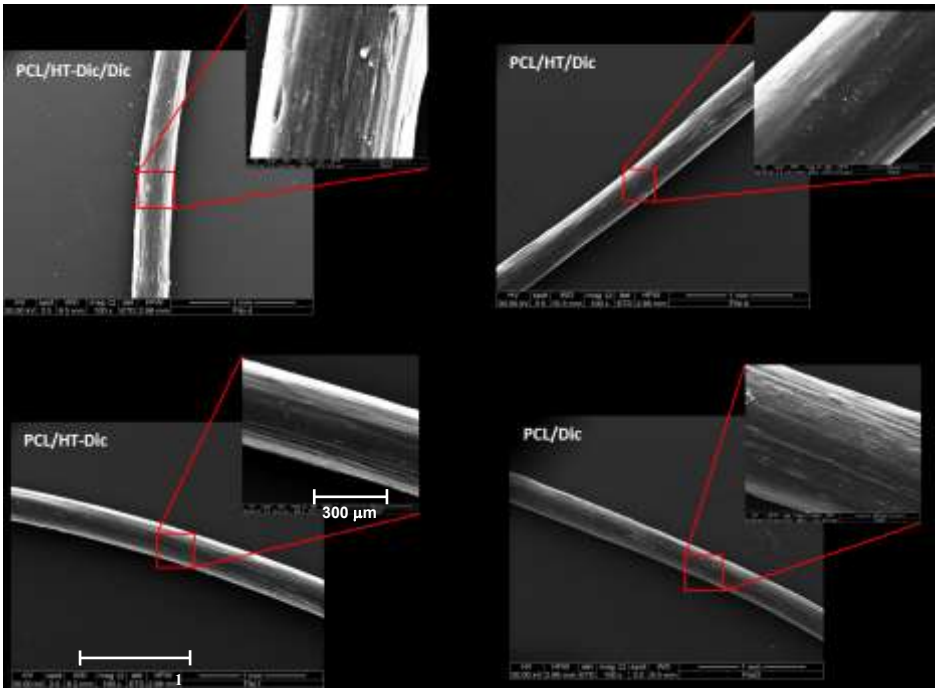
Table 11. Composition of monofilaments.

Fiber	*PCL (%)*	*HT-Dic (%)*	*HT (%)*	*Dic (%)*	*Dic (mg/cm)*	*Diameter (µm)*
PCL/HT-Dic	82	18	-	-	0.14	315±30
PCL/Dic	82	-	-	18	0.17	324±21
PCL/HT-Dic/Dic	75	17		8	0.22	328±67
PCL/HT/Dic	75	-	8	17	0.12	264±44

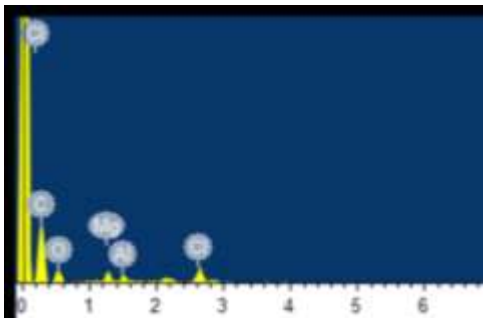
After extrusion, fibers were analysed by scanning electron microscopy (SEM) to evaluate size and surface morphology (Figure 3). In details, as spun fiber of PCL formed at 100°C die temperature showed extruding lines on the surface (Figure 3a), likely due to polymer curing in the applied processing conditions. Nevertheless, the presence of some particles on the surface of the fibers containing HT or HT-Dic was observed. To investigate the nature of these particles, fiber surface was analysed by Energy Dispersive X-ray spectroscopy (EDS). Particles were composed mainly by magnesium and aluminium, thereby suggesting that HT-Dic particles locate also on the filament surface during processing (Figure 3b).

a

[325] Sareen S., Mathew G. and Joseph L. Int J.Pharm.Investig. 2012



b



	Mean (%)	*Standard Deviation*
C	66.74	0.20
O	26.16	0.18
Mg	2.06	0.21
Al	1.21	0.07
Cl	3.83	0.10

Figure 48. SEM images of monofilament surface (a) and EDS analysis of PCL/HT-Dic monofilament surface (b). The sample is representative of outer monofilament portion.

To evaluate the inner morphology of the fibers, SEM images on fiber sections were taken (Figure 49). All the fibers had round cross-sectional geometry and non-porous morphology, with an average diameter around 0.3 mm comparable with those measured by a calibre (table 11). Micrographs indicated a rather homogeneous dispersion of included components with the presence of small internal voids due to a decrease of feeding load during extrusion.

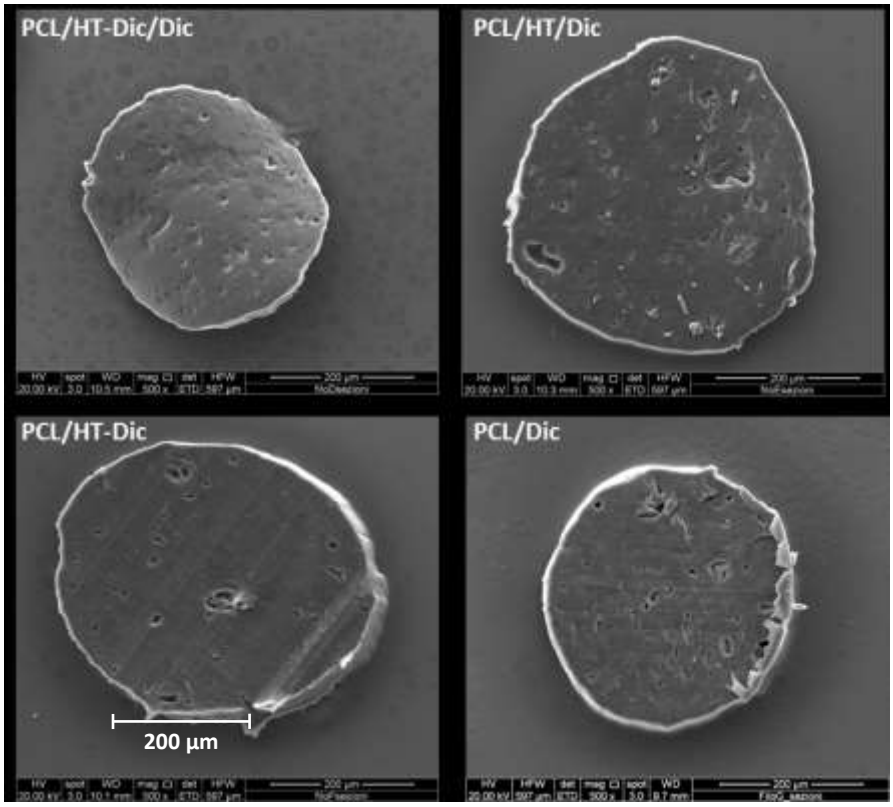


Figure 49. SEM images of monofilament section. The sample is representative of other inner section.

3.2. Mechanical properties

Given that the use of sutures strongly depends on mechanical properties, their strength is the most frequently reported parameter.[326] There should be a proper match between suture strength and tissue strength [327] and for this reason the selection of proper suture depends also from the tissue involved. Furthermore, tensile properties of sutures are important when making a knot. If the material is too weak and the knotting force is stronger than tensile strength of suture material, suture can easily break while tightening the knot.[328,329]

Results of tensile tests performed on melt-spun straight and knotted filaments are shown in Figure 50 and Figure 51.

[326] Pillai, 2010
[327] Patel KA. and Thomas W.E.G. Surgery. 2008
[328] Kim J.C., Lee Y.K., Lim B.S., Rhee S.H. *et al.* J.Mater.Sci.Mater.Med. 2007
[329] Freudenberg S., Rewerk S., Kaess M., Weiss C. *et al.* Eur.Surg.Res. 2004

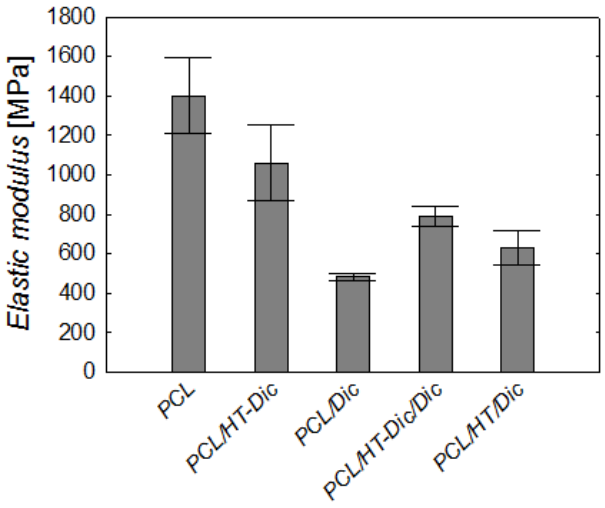
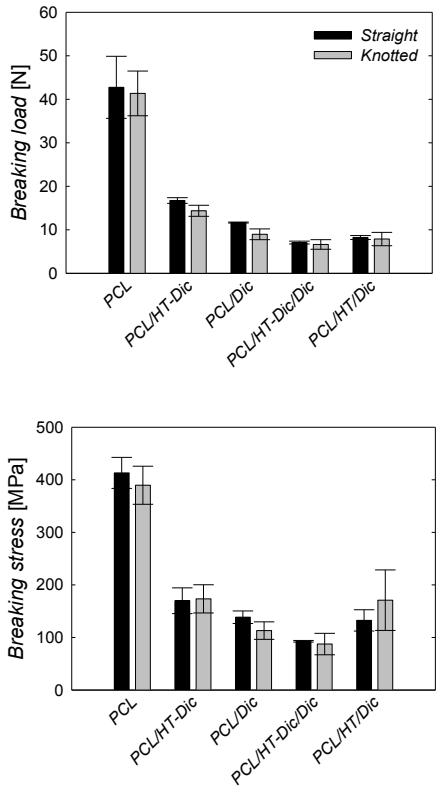


Figure 50. Elastic modulus of monofilaments with different composition. Results are the mean of five measurements, error bars are standard deviations.



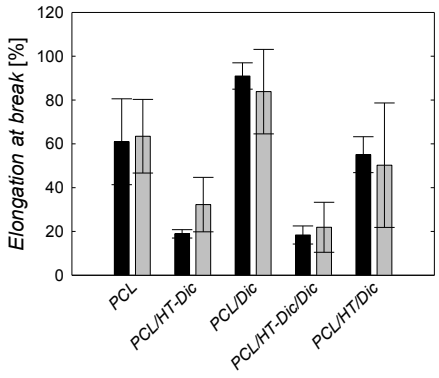


Figure 51. Mechanical properties of straight and knotted monofilaments: breaking load (a); breaking stress (b); elongation at break (c). Results are the mean of five measurements, error bars are standard deviations

The elastic modulus data (Figure 51) indicated that the inclusion of particulate components decreased the stiffness of the fiber with respect to pure PCL. In particular, the addition of HT-Dic caused a reduction of tensile modulus from 1400 MPa to about 1100 MPa (-25%) while the inclusion of Dic caused a reduction of the modulus up to about 480 MPa (corresponding to -66%). Systems including both Dic and HT as well as Dic and HT-Dic also showed a significant decrease in modulus. Tensile properties (breaking stress and elongationa at break) of straight and knotted fibers are shown in Figure 51. Data indicated that, whitin esperimental errors:

- the knotted fibers had the same breaking stress and the same elongation at break of straight fibers thus indicating that the knot does not represent a weakness to the fiber;

- composite fibers showed a three-fold decrease with respect to pure PCL fibers in breaking stress;

- fibers containing HT-Dic showed fragile behaviour with elongations at break around 20% (other systems being around 70%) and a tenacity in the range 10÷17 MJ/m^3 (other systems being around 70 MJ/m^3).

The effect of knotting on the strength of various sutures was studied by Kim et al.,[330] who concluded that the knotting of a suture reduced its tensile strength. In the present case the knots do have only a marginal influence on the mechanical behaviour of the fibers, and this is an advantage for their use in slow-healing tissue (skin, fascia and tendons).

[330] Kim, 2007

3.3. In vitro release and degradation

The release profile of Dic from fibers was assessed in PBS at pH 7.4 and 37 °C, simulating physiological conditions (Figure 7). In all the samples, an initial burst of Dic release was observed. The control fiber containing free HT and Dic (PCL/HT/Dic) completely eluted its Dic content in around 14 days, thus showing a poor control over release rate. The fibers containing HT-Dic (PCL/HT-Dic) reached around 80% release after 70 days thus providing an effective release control. In the fiber containing both HT-Dic and a fraction of free Dic (PCL/HT-Dic/Dic) the release rate was slowed down further, giving an amount of released Dic around 40% after 60 days. These data suggest that a fine tuning of release profiles can be accomplished playing on reciprocal HT-Dic/Dic ratio and optimal rate may be selected according to the therapeutic needs. Dic amount released from PCL fiber (PCL/Dic) was comparable to that released from PCL/HT-Dic although poorly modulated in the initial stage. After 70 days Dic was still released in native form indicating the protective effect of PCL and HT on its chemical structure.

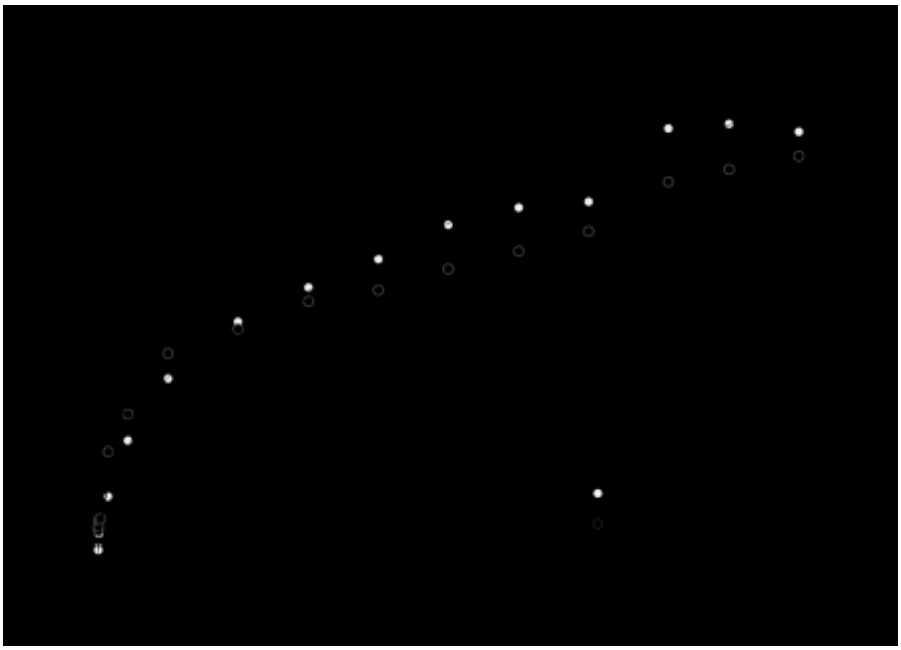


Figure 52. Dic release from monofilaments in PBS (10mM) at pH 7.4 and 37 °C. Results are reported as mean ± standard deviation of three measurements. Lines through data points are to guide the eye.

In order to evaluate the mode of degradation, fiber morphology was analysed after 70 days of release in PBS at pH 7.4 at 37 °C (Figure 52). Surface modification of the fiber, likely

due to slow polymer progressive degradation, was observed without the occurrence of fractures. As expected, the extent of polymer degradation was very limited and confined to the surface, confirming that this kind of system behaves mainly as a surface eroding system. Nevertheless, surface degradation of PCL determined the outcrop on the surface of HT and HT-Dic as shown by EDS analysis. This phenomenon was particularly evident in the fiber PCL/HT/Dic.

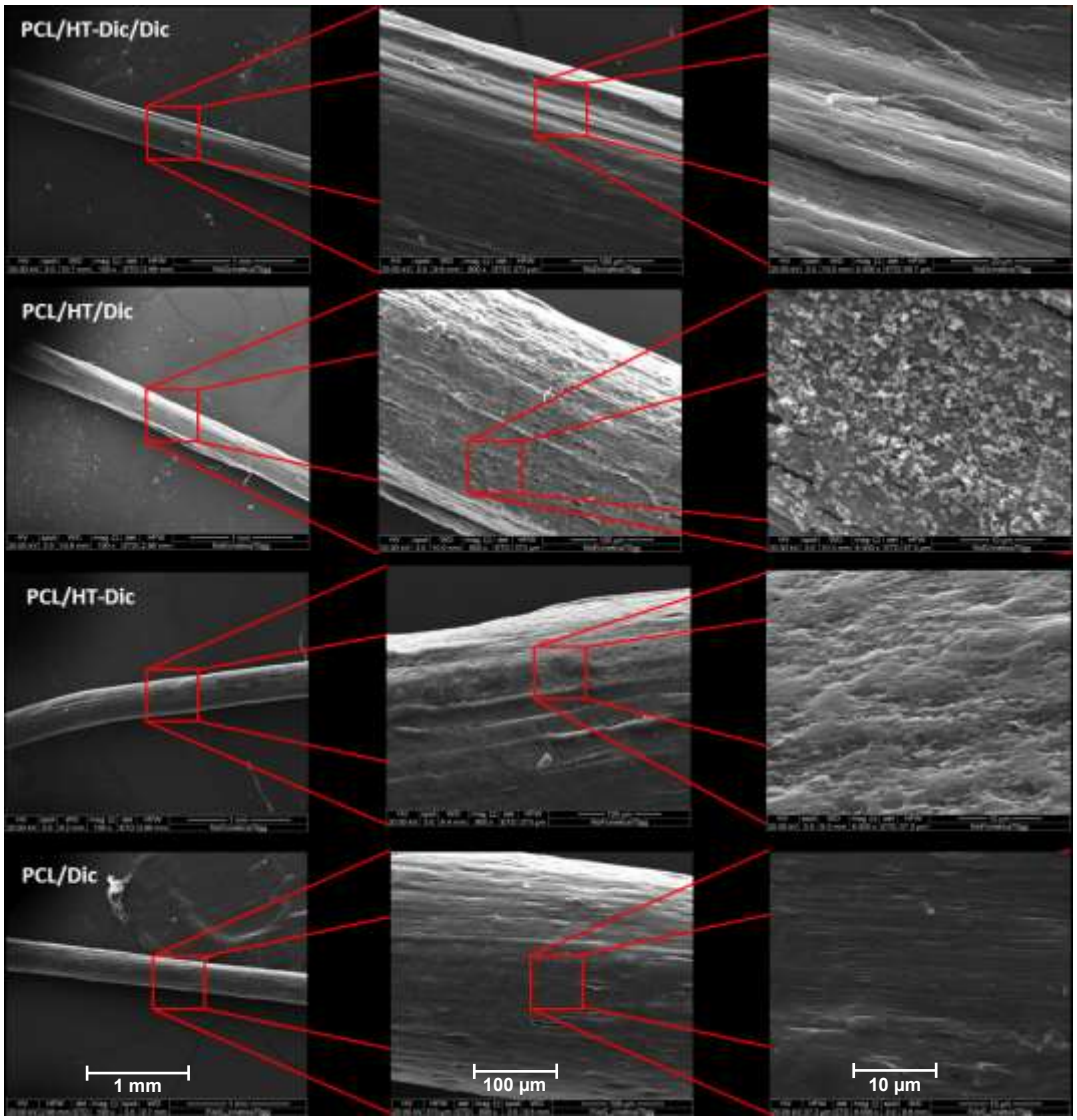


Figure 53. SEM images of monofilaments after 70 days of incubation in PBS (10 mM) at pH 7,4and 37°C.

This degradation mode suggests that DIC release from fiber is predominantly controlled by drug dissolution and diffusion. In the case of PCL/Dic, drug diffusion in the rubbery PCL phase as well as in polymer mesopores is the prevailing mechanism. When the fiber contains

HT-Dic nanohybrid, Dic needs to diffuse out of the lamellar structure and then through the polymer matrix, which adds a supplementary process contributing to the overall release.

Sustained Dic release may represent a clinically relevant therapeutic modality. It is worth of note that besides anti-inflammatory effect, sustained Dic release can have an impact also in the management of postoperative pain, thus minimizing the dose of medications to lessen side effects while still providing adequate analgesia. To date, perioperative administration of a single local dose of nonsteroidal anti-inflammatory drugs has shown inconclusive efficacy.[331] Rather than a single bolus, *Lavand'homme et al.*[332] found that continuous intrawound infusion of Dic demonstrated a greater opioid-sparing effect and better postoperative analgesia than the same dose administered as an intermittent intravenous bolus. Thus, Dic-eluting sutures can combine delivery concepts to a biomedical device to exert both effective control of inflammatory phase and alleviation of post-operative pain.

3.4. In vivo effects

To assess in vivo anti-inflammatory effect and to relate it to fiber composition, sutures were employed to close a traumatic wound in mice. Two histological parameters were evaluated in each sample: inflammatory infiltrate and granulation tissue (Table 12).

Table 12. Inflammatory infiltrate and granulation tissue in specimens.

	Inflammatory Infiltrate		Granulation Tissue	
Fiber	CUTANEOUS TISSUE	SUB-CUTANEOUS TISSUE	CUTANEOUS TISSUE	SUB-CUTANEOUS TISSUE
PCL/Dic	++	++/+++	-	-
PCL	+/++	+/++	-	-
PCL/HT-Dic/Dic	+	++	-	-
PCL/HT/Dic	+/-	+	-	-
PCL/HT-Dic	+	++	-	+

+ = weak inflammatory infiltrate / low amount of granulation tissue

++ = moderate inflammatory infiltrate / moderate amount of granulation tissue

+++ = intense inflammatory infiltrate / intense amount of granulation tissue

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[332] Lavand'homme P.M., Roelants F., Waterloos H. and De Kock M.F. Anesthesiology. 2007

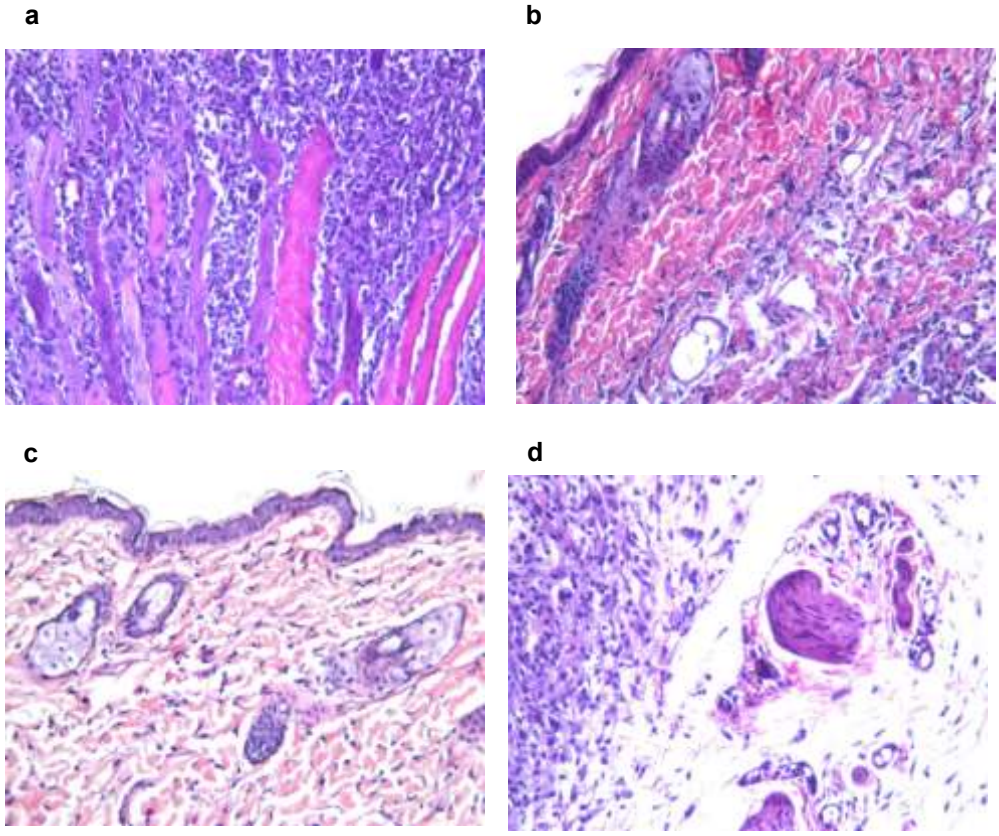

Figure 54. Hematoxylin and eosin-stained sections of sub-cutaneous tissue from mice at suture site (H&E 20x). a) PCL/Dic; b) PCL; c) PCL/HT/Dic; d) PCL/HT-Dic

In samples containing PCL/Dic an inflammatory infiltrate variable from moderate to intense was observed, especially at the level of the sub-cutaneous tissue (Figure 9a), without formation of granulation tissue. For PCL fiber, the inflammatory response appeared to be less intense (Figure 9b), again without the formation of granulation tissue. In all the other specimens no variations in the inflammatory response was observed, except for PCL/HT/Dic where this reaction was found to be less intense (Figure 9c). It is worth of note that the presence of granulation tissue was observed only in the sub-cutaneous tissue of PCL/HT-Dic sample (Figure 9d). Regardless of the surgical site, in the injured tissue the inflammatory reaction reduces the damage, removes damaged tissue components and stimulates the deposition of extracellular matrix, inducing angiogenesis. Therefore the observation that a granulation tissue is present can be related to an expression of a more effective and early reparative process in progress.

Conclusions

In summary, we have developed a drug-eluting anti-inflammatory suture that could have the dual function of closing the site of wound excision while providing sustained localized delivery of Diclofenac. The melt spun fiber, containing HT-Dic, has shown a homogenous distribution of fillers, good mechanical properties, tuneable release rate as a function of composition and *in vivo* activity that suggests their use in surgical practice. The strategy proposed to produce drug-eluting fibers is of great potential and versatility. Indeed, melt-spinning is a simple and reproducible process that can be scaled-up at industrial level and is adaptable to incorporate a large variety of drugs. On the other hand, control of fiber properties by nanohybrid should help to extend the applicability of our concept to other bioactive drugs.

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