POLYVINYL ALCOHOL HYDROGELS LOADED WITH ANTIBACTERIAL CONSTITUENTS FOR BURN HEALING APPLICATIONS

Renata Nunes Oliveira

Tese de Doutorado apresentada ao Programa de Pós-graduação em Engenharia Metalúrgica e de Materiais, COPPE, da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Engenharia Metalúrgica e de Materiais.

Orientadores: Rossana Mara da Silva Moreira Thiré
Gloria Dulce de Almeida Soares
Garrett Brian McGuinness

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Examinada por:

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Prof. Rossana Mara da Silva Moreira Thiré, D.Sc.

_____

Prof. Marysilvia Ferreira da Costa, D.Sc.

_____

Prof. Fernando Luiz Bastian, D.Sc.

_____

Prof. Marisa Masumi Beppu, D.Sc.

_____

Dr[a]. Elena Mavropoulos Oliveira Tude, D.Sc.

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Orientadores: Rossana Mara da Silva Moreira Thiré

Gloria Dulce de Almeida Soares

Garrett Brian McGuinness

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Dedication

I'd like to dedicate this thesis to three remarkable women, Luiza V. Pinto (grandma - in memorium), Katia N. Pinto (mom) and Gloria D.A. Soares. They taught me the most important lesson of my life, they taught me how to be brave.

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Resumo da Tese apresentada à COPPE/UFRJ como parte dos requisitos necessários para a obtenção do grau de Doutor em Ciências (D.Sc.)

HIDROGÉIS DE POLI(ÁLCOOL VINÍLICO) CARREGADOS COM AGENTES BACTERICIDAS PARA APLICAÇÃO EM TRATAMENTO DE QUEIMADURAS

Renata Nunes Oliveira

Fevereiro/2014

Orientadores: Rossana Mara da Silva Moreira Thiré
Gloria Dulce de Almeida Soares
Garrett Brian McGuinness

Programa: Engenharia Metalúrgica e de Materiais

Queimaduras são causa de morte de milhares de pessoas por ano, principalmente devido a infecções. De forma a estimular a cura, ambiente úmido seria favorável. Hidrogéis, especialmente hidrogéis de PVA, apresentam muitas das características do curativo ideal, mas não têm propriedades bactericidas que auxiliariam no manejo de infecções. Dentre os agentes bactericidas usados em feridas, prata (Ag) e própolis têm sido usados por décadas. O objetivo do presente trabalho foi desenvolver e caracterizar hidrogéis de PVA carregados com Ag e própolis. Ambos os fármacos foram incorporados com sucesso aos géis de PVA. Os géis de PVA-Ag apresentaram propriedades mecânicas e de intumescimento adequadas para a aplicação, apresentaram atividade contra os microorganismos estudados e foram atóxicos a queratinócitos. As amostras de PVA-própolis com quantidade de própolis de 0,15 e 0,45% própolis/placa de petri apresentaram propriedades mecânicas e de intumescimento adequadas; apresentaram atividade contra *S. aureus* apenas e foram citotóxicos a queratinócitos humanos.

Abstract of Thesis presented to COPPE/UFRJ as a partial fulfilment of the requirements for the degree of Doctor of Science (D.Sc.)

POLYVINYL ALCOHOL HYDROGELS LOADED WITH ANTIBACTERIAL CONSTITUENTS FOR BURN HEALING APPLICATIONS

Renata Nunes Oliveira

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Advisors: Rossana Mara da Silva Moreira Thiré
Gloria Dulce de Almeida Soares
Garrett Brian McGuinness

Department: Metallurgical and Materials Engineering

Burns are the cause of death of thousands of people per year, mainly due to infection. In order to improve healing a moisturized environment should be promoted. Hydrogels, especially PVA ones, present most of the characteristics of the ideal dressing, but they do not present any bactericide property, which could help the management of infections. Among the bactericide agents used for wound healing, silver and propolis have been used successfully for decades. The goal of the present work is to develop and characterize PVA hydrogels loaded with Ag and with propolis. Both drugs were successfully incorporated to the PVA gels. The PVA-Ag gels of the present work presented mechanical and swelling properties adequate to the application; they presented activity against the microorganisms studied and non-cytotoxicity to human keratinocytes. The PVA-propolis samples with 0.15 and 0.45% propolis/Petri dish presented mechanical and swelling properties adequate to the application; however they presented activity against *S. aureus* only and they were cytotoxic to human keratinocytes.

Summary

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1. Introduction

Burns are common injuries, which can be caused by electricity, heat, chemicals and radiation. Burns are responsible for thousands of deaths per year and burns infection is the main cause of death related to burnt patients.

Burns treatment has changed over time and the latest developments consider that a moist environment improves the healing. Several dressings have been developed to fulfil this requirement, including many hydrogels. Hydrogels present several characteristics of the ideal dressing, but they do not always have bactericidal properties.

Among the positive characteristics of the Polyvinyl alcohol (PVA) hydrogels, there is the transparency, which allows monitoring of the progress of healing, potentially diminishing the frequency of dressing changes. They present high swelling capacity, keeping a moisturized wound environment, and they are biocompatible. However, they do not control or hamper the infection process.

It is possible to prepare PVA hydrogels loaded with antimicrobial agents to incorporate the bactericide property to the hydrogels intrinsic characteristics. The presence of these substances might interfere with the gels properties and thorough characterization is necessary to guarantee the necessary characteristics for the application.

Silver is a well-established bactericide agent that has been successfully used in burns for decades. PVA hydrogels have been loaded with silver before, since PVA is a well established capping agent for silver nanoparticles production. However, if PVA gels are intended for wound healing applications, the amount of silver would need to be carefully adjusted to obtain bactericidal properties coupled with non-toxicity to human cells.

Propolis on the other hand is a natural product used in folk medicine for centuries. Propolis presents some bactericide properties and it is used in wound healing for at least some decades. The propolis characteristics vary considerably, since its properties are defined by its geographic region and its botanical origin.

The loading of propolis into hydrogels has been considered a matter of study recently. The gels developed so far present favourable characteristics, such as inhibition

of microorganisms and also improving the wounds healing in *in-vivo* tests. However, only a few polymeric systems loaded with propolis have been studied so far and there is a lack of cytotoxicity analysis.

The goal of the present work is to prepare PVA hydrogels loaded with silver and with propolis to obtain dressings that keep a moisturized environment while presenting bactericidal characteristics. The gels were characterized microstructural and morphologically, the swelling and the mechanical properties of the swollen samples were evaluated and antimicrobial and in-vitro tests were performed.

2. Literature review

2.1. Burns

Burns can be caused by heat, electricity, radiation and chemicals, but the most common are the thermal injuries that damage the skin (thermal burns). Besides being the cause of disabilities and disfigurement, in 2008, burns were considered as being the cause of death of approximately 300 thousand people a year globally[1] and, in 2004, nearly 11 million burnt people required medical treatment[2].

Burns can be classified into different groups, first degree burns, which affect only the epidermis (superficial); second degree burns, which affect the epidermis and the dermis (partial thickness); third degree burns, which affect the full depth of the skin and underlying tissues, including nerves (full thickness). The first two types of burns are red and painful and the second degree burns can be blistered and swollen. Third degree burns can be painful or not (if there were damage of the nerves) and whitish in colour[3-6].

On the 18th century, burns treatments were based on drying the site and the formation of a scab, preventing the inflammation and allowing the tissue under it to heal[7]. This treatment has been used since then; however, if there are microorganisms on the burn site, they can lead to infection. Infection is the most common cause of death of burnt patients[3]. If not fatal, infection, at least, delays the burn healing. Burns contractures and disfigurement, besides physical impairment, according to fire burn survivors can lead to handicaps and reduce their chance of living social and economically productive lives[1].

Infection is main cause of deaths related to burns[3,8]. The infection can be caused by the patient's endogenous flora, by the microorganisms present in the locality of treatment (bacteria, fungi) or by the patient's sepsis. Typically, burns are colonized by gram-positive bacteria and, in a week, these can be replaced by gram-negative organisms[9,10].

Gram positive and gram negative bacteria can be distinguished by their membrane structures: gram negative bacteria have a peptidoglycan layer between the cytoplasmic membrane and the outer membrane of 2-3 nm, while gram positive ones

lack the outer membrane and they have a peptidoglycan layer of ~30 nm[11]. Aerobic and anaerobic bacteria, as well as fungi, are encountered on burn sites.

Among the most common bacteria, there are the aerobic bacteria [*Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. Aeruginosa*), *Escherichia coli* (*E. Coli*), *Klebsiella sp.*, *Proteus sp.* etc], the anaerobic bacteria [*Pigmented Bacteroides, Bacteroides fragilis, Peptostveptococcus anaerobius* etc] and the fungi [*Aspergillus niger, Candida sp*., *Zygomycetes* etc]. Among aerobic bacteria, the common gram negative bacteria in burns are *P. aeruginosa*, *Klebsiella sp, Proteus sp, E. coli* etc, and the common gram positive ones are *S. aureus*, *Staphylococcus epidermidis* etc[10,12-14].

Since different microorganisms are present on burn sites, materials studied to cover burns should be tested in contact with these different microorganisms. In addition, if the burnt site is healed without medical treatment, scarring takes place and the new skin formed does not present normal dermis, nerves, vessels, and adnexa, and it is less elastic[15]. Burns treatment should avoid infection and promote the healing, leading to the formation of newly normal skin.

2.2. Burns treatment

Burns have been treated for centuries using different approaches according to what was known at the different times. Besides the evidence from Neanderthals (drawings in their caves showed some care with burns, 3500 BC[16]), the Egyptians (1500 BC) applied salve resin comprised of honey, mud, oil and plant extract, to treat burns[7]. Celsus used wine and myrrh in the first century AD to treat burns, probably related to their bacteriostatic properties[16].

Theophrastus (371-287AD) was the first one that documented the use of the concept of cooling the burnt site and this concept was applied for centuries. However, in the 18th century, according to the anatomist John Hunter, exposure and dry treatment would be the best ones. They would prevent inflammation and infection by creating an inappropriate environment for bacteria colonization, permitting a scab to form which allows tissues under it to heal. Hunter also realized that, although cooling the site would relieve pain while doing it, the symptoms would become worse after removing it[7].

In the late 18th century, the surgeon Sir James Earle proposed a treatment for burnt patients with cold water or ice as soon as possible for several days, which diminished pain and the incidence of blisters and prevented the progression of inflammation[7]. Although applying water to cool the burn had been used, the risk of hypothermia led to the use of warm body temperature water[17].

Even though applying water brought some promising results in burn healing, the traditional treatment used in the 20th century was still related to dry treatment. This is reflected in "the primary aims in the treatment of burns" stated by Kaye[18] in 1956:

> The primary aims in the treatment of burns are: (1) prevention and treatment of shock, (2) prevention and treatment of infection, (3) utilization of a local therapy that will insure drying of the burn wound and permit early skin grafting, (4) maintenance of adequate nutrition and hemoglobin level during the healing stage, and (5) prevention and correction of contractural deformities. (Kaye, 1956, p.123)

Cold water relieves pain, however the first action should be the cleansing of the site. The cleansing should be done with the help of flowing water or warm saline solution (debridement), according to the injury extension to remove dirt and devitalized tissue, since they interfere with the tissue repair process[19-21].

According to Rawlins[22] the size and thickness of the burn determines the requirements for adequate burn management. Epidermal burns heal normally. After debridement, the first degree burns should be treated cooling the site with water and applying aqueous emulsions, foam sprays and lotions[23]. In second degree burns, dressings should be used. The use of improved dressings, topical antimicrobials and early wound excision has reduced the incidence of infection[8]. Dressings also prevent fluid losses, diminish pain and improve the healing[24]. Third degree burns would require a set of dressings and medications to prevent infection (groups of topical medications and antibiotics, e.g. mefenamide, silver sulfadiazine). They can also require skin grafts[25,26].

Many new dressing systems for burns coverage have been developed in recent decades. According to Quinn and collaborators[27], 1985, p.370 "In the clinic, burn wound management falls into two categories - the open or exposure method and the closed method, i.e. the application of dressings (…)". The same authors state that

opened burns form a natural dressing, the crust. The re-epithelialization occurs under it and when it falls off, the site is healed.

With regard to burn dressings, the characteristics of the ideal dressing would be to keep a moisturized environment, to be transparent, to absorb excess of exudates, to eliminate any empty space, to be pain free, to promote thermal insulation, and to be barrier to microorganisms, besides being conformable, elastic, sterile, non toxic and present water vapour transmissibility[27-29]. Most of these characteristics can be found in hydrogels.

2.3. Hydrogels

In 1960, Wichterle and Lim[30] reported the possibility of permanent use of plastics in contact with living tissue and the difficulties related to this use. These plastics presented structural similarity to the body, but unfavourable physiological effects were observed in most cases due to foreign body reaction. Polyvinyl alcohol (PVA) would be an exception so far, but it had the disadvantage of being absorbable. Wichterle and Lim[30], 1960, p.118 argued that the plastic for this application would have:

> "(1) a structure permitting the desired water content; (2) inertness to normal biological processes (including resistance to the degradation of the polymer and to reactions unfavourable to the organism); (3) permeability for metabolites. Materials with these properties must have hydrophilic groups. Further, they must have a three-dimensional structure with at least enough cross-linkages to prevent absorption".

These authors developed a crosslinked network of glycolmonomethacrylate, a transparent porous structure. When dried, these gels were transparent solid materials and when immersed in water, they presented high water uptake. No irritating reactions were observed when tested in living tissue[30]. This could be considered the first development of hydrogels[31].

Hydrogels are tridimensional networks based on crosslinked hydrophilic polymers that swell in contact with aqueous solutions while they keep their structural integrity (due to the crosslinks)[29,32-34].

Among the hydrophilic polymers most used in hydrogels, there are biocompatible natural and synthetic polymers. Among the natural polymers, there are: anionic polymers, e.g. pectin, carrageenan and chondroitin sulphate; cationic polymers, e.g. chitosan and polylysine; amphipathic polymers, e.g. collagen (and gelatin), carboxymethyl chitin and fibrin; and neutral polymers, e.g. dextran and agarose. Among the synthetic polymers, there are: polymethacrylates, polyesters, e.g. PEG-PLA-PEG, PEG-PLGA-PEG, PEG-PCL-PEG, PLA-PEG-PLA, PHB; and other polymers, e.g. PEG-bis-(PLA-acrylate), PAAm, P(NIPAAm-co-AAc), P(NIPAAm-co-EMA), PVAc/PVA, etc[31,35].

PVA is a semicrystalline polymer, with a simple chemical structure in which there are hydroxyl pendant groups[36]. Its chains can present inter and intra-molecular bonds[37]. PVA hydrogels are transparent, malleable, bio-inert and biocompatible. They have been successfully applied as contact lens, in artificial hearts, as drug delivery systems, as articular cartilage, in catheters, in dialysis membranes, as burn dressings and as temporary skin substitutes[38,39].

PVA hydrogels present high swelling degree in water and in biological fluids, with elastic or rubbery characteristics, which make them suitable to mimic organic tissues[36].

PVA hydrogels can be crosslinked via chemical or physical routes. Among chemical routes, there are irradiation processes and processes that use cross-linking agents[40]. The second type refers to the addition of chemicals, e.g. glutaraldehyde and formaldehyde, which react with PVA chains connecting them via covalent bonds[39]. However, if residual chemicals remain within the matrix, when in contact with the body, they can be delivered, being potentially hazardous[41].

Radiation techniques are clean and effective, they crosslink the PVA chains through covalent bonds that are formed between the groups originally in the polymer chains and they also sterilize the gels. Among the advantages of this technique, there are the high gelatinization and the low formation of sub-products[42].

When the PVA is submitted to gamma radiation, polymeric radicals are formed, $-(CH_2-C^{\cdot}HO)-$ and / or $-(CH_2-CHO^{\cdot})-$ and these radicals interact with each other through combination and disproportionation to form inter and intramolecular bonds[42,43].

These are the crosslinking points and the presence of crosslinkages increases the gels mechanical strength[33].

For wound healing applications, the mechanical strength should be studied carefully. If it is not high enough for the wound healing application and if the gels present high stiffness when they are dry, the gels could damage the wound and the new formed skin and also they could increase the need of dressing changes[44,45].

Physical crosslinking does not require any chemical bond formation and its crosslinking method differs from the chemical routes. Usually, to physically crosslink PVA, an aqueous solution is prepared by heating the solution up to temperatures higher than the glass transition temperature (Tg), in which the amorphous chains have mobility. When this solution is frozen, ice crystals are formed and, for that, the polymer chains are pushed out from these regions and pressed together in the regions without ice crystals. When the chains are close together they can pack into crystallites and hydrogen bonds between chains occur. When thawed, the ice crystals melt, leaving macropores and, the phase rich in PVA prevents the structural collapse of the gel[37,46-49]. The crystallites act as physical crosslinking points, resulting in a non-degradable 3D structured – a cryogel[50].

These PVA cryogels are non-toxic, they present high mechanical strength, e.g. 40 kPa of mechanical strength, and high swelling capacity in aqueous solutions[47,49,51]. The cryogels mechanical properties depend on the time and on the temperature of freeze-thawing, besides the dependence on the solution concentration and on the molecular weight of the polymer. For PVA hydrogels, freezing cycles as short as 1h are able to make insoluble gels with high swelling capacity (important to keep a moisturized environment that improves the healing) and, although long cycles resulted in high mechanical strength, they also contributed to low swelling capacity[49,52,53].

Besides the beneficial properties, PVA hydrogels do not present any intrinsic anti-inflammatory or antimicrobial property. To overcome this limitation, PVA hydrogels have been studied as drug delivery systems for some decades[54,55].

Shaheen and collaborators[56] studied PVA hydrogels loaded with theophylline for asthma treatment. 100% of the drug delivery occurred in 5h of immersion in water. Kenawy and collaborators[57] studied the drug delivery of PVA hydrogels loaded with atenolol for the treatment of hypertension. They found out that there was a high drug

delivery to phosphate buffer in the first hours, probably due to the release of the drug on the surface followed by a slow delivery. Total delivery occurred in 30h.

Regarding the wound healing application, Hwang and collaborators[58] studied PVA/dextran hydrogels loaded with gentamicin, a drug used in the treatment of skin infections due to its bactericide effect. They concluded that the gels with the drug contributed positively to the healing in the *in-vivo* tests.

Singh and Pal[59] studied the release of tetracycline HCl and gentamicin by PVA hydrogels to prevent wound dehiscence and reduce bacterial contamination. They analyzed the drug delivery for 1 day to different media, including pH 2.2 buffer and pH 7.4 buffer solutions. There was a high delivery in the first hours followed by a slow delivery, due to the drugs concentration gradient. The drugs were released to all media and Singh and Pal[59], 2012, p.20 concluded that these "dressings could be applied to all the stages of wound healing".

Among the several additives that would be useful for the wound healing application, silver is a well-established bactericide agent used in burn healing for centuries[60]. On the other hand, propolis is a natural product that has been used in medicine through thousands of years and it was used in wounds treatment during World War II[61]. The next sections are related to these two substances which are established for the wound healing applications, with a view to incorporation into PVA gels to make bactericidal membranes.

2.4.Silver

Silver has been used in wounds healing for at least three centuries[62]. Stromeyer, in 1844, stated that burns healing should be treated according to four main steps: the treatment should be anti-inflammatory; the burns should be protected from the external environment, a crust should be quickly formed and then the scarring should be promoted[60].

The first successful record of the use of silver on burns was the one related to Moyer, 1960. Moyer applied a 0.5% $AgNO_3$ aqueous solution on burns resulting in

bactericide effect against *S. aureus*, *P. Aeruginosa*, *E. Coli* and it also did not interfere with the skin cells proliferation[62].

Yamanaka and collaborators[63] studied the mechanism of action of Ag^+ on *E. Coli* and they concluded that the ions penetrate the cells through ionic channels without damages to the membrane, the ions denature the ribosome suppressing the enzyme and the protein expression which are essential to the ATP production, resulting in the membrane collapse.

According to the literature, there are number mechanisms of action of Ag^+ on bacteria[62,64]. The first one would be the Ag^+ interacting with the thiol groups of proteins, denaturing the enzymatic activity. Besides that, when Ag^+ attaches to the proteins of the tissue, there are structural changes on the bacteria's wall. In addition, the Ag^+ reaction with sulphur and phosphorous groups of the bacteria's DNA hampers the bacteria replication and leads to death. In another mechanism, the Ag^+ attacks the bacteria, resulting in some protein being produced by the bacteria. These proteins would locate themselves around the bacteria DNA, compressing it and inhibiting the bacteria replication[62,64-66].

Metallic silver (Ag^0) should be considered an inert substance. However, when on the skin, it reacts with the wounds fluids and the silver oxidizes ($Ag^0 \rightarrow Ag^+ + \bar{e}$)[62]. Nano-silver has been used for at least a century and it was registered as a biocidal material in 1954. Nano-Ag was used in the treatment of wound infections in the past but it turned back as a medicine treatment in the 1990s[67].

The mechanism of action of nano-Ag on bacteria differs from the Ag^+ mechanism. The nano-Ag particles bind themselves to the bacteria membrane altering the bacteria's permeability and respiration. The nutrient transport through the membrane becomes uncontrolled, leading to the cells death. The nano-Ag also allows the interaction of silver with the P and S groups of the bacteria's DNA, inhibiting the bacteria[66,68,69].

According to Singh and Pal[64] the nano-Ag mechanism of action could be summarized in three steps:

1. the Ag nanoparticles bind themselves to the membrane, altering bacteria's permeation and respiration;

2. the nano-Ag penetrate the bacteria and the Ag^+ react with the P and S groups of the DNA;
3. nano-Ag release Ag^+ (they work as a source of Ag^+), additional contribution to the bactericide effect.

The nano-Ag effect is more pronounced in gram-negative organisms, since these bacteria present a peptidoglycan layer of 2-3nm thick while the gram-negative ones, the peptidoglycan layer thickness is of ~30 nm[11]. The thicker the layer, more difficult it is for the nano-Ag to penetrate, their effectiveness then decreases.

High levels of silver could hamper the re-epithelialization and also it could be toxic to human keratinocytes[70,71]. However, there is a difference between eukaryotic (human cells) and prokaryotic cells (bacteria). Eukaryotic cells are bigger and more complex organisms than bacteria. The silver concentration necessary to cause toxicity to human cells is considerably higher than the silver concentration necessary to cause toxicity to bacteria, establishing a therapeutical window. So, there is a range of silver high enough to inhibit bacteria with no damage to eukaryotic cells[72].

Some dressings loaded with silver have been considered an alternative to antibiotics in the wounds healing management. These dressings deliver silver which interacts with bacteria preventing infection[73,74].

2.5. Hydrogels loaded with silver

The use of silver in dressings should result in their having antimicrobial activity. Several types of hydrogel can be loaded with silver for wound healing application, e.g. the incorporation of silver into alginate dressings[75]; carboxymethyl cellulose hydrogels loaded with nano-Ag[76]; etc. Besides these academic studies, there are the commercial dressings loaded with Ag available, e.g. SilvaSorb®, Acticoat® Absorbent, AQUACEL® Ag, SILVERCEL®, PolyMem® Silver, all of which are approved by the FDA - US Food and Drug Administration[77].

PVA hydrogels / solutions loaded with a silver precursor, e.g. $AgNO_3$, have been studied for nanoparticles production. The silver nanoparticles can be obtained via the addition of a reduction agent, via thermal reduction or via radiation, e.g. gamma

irradiation. PVA is a successful capping agent for silver nanoparticles. There is a colour change of the material from transparent towards the yellow/brown colour when the silver reduction occurs[78-81].

Besides nano-Ag production, there is interest in metalo-polymeric nanocomposites. These can be obtained through two routes: *ex situ* (polymerization and synthesis of nanoparticles separately) or *in situ* (the polymer and the silver precursor are mixed together and the nanoparticles are synthesized inside the polymeric matrix, which also acts as capping agent)[82].

When the system PVA-silver precursor is submitted to radiation, two main reactions take place, the PVA crosslinkage and the silver reduction. The PVA crosslinkage would take place through the PVA radicals [-(CH_2-C˙HO)- and/or -(CH_2-CHO˙)-] formed under radiation[42,43]. The silver reduction would mainly occur in two steps, first the formation of the chelate PVA-Ag^+, followed by the nano-Ag formation[81,83,84].

Studies on PVA blends that were gamma-irradiated and loaded with silver to be used in wound healing have been developed recently[40,85]. PVA-Ag systems can be crosslinked and nano-Ag produced when these systems are exposed to doses of gamma radiation as low as 1 kGy[86]. In addition, to obtain sterilized gels, doses around 25kGy would be sufficient[87].

Among the challenges related to PVA-silver systems produced via gamma radiation for wound healing, there is the need to establish effective silver dose levels that are also within the therapeutical window. In addition, the obtained gels must have proper mechanical characteristics when hydrated. The gamma dose used should be the one that sterilize and crosslink the PVA while reducing the silver.

2.6. Propolis

Propolis is a resinous substance produced by bees to protect the hive from bacteria, fungi, parasites and invasive insects[88,89]. Propolis has antibacterial, anti-fungal, anti-viral and anti-inflammatory activities[90,91]. Propolis is collected by *Apis Mellifera* bees from sprouts, flowers and resinous exudates, adding wax and the enzyme 13-

glucosidase to it[92,93]. The propolis composition varies according to the geographic location, botanical origin (type of flowers / sprouts / leaves / trees), etc[88,94,95].

Propolis has been used in folk medicine for centuries96. Regarding this resinous substance, according to Adewumi and Ogunjinmi[88], 2011, p. S55 the "propolis constituents generally include about 10% essential oils, 5% pollen, and 15% various organic polyphenolic compounds including, flavonoids and phenolic acids".

Propolis is a combination of a large number of constituents, more than 160 according to Mirzoeva *et al*[97]. There is some divergence regarding the amount of substances in propolis. According to Pereira and collaborators[90], 2008, p. 2580 "at least 200 compounds were identified in different propolis samples, with more than 100 in each", however according to Ramos and Miranda[98] there are more than 300 compounds in propolis.

Marcucci[99] compiled the different groups that compose propolis: alcohols, aldehydes, aliphatic acids and aliphatic esters, amino acids, aromatic acids, aromatic esters (e.g. caffeic acid phenethyl ester - CAPE), chalcones and dihydrochalcones, flavonones, flavones and flavonols (flavonoids), hydrocarbons esters, hydrocarbons ethers, hydroxyl and keto waxes, waxy acids, ketones, terpenoids and other compounds, steroids and sugars.

There are several flavonoids in propolis. These compounds besides phenolic substances, e.g. CAPE, and cinnamic acids derivatives, would be the ones responsible for the antioxidant, anti-inflammatory, anticancer and antiviral activities of propolis[95,97,100]. Their mechanism of action against bacteria would be altering the membrane permeability, inhibition of cell division and inhibition of the synthesis of some proteins[94,101].

Propolis has antimicrobial activity against gram-positive bacteria, e.g. *Staphylococcus aureus* and *Staphylococcus epidermis*, but limited action against gram-negative bacteria and also against some fungi, e.g. *Candida albicans*[95,97,102,103]. Propolis antibacterial activity is bacteriostatic and, in high concentration, it is bactericidal[90].

Propolis is a well established bactericide and it has been used for long in folk medicine, however it should be pointed out that propolis can cause some allergenic effects, usually related to the presence of esters of aromatic acids and of some flavonoids. These constituents can be found mostly in green propolis and care should be

taken in use, regarded to these effects[104]. In addition, high amounts of Brazilian green propolis presented mutagenic effects while anti-mutagenic effects are related to low amounts of this propolis[90].

The geographic location of the propolis alters its composition and, as a consequence, its properties. European propolis contains mainly flavonoids, aromatic acids and esters; Mediterranean propolis contains mostly diterpenes. The main constituents of propolis from Taiwan are prenylated flavanones and the main constituents of Australian propolis are prenylated stilbenes. Tropical propolis contains various phenolics (prenylated cinnamic acid derivatives, flavonoids, polyprenylated benzophenones and lignans)[105].

The change of the geographical location means different flora, different sources of propolis, and as a consequence, different properties. Regarding Brazilian propolis, Brazilian red, green and brown propolis are derived from *Dalbergia ecastophylum*, *Baccharis dracucunlifolia* and *Copaifera sp*., respectively[96].

The red Brazilian propolis consists of chalcones, pterocarpans, and other isoflavonoids. The green propolis is the one most common in Brazil and it contains mainly phenylpropanoids, prenylated phenylpropanoids (e.g., artepillin C), and sesqui- and diterpenoids. Green Brazilian propolis usually presents high level of phenolic compounds. Nonetheless, there are variations in the propolis composition even for slight changes in the location / flora of the source. Propolis from the Brazilian southeast is usually green propolis[105].

2.7. Hydrogels loaded with propolis

Some membranes with propolis have been developed recently. Biocellulose membranes were prepared by Barud and collaborators[103] and the membranes were then immersed in propolis to obtain bactericide dressings. Preliminary studies revealed that these membranes were effective against *Staphylococcus* species and also that they promoted a better tissue repair in the early periods of the healing in the *in-vivo* tests.

Silva and collaborators[102] developed latex membranes loaded with propolis for biomedical applications. They found out that the propolis was successfully incorporated

to the latex membranes and that these membranes inhibited the growth of *C. Albicans* colonies.

Collagen films loaded with green and red propolis were studied by Almeida and collaborators[104] to be applied in dermal burn healing. They realized that the amount of flavonoids in the green propolis was different from the amount of flavonoids in the red propolis, ~0.95% and ~1.87% flavonoids, respectively. However, both propolis types were satisfactory for the application. The collagen-propolis films decreased the severity of the burns inflammation and they also improved the epithelialisation rates.

Some studies compared propolis formulations with silver formulations on *in-vivo* wound healing and they observed that the wounds healed completely faster when treated with propolis[106,107].

3. Objectives

3.1. Main Objective

The main goal of the present work was the development of PVA hydrogels loaded with antimicrobial constituents (silver and propolis) in order to get antimicrobial dressings.

3.2. Specific Objectives

The hydrogels are usually applied in the swollen state and the presence of fluids in these gels alters their mechanical properties. The health professional would have to swell these gels prior to their use. To analyse the gels behaviour in the swollen state, three media were used: (i) saline solution, common media used in hospitals to swell these dry gels, (ii) PBS, which mimics the inorganic part of human plasma and (iii) Solution pH 4.0, which simulates the inflammatory environment - standard ISO 10993-9:1994.

Regarding the PVA-propolis gels, these are materials not reported in the literature so far, so the present work intends to clarify the behaviour of these gels and to determine if they are suitable for the wound healing application.

The specific goals of this work were:

- ✓ Microstructural characterization of the gels;
- ✓ Morphological characterization;
- ✓ Study of the swelling behaviour of the gels in the different media;
- ✓ Analysis of the drug (silver, propolis) delivery to the media;
- ✓ Mechanical tests of the samples swollen in the different media;
- ✓ Antimicrobial tests;
- ✓ In-vitro (cytotoxicity) analysis of the gels.

4. Materials and Methods

The PVA-Ag and the PVA-propolis samples were produced and characterized morphologically, thermally and microstructurally in order to identify the interactions and the effect of the Ag and of the propolis on the PVA hydrogels. The swelling capacity of the gels was also analyzed, since the hydrogels for dressing applications should keep a moisturized environment.

The gels mechanical properties were evaluated using swollen hydrogels, since the hydrogels would be applied hydrated. The antimicrobial tests and the in-vitro tests were useful to determine the efficacy and the safety of the gels so far. The methodology used in each step of the samples production and of the samples analysis is displayed in this section.

4.1.Samples manufacturing

The PVA-Ag and the PVA-propolis hydrogels were prepared via different techniques; however, they resulted in gels with similar thickness. Poly(vinyl alcohol) - PVA, Mw 85.000 - 124.000 and degree of hydrolysis 99+%; Silver Nitrate - $AgNO_3$; were purchased from Sigma-Aldrich and they were used without further purification. The green propolis extract, Extrato de Própolis Makrovit, 12% propolis / alcohol, was produced by W. Wenzel Ind. e Com. de Produtos Apícolas Ltda, São Paulo, Brazil. The methodology regarding the PVA-Ag samples and the PVA-propolis samples will be displayed separately.

4.2.PVA-Ag samples

PVA solutions were obtained dissolving 10g PVA in 70ml of distilled and deionized water at approximately 90°C for 2h, under mechanical stirring. After dissolution, the solutions remained under stirring until they reached environmental temperature. In parallel, the $AgNO_3$ solutions were prepared, dissolving the correct amount of silver nitrate in 30ml of distilled and deionized water in the dark, under magnetic stirring, at environmental temperature, for 2h.

The samples with different amounts of $AgNO_3$ [0.00, 0.25 (1.47 mM $AgNO_3$) and 0.50% $AgNO_3$ (2.94 mM $AgNO_3$), related to the weight of the polymer] were prepared by mixing the PVA solution with the $AgNO_3$ solutions, under mechanical stirring, in the dark, for 30 minutes. After that, the samples were submitted to ultrasonic waves for 30 minutes in order to remove the solution bubbles. Then, 20ml of each sample were poured into petri dishes (150 mm of diameter) and dried in the dark, at environmental temperature, under constant air flow, for 48h. The last procedure was the irradiation (gamma radiation, Co60 source, 15 kGy, rate of 1.5 kGy/h) to crosslink the polymer and sterilize the samples.

The samples without silver (10g of PVA in 100ml H_2O) were called "PVA"; the samples with 0.25% $AgNO_3$ (10g PVA, 0.025g $AgNO_3$, 100ml H_2O) were called "0.25" and the samples with 0.50% $AgNO_3$ (10g PVA, 0.05g $AgNO_3$, 100ml H_2O) were called "0.50". For the macroscopic analysis, the samples were photographed, in order to analyze the colour change, using the SAMSUNG PL221 camera.

4.2.1. Microstructural characterization

The samples were analyzed by X-ray diffraction - XRD. The hydrogels analysis was performed using a XRD 6000 Shimadzu Diffractometer with Cuκα at 30 kV and 30 mA and step length of 0.02° with step time of 1s. The diffraction angle was set between 5° and 60°. In order to calculate the PVA crystallite sizes of each sample, the Scherrer equation was used[108], Equation 1.

$$t = \frac{\lambda}{\beta \cos(\theta)} \quad \text{Eq. 1}$$

where t is the crystallite size, λ is the X-ray characteristic wavelength (Cuκα, λ = 1.54 Å), θ is the degree of the PVA's main peak and β is the width of the half height related to the PVA's main peak.

The Fourier-transform Infrared Spectroscopy - FTIR revealed the altering in the binding vibrations according to the composition of the samples. The hydrogels were analyzed using a NICOLET 6700 Spectrometer with 32 scans per sample in the region of 550 - 4000 cm^{-1}. The results after swelling are displayed in Annex I.

4.2.2. Surface analysis

The morphology of the PVA and 0.25 films was evaluated by atomic force microscopy (AFM) using a JPK Nanowizard instrument. The tips (MikroMasch™ NSC16) were made of silicon and mounted on a cantilever with a spring constant of ca. 40 N/m and resonance frequencies of 170 kHz range. Scanning was carried out at the free cantilever oscillation frequency and different amplitudes, depending on the stability and contrast obtained. The amplitude was set higher than 80 nm, and the set point was fixed at 10-30% of the free oscillation amplitude to guarantee that the microscope was operating in intermittent contact mode. Samples were fixed on double sided adhesive tapes, and the AFM images of the external surface were obtained in air. The Image J software was used to process images[109].

4.2.3. Thermal tests

The samples were analyzed via differential scanning calorimetry (DSC), equipment Perkin Elmer, DSC 8000. Approximately 10 mg of each sample was submitted to heating rate of 10°C/min from room temperature to 250°C. To overcome the thermal history of the samples, the second heating cycle was used to obtain the gels properties: glass temperature (Tg) and melting temperature (Tm). The degree of crystallinity (Xc) was calculated according to the Equation 2, where ΔH is the melting enthalpy, obtained experimentally, φ is the weight fraction of the filler, and the $\Delta H_{100\%}$ is the melting enthalpy of the PVA 100% crystalline[110], 138.6 J/g.

$$Xc = 100\ \frac{\Delta H}{(1-\varphi)\Delta H_{100\%}}\ (\%) \quad \text{Eq. 2}$$

4.2.4. Swelling, degradation and Ag delivery tests

Fluid absorption studies (swelling tests) were performed using three different media: sterile saline solution, Sigma Aldrich (0.9% NaCl), since it is the media used in hospitals to swell hydrogels before the application in burns[111]; Phosphate Buffered Saline - PBS, Sigma Aldrich (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl), since it can be considered an approach to the inorganic phase of body fluid; Phosphate Buffered Saline with reduced pH (pH 4.0, the pH was lowered using Lactic Acid, Sigma Aldrich) - Solution pH 4.0, which intended to simulate the local inflammatory environment, characteristic of the wounds (standard ISO 10993-9:1994).

The swelling/degradation tests followed the standard ISO 10993-9, where samples of approximately 5cm^2, weight normalized, were placed in 5ml of each media in a water bath at 37°C. The samples remained in the media at 37°C for 4 days, being weighed at regular time intervals (30 min, 1h, 2h, 3h, 4h, 1 day, 2 days, 3 days and 4 days). After 4 days, the samples were dried and weighed in order to calculate the weight loss.

The fluid absorption (swelling degree - SD) of each sample was calculated according to Equation 3, where W_W is the wet weight (weight of the sample at each time interval) and W_D is the dry weight before swelling[112]. After 4 days of immersion, the samples were dried and weighed (standard ISO 10993-9) in order to calculate the weight loss (WL), Equation 4, where W_{DS} is the weight of the dried samples after swelling tests and W_D, the weight of dried samples before it.

$$SD = 100\ \frac{W_W - W_D}{W_D} \quad (\%) \quad \text{Eq. 3}$$

$$WL = 100\ \frac{W_D - W_{DS}}{W_D}\ (\%) \quad \text{Eq. 4}$$

The swelling media (saline, PBS and Solution pH 4.0) after 4 days was analyzed via UV-Vis spectroscopy, equipment Cary 50 version 3, operated in the wavelength range 300-500 nm with the resolution of 1 nm, using polyestirene cuvettes. If silver nanoparticles were delivered to the media, then a characteristic band around 420 nm would be present in the spectra, due to the surface Plasmon resonance effect[113-115].

4.2.5. Tensile tests on swelled samples

At least 10 samples of each composition (PVA, 0.25, 0.50) were cut in dog-bone shape from the dried hydrogels. After 1 day of swelling in the three different media (Saline solution, PBS and Solution pH 4.0), the samples were submitted to tensile tests at room temperature. Three measurements of the cross-section area, in the same swollen sample, were made. The samples were then attached to the grips (Zwick Z005 Tensile Test Machine) with the help of sand paper (the back of the sand paper in contact with the sample). The tests were performed using a 500 N load cell, cross-head rate of 10 mm/min until failure.

According to the ASTM D882-00 standard, the samples could be considered isotropic. The results of at least 10 samples were used to obtain the fracture strength values (σ_F) and the Secant modulus values (E). The Secant modulus of each sample was calculated at the strain of 50%. The average tensile curves for each condition were plotted.

4.2.6. Antimicrobial tests

Antimicrobial activity of the PVA and of PVA-$AgNO_3$ hydrogels swollen in PBS against *E. coli* (ATTC 25992), *S.aureus* (ATTC 6538) and *C. albicans* (ATTC 10231) were evaluated using the disc diffusion method[40]. Overnight grown cultures of *E. coli*, *S aureus* and *C. albicans* were individually diluted and plated on Mueller Hinton agar inoculated with approximately 10^8 colony forming unit / ml. The hydrogel samples consisting of different compositions (PVA, 0.25 and 0.50) were cut (circular,

ϕ1.80 cm), kept in 5 ml of PBS overnight and then placed on the plates. The plates were incubated at 37°C for 18h and the zones of inhibition were measured.

For the antimicrobial penetration test, autoclaved test tubes with10 ml of nutrient broth were covered with the hydrogels, a sealed tube was used as the negative control and an open tube was used as the positive control. The turbidity of the media was observed for up to one month.

4.2.7. Cytotoxicity analysis

The cytotoxicity test was performed according to the Alamar Blue Assay[116], to evaluate the cell viability. Human keratynocites, HACAT cells, were obtained in the cell bank of the DCU, Dublin, Ireland. The cells were incubated in a sterile 48 well-plate by adding $5x10^4$ cells / ml of DMEM (10% fetal bovine serum - FBS, 1% penicillin / streptomycin) in each well until they reached sub-confluency. The media was then removed and 1 ml of the samples extracts was added. The negative control was wells (sub-confluency) in which 1 ml of DMEM were added and the positive control was empty wells. Extracts were obtained after immersion of 2 cm^2 of each sample (PVA, 0.25 and 0.50, triplicates) in 2 ml of Dulbecco's modified Eagle's medium (DMEM) for 24h at 37°C in incubator.

The HACAT cells remained in contact with 1 ml of each samples' extract in the incubator, humidified 5% CO_2 atmosphere for 24h at 37°C. After the incubation, 1ml of solution 10% alamar blue in DMEM was added to each well and the plate remained for 5h in the incubator. 200 μl of each media in each well was placed in the wells of a 96 well-plate (no centrifugation) and they were analyzed in a UV-Vis spectrometer (Nanoquant Infinite m200, Tecan). The absorbance was measured at 570 nm and at 600 nm, wavelengths to evaluate the cells viability, since the cells growth causes a reduction of the alamar blue, where the cells continued growth maintains a reduced environment (red colour) and the inhibition of growth, an oxidized environment, blue colour. The material is considered non toxic if at least 75% of the cells survived.

4.2.8. Statistical analysis

To analyze the significance of the two factors (medium type and amount of silver) and three levels of each factor (saline, PBS and PBS pH4; 0%, 0.25% and 0.5% Ag, respectively) in some results, two-way ANOVA analysis, program Origin Pro 8®, significance level of 95% ($\alpha = 0.05$), was used. A Tukey test was conducted to find out the difference between the levels on the swelling degree (at room temperature and at 37°C); on the weight loss after the degradation test; on the secant modulus and fracture strength results and on the UV-Vis results.

The ANOVA 1-way analysis, significance level of 95%, was used to analyze the significance of the factor amount of Ag on the gels' antimicrobial and cytotoxicity results. For the antimicrobial results three levels were used: PVA, 0.25 and 0.50, while for the cytotoxicity results, 5 levels were used, (+) control, (-) control, PVA, 0.25 and 0.50. When the factor was significant, a Tukey test was conducted to find out if there was a significant difference between the levels.

4.3. PVA-propolis samples

PVA solutions were obtained by dissolving 10g PVA in 100ml of deionized water at approximately 90°C for 6h, under mechanical stirring. After dissolution, the solutions remained under stirring until they reached environmental temperature. For the PVA-propolis samples, different amounts of Propolis extract were added under mechanical stirring, 0.075% (1.5ml of propolis/petri dish), 0.15% (3ml of propolis/petri dish), since these were the dose recommended for topical application (1 to 3 ml or 500 to 1000 mg daily)[117], and some extrapolations to high doses were also prepared, 0.45% (9ml of propolis/petri dish) and 0.90% (18ml of propolis/petri dish) of Propolis related to the volume of the PVA solution. The amount of PVA solution in each petri dish (ϕ140 mm) was fixed in 20 ml/dish (the added propolis volume was considered an extra amount to each dish).

The samples were then freeze-thawed, for 16h at -18°C followed by 5 cycles of 30 min at room temperature and 1h at -18°C. The samples were dried in environmental conditions and they were exposed to 30 min of UVB radiation to sterilize them.

4.3.1. Microstructural characterization

The samples were analyzed by X-ray diffraction – XRD, using the same equipment and conditions as the analysis of the PVA-Ag samples, except that the range for PVA-propolis samples was set between 5° and 50°. The FTIR analysis was similar (same equipment and range used for the PVA-Ag samples), with the exception that the number of scans was 64 scans per sample. The FTIR results of the samples after swelling were displayed in Annex II.

4.3.2. Swelling, degradation and drug delivery tests

Triplicates of each samples composition (~2cm^2, weight normalized) for each time interaval studied (1, 2, 4, 24 and 96 h) were immersed in 2ml of different fluids at 37°C. Two different media were used, standard ISO 10993-9, Phosphate Buffered

Saline - PBS, Sigma Aldrich, an approach to the inorganic phase of body fluid; Phosphate Buffered Saline with reduced pH (pH 4.0, the pH was lowered using Lactic Acid, Sigma Aldrich), intended to simulate the local inflammatory environment, characteristic of the wounds. The samples were removed after each time interval to calculate the swelling degree, the weight loss and to allow the analysis of the propolis delivery at each time interval.

The fluid absorption (swelling degree - SD) of each sample was calculated according to Equation 3[112]. After 4 days of immersion, the samples were dried and weighed in order to calculate the weight loss (WL), Equation 4.

The swelling media was analyzed after fixed time intervals (1h, 2h, 4h, 24h and 96h) and analyzed via UV-Vis spectrometer, from 300 nm to 800 nm, using polyestirene cuvettes. For quantification of the propolis delivered, a standard curve was prepared by analyzing controlled dilutions of propolis in isopropyl alcohol. The propolis dilutions (0.30×10^{-5}, 0.60×10^{-5}, 1.80×10^{-5} and 2.40×10^{-5} g/ml). The amount of propolis delivered was obtained via comparison with the standard curve. The detailed results were displayed in Annex III.

4.3.3. Tensile tests on swelled samples

The tensile tests were performed and analyzed according to the same methodology described for the PVA-Ag samples tensile tests, exception for the load cell used, the PVA-propolis tests were performed using a 5 kN load cell.

4.3.4. Thermal tests

The DSC thermal analysis was performed and analyzed according to the methodology described for the PVA-Ag samples thermal analysis.

4.3.5. Antimicrobial tests

The antimicrobial analysis was performed and analyzed according to the methodology described for the PVA-Ag samples antimicrobial analysis.

4.3.6. Cytotoxicity analysis

The cytotoxicity analysis was performed and analyzed according to the methodology described for the PVA-Ag samples cytotoxicity analysis.

4.3.7. Statistical analysis

The ANOVA two-way analysis, significance level of $\alpha = 0.05$, was used to analyze the significance of two factors, type of media and amount of propolis on the gels' swelling capacity, weight loss and mechanical properties. For the factor type of medium ("Media"), two levels were used: PBS and Solution pH 4.0 (named "pH4"). For the factor amount of propolis ("% Propolis") five levels were used: 0 (PVA), 0.075%, 0.15%, 0.45% and 0.90% of Propolis, program Origin Pro 8®. For the drug delivery analysis, 4 levels were used for the factor amount of propolis instead of 5 levels, 0.075%, 0.15%, 0.45% and 0.90% of Propolis. When the factors were significant for the property analyzed, a Tukey test ($\alpha = 0.05$) was conducted to find out if there was a significant difference between the levels.

The ANOVA 1-way analysis, significance level of $\alpha = 0.05$, was used to analyze the significance of the amount of propolis on the gels' antimicrobial properties and cytotoxicity. Five levels were used: 0 (PVA), 0.075%, 0.15%, 0.45% and 0.90% of Propolis for the analysis of the antimicrobial activity and 7 levels were used for the cytotoxicity results analysis, negative control (-), positive control (+), 0 (PVA), 0.075%, 0.15%, 0.45% and 0.90% of Propolis. When the factor was significant, a Tukey test ($\alpha = 0.05$) was conducted to find out if there was a significant difference between the levels.

5. Results and Discussion

5.1. PVA-Ag - Results and Discussion

The results obtained for the PVA-Ag samples will be presented in the following sections.

5.1.1. Macroscopic analysis

The colour of the PVA, 0.25 and 0.50 samples were considerably different from each other (Figure 5.1.1), where the yellow/brown colour observed on the gamma irradiated PVA-Ag samples is an indicative that the $AgNO_3$ was reduced to metallic Ag[118]. Saion's group[119] showed that when PVA-$AgNO_3$ samples are submitted to gamma radiation, the first step of silver reduction would be: PVA-$AgNO_3$ → PVA-$\bar{e}$ + $AgNO_3$; then, the reduction itself takes place: $\bar{e} + Ag^+ \rightarrow Ag^0$ (atoms); finally, agglomeration occurs: $n\ Ag^0 + Ag^0 \rightarrow (n+1)\ Ag^0$ (nanoparticles). According to them, the yellow colour would be related to the formation of silver nanoparticles, however they also observed that the samples got darker with the increase of the gamma dose. Moreover, higher doses resulted in smaller silver particles.

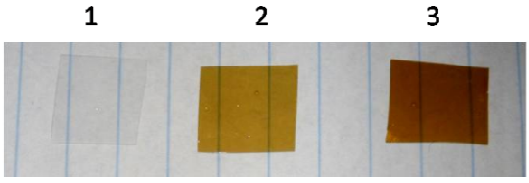


Figure 5.1.1 - Image of the samples (1) PVA, (2) 0.25 and (3) 0.50

5.1.2. Surface analysis

To confirm the presence of a nanoparticle phase on the gels surface, some samples were analyzed by AFM, Figure 5.1.1. Comparing the cross-section plot of the

topographic images of PVA and of 0.25, Figure 5.1.1 (a) and (c), higher and wider peaks were present in 0.25 plot, which can be related to the expected silver nanoparticles. Based on the image analysis of 0.25 topographic image (not shown), the nanoparticles have approximately (89 ± 32) nm in diameter.

AFM phase contrast images are produced by changes in phase angle of the cantilever probe and have been shown to be sensitive to stiffness, viscoelasticity and chemical composition of materials surface[120]. The PVA contrast phase image shows a homogeneous material, Figure 5.1.1 (b), where only a polymeric matrix is observed. A second phase, different from the polymeric matrix, is observed in the image of the sample with silver, Figure 5.1.1 (d). The dark regions in the phase image of the 0.25 sample are probably related to silver nanoparticles surrounded by PVA matrix (clearer regions).

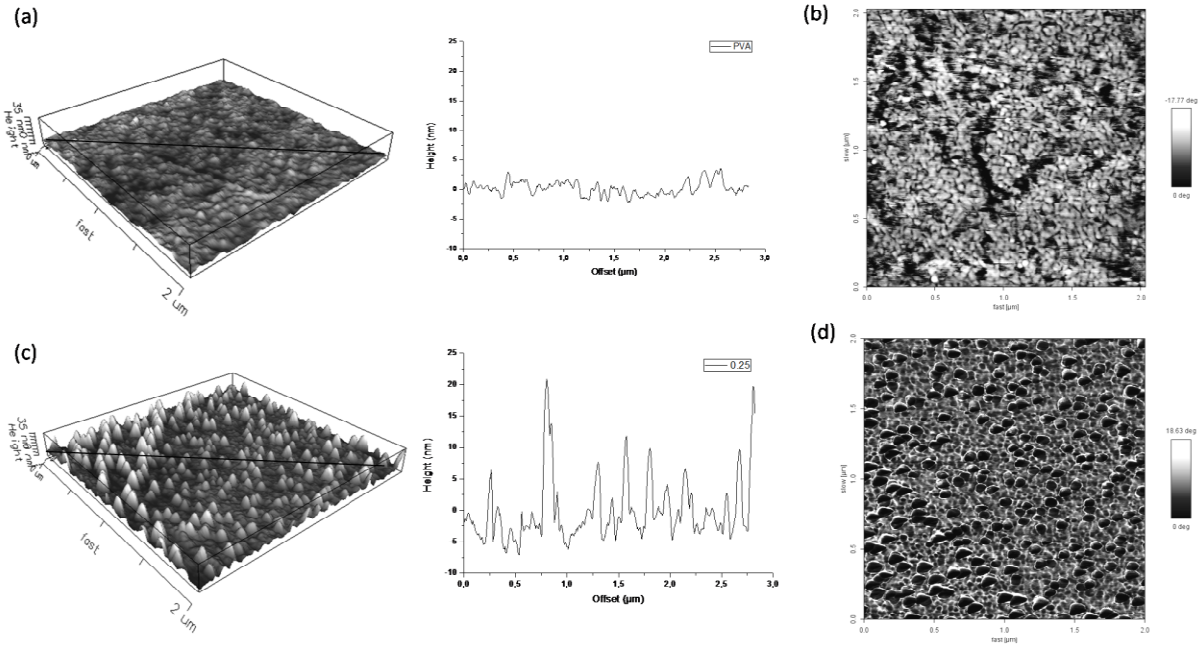


Figure 5.1.1 - AFM images, (a) topographic 3D image of PVA and line profile of the PVA sample, (b) phase image of PVA, (c) topographic 3D image of 0.25 and line profile of the 0.25 sample, (d) phase image of 0.25.

5.1.3. Microstructural analysis

The diffraction patterns of the hydrogel samples are displayed in Figure 5.1.2. The PVA characteristic peak (at 2θ = 19.6°) was observed in all samples, however this peak intensity was reduced in the composite samples, first indicative of the interference of silver in the polymer crystallization. The composite samples also presented peaks at 2θ = 14.0° and at 2θ = 16.8°. These peaks were not related to metallic silver (the peaks of Ag^0 appear after 2θ = 30°, JCPDS 41-1402) nor to the chelate[121] PVA-Ag^+.

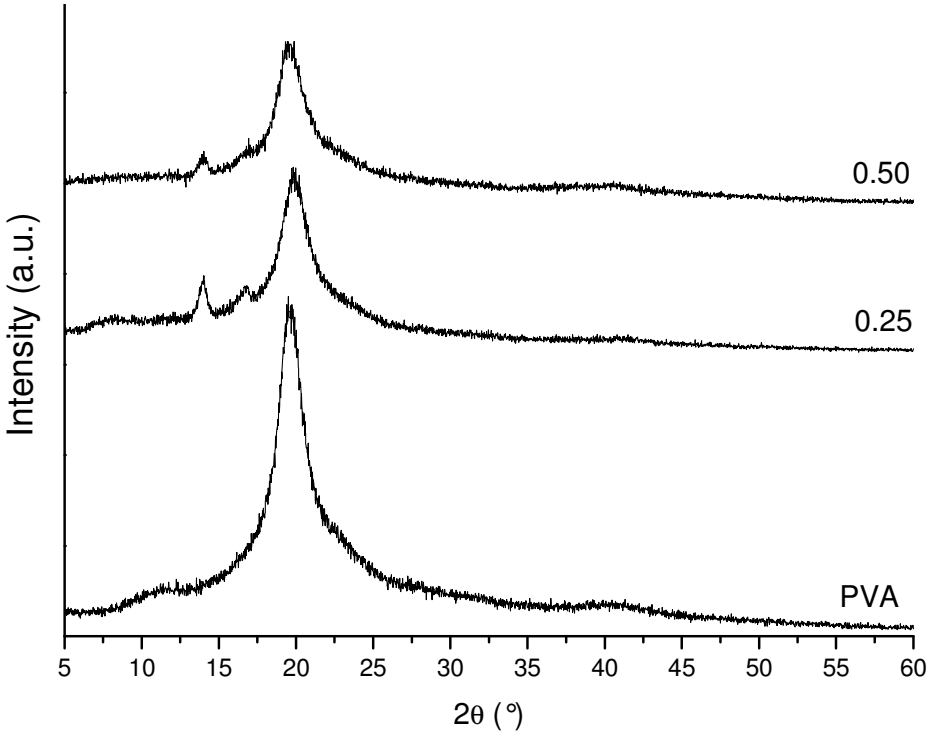


Figure 5.1.2 - Diffraction patterns of hydrogel samples.

Based on the XRD results it was possible to calculate the crystallite size of each sample, based on the Sherrer equation[108]. The crystallite sizes were 7.70 nm, 7.41 nm and 6.98 nm for PVA, 0.25 and 0.50 samples respectively. It can be noticed that the increase of the amount of silver led to smaller crystallites, showing that the presence of nano-Ag interferes with the PVA crystallization.

The FTIR spectra of the hydrogels samples did not show considerable differences, Figure 5.1.3. If the silver is present as nanoparticles instead of in the chelate form (in chelate, the silver is attached to the polymer, where it could interfere with some of the polymer binding vibration modes), the nanoparticles could be close to the PVA chains with no interference with the polymer bonds vibration modes[122-124].

The bands and respective vibration modes found in the original samples, Figure 5.1.3 (a), according to different groups studying PVA and its interactions, were related to PVA, Table 5.1.1.

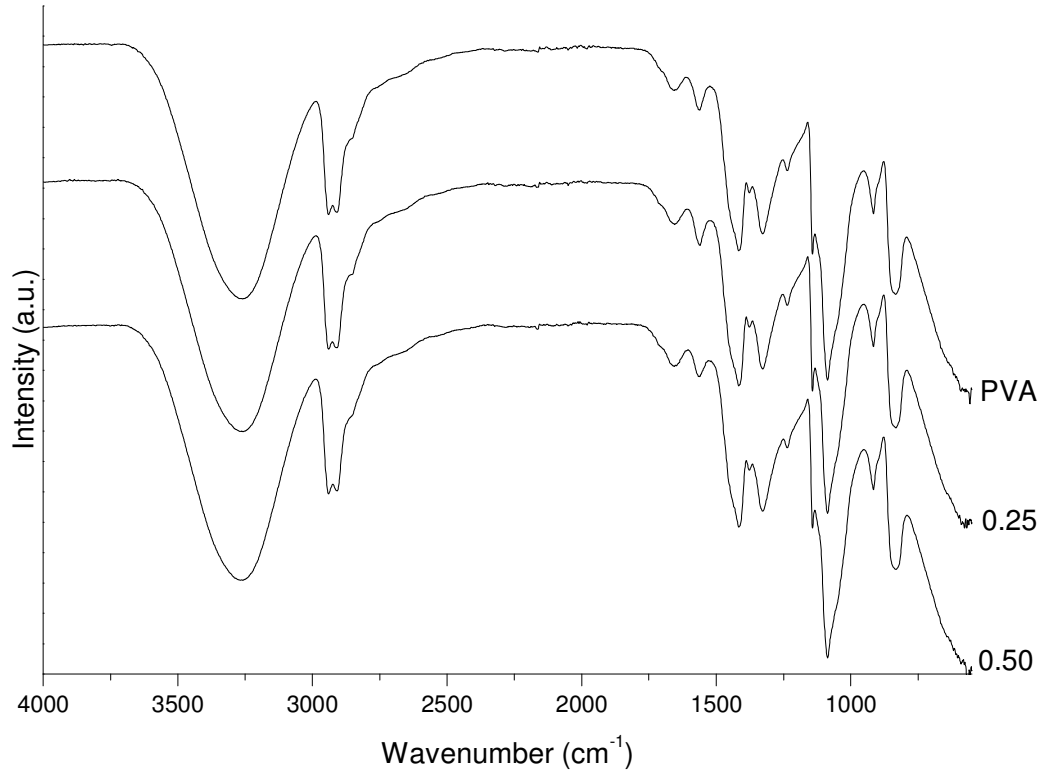


Figure 5.1.3 - FTIR spectra of the samples.

The crosslinking can be observed due to the band at 1143-1142 cm^{-1}. It is related to the stretching vibration of C-O-C, this binding was probably formed when the radiation reached the polymer chains, forming free radicals (for example, ~CH_2-$CHO^{\cdot}$-CH_2~) which react with other chains (or with other parts of the same chain), forming bindings like C-O-C, and eliminating small molecules, like water[125].

Table 5.1.1 - PVA bands vibration.

PVA Bands (cm^{-1})	*PVA groups' vibration modes*[41,113,114,126-135]
3290-3266	υ(O-H)
2936-2931	υ(C-H) in CH_2 groups
2923-2909	
2852	
1659-1649	υ(C=O) and/or υ(C=C)
1564-1562	υ(C=C) of the acetate groups non-hydrolyzed
1418-1416	δ(CH_2), wagging (C-H), symmetrical υ(C-O-C)
1375-1378	coupling of O-H in plane vibration with C-H wagging vibration
1330-1325	bending (CH + OH)
1237-1234	υ(C-C)
1143-1142	υ(C-O-C)
1090-1088	υ(C-O)
918-915	υ(CH_2) and out-of-plane bending (C-H)
841-836	υ and pendular mode of (C-C)

No increase was observed in the transmittance of the band at 1375 cm^{-1} compared to the band at 1420 cm^{-1}, in samples with Ag. If there were an increase of the band at 1375 cm^{-1}, it could indicate a decoupling between OH and CH vibrations due to the interaction of nano-Ag and OH groups[130,131]. No chemical interaction between Ag and PVA was observed in this region of the PVA-Ag spectra.

In addition, a small decrease in the intensity of the band at 1141 cm^{-1} would be related to the incorporation of nano-Ag in the PVA matrix, indicating a decrease in the PVA crystalline phase[131]. No decrease at ~1140 cm^{-1} was observed, although the reduction of the PVA crystalline phase due to the presence of nano-Ag was indicated by the XRD analysis. No evidence of chemical interaction between Ag and PVA was encountered.

The FTIR profiles of the samples after immersion in saline solution, in PBS and in Solution pH 4.0 for 4 days are displayed in Annex I. It can be noticed that in PVA swelled samples, the band related to the acetate groups non-hydrolyzed (1566 cm^{-1}) disappears after swelling, indicating some leaching of the acetate groups[113,126-128]. The shoulder at ~2850 cm^{-1}, C-H stretching vibration from alkyl groups, becomes a band after the immersion in Solution pH 4.0, may be due to a contribution of the vibration of alkyl groups from remaining lactic acid in the polymer network[129,132]. After PVA immersion in acidic media, the band at ~1650 cm^{-1} splits in two, 1650cm^{-1}, C=C stretching, and ~1712 cm^{-1}, related to the C=O stretching band of lactic acid[113,134,136-138].

The PVA-Ag samples after swelling revealed that the band at 1566 cm^{-1} in the original samples disappears after swelling, indicating some leaching of acetate groups. The PVA bands presented low intensity after immersion in Solution pH 4.0. Also, in Solution pH 4.0, the band at ~1650 cm^{-1} splits in two bands, at 1648 cm^{-1} and at 1712 cm^{-1}, the last one related to lactic acid.

5.1.4. Thermal analysis

The DSC analysis, Figure 5.1.4, shows that the parameters related to the crystalline phase of PVA [degree of crystallinity (Xc) and melting temperature (Tm)] are affected by the presence of Ag, although no trend was observed. For the sample 0.50, low degree of crystallinity (Xc) and low Tm were observed, indicating an influence of the silver nanoparticles on the chain packing, data that corroborate the XRD indication of lower crystallinity in the presence of silver.

Regarding the amorphous phase, the glass temperature (Tg) of the polymer increases with the increase of silver concentration. The Tg values were 71.77°C, 72.72°C and 74.61°C for PVA, 0.25 and 0.50 samples respectively. The nanoparticles might anchor the PVA chains of the amorphous phase, which difficult the chains movement, increasing the Tg.

According to Kareen and Kaliani[139], 2011, p. 330, whose studies are related to PVA-Ag systems, there is an increase in the Tg due to the presence of Ag, “the reason for shifting in Tg towards higher temperature region as it is because of the increased lateral forces in the bulk state due to the restricted steric effect of –OH groups by the branching of Ag+ ions with the chain of PVA molecules”.

Vodnik and collaborators[140], 2013, p. 56, studied similar systems and they observed a shift in the Tg in samples with Ag, “The nanocomposites are found to have a slightly higher glass transition temperatures (Tg) (about 4–8 °C) corresponding to different segmental relaxations as a results from micro-Brownian motion of the main-chain backbone, compared to the pure PVA”. They also observed similar values of the PVA crystalline phase parameters for the samples with different amounts of Ag.

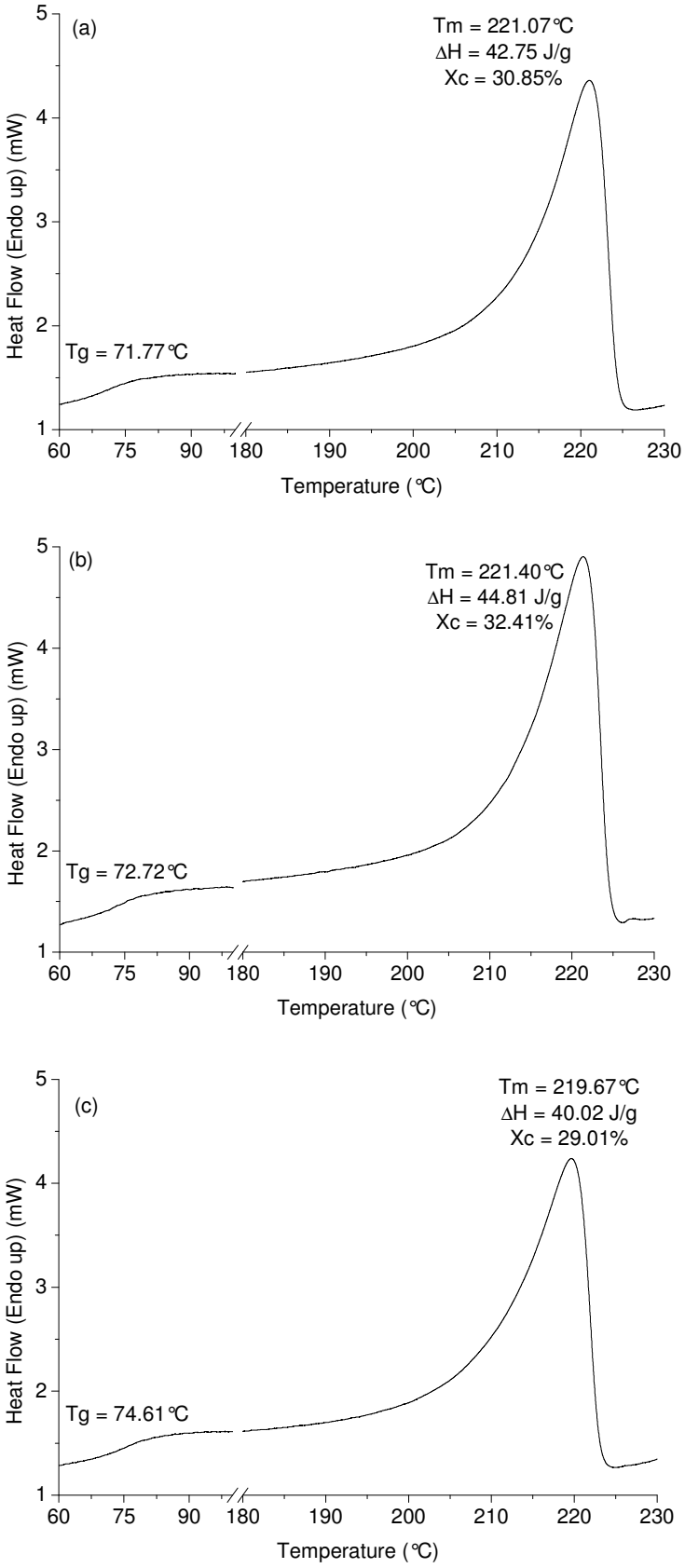


Figure 5.1.4 - DSC analysis of (a) PVA, (b) 0.25 and (c) 0.50, where ΔH is the enthalpy, Tm is the melting temperature and Xc is the degree of crystallinity.

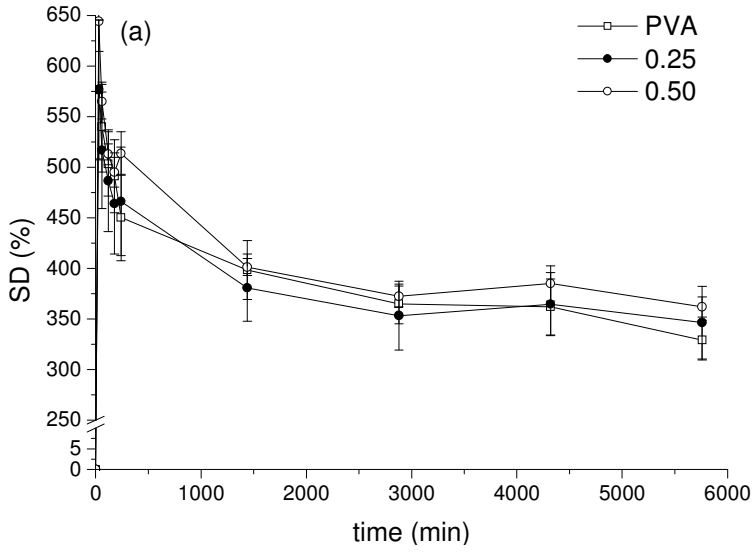
5.1.5. Swelling, degradation and drug delivery tests

a. Swelling tests

The swelling degree of the samples immersed in different solutions is shown in Figure 5.1.5. The equilibrium of swelling degree, after 1 day in saline solution, the most common swelling media available in hospitals to hydrate dry hydrogels, was (398 ± 29)% for the PVA samples, (380 ± 33)% for the 0.25% Ag samples and (401 ± 8)% for the 0.50% Ag samples [Figure 5.1.5(a)].

The samples tested in PBS, a medium that mimics the inorganic part of human plasma, Figure 5.1.5(b), at 37°C showed behaviour slightly different from that of the samples swollen in saline solution. In PBS, the higher the amount of silver in the samples, the higher was the swelling degree. The equilibrium of swelling degree, after 1 day of immersion, was (340 ± 33)% for PVA samples, (409 ± 41)% for 0.25% Ag samples and (481 ± 37)% for 0.50% Ag samples.

The samples swollen in Solution pH 4.0 at 37°C presented a swelling behaviour close to each other, Figure 5.1.5(c). The equilibrium of swelling degree, after 1 day, for all samples was: (309 ± 25)% for PVA samples, (304 ± 13)% for 0.25% Ag samples and (333 ± 22)% for 0.50% Ag samples.



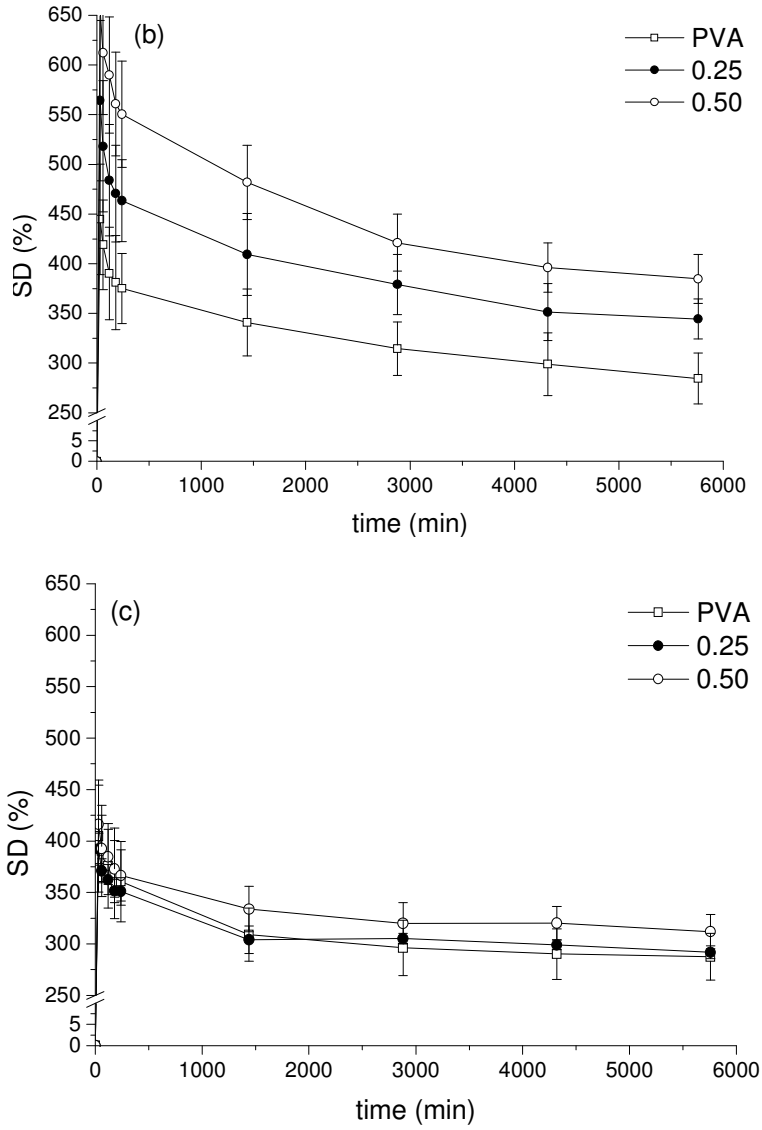


Figure 5.1.5 - Swelling degree of the samples swelled at 37°C in (a) saline solution, (b) PBS and (c) Solution pH 4.0.

Based on the statistical analysis, 2-way ANOVA analysis, $\alpha < 0.05$ (Annex IV, Table IV.1), it can be noticed that all factors (medium and %Ag) and their interaction are significant to the swelling behaviour at 37°C. However, with regard to the amount of silver, there is a relevant difference between the samples with 0.50% Ag and the others. More silver meant higher swelling.

In all media, the highest amount of silver led to highest swelling. The silver precursor, $AgNO_3$, allows the presence of Ag^+, which interacts with the hydroxyl groups of the PVA. The gamma radiation crosslinks the PVA and also reduces the silver. According to Nghiep and collaborators[141], who submitted aqueous solutions of

PVA to gamma radiation, in the absence of oxygen, suggested the crosslinking would occur due to the formation of radicals in the PVA chains when irradiated:

$-(CH_2 - CHOH - CH_2 - CHOH)- \rightarrow -(CH_2 - C^{\cdot}HO - CH_2 - CHOH)-$

$-(CH_2 - CHOH - CH_2 - CHOH)- \rightarrow -(CH_2 - CHO^{\cdot} - CH_2 - CHOH)-$

According to Gautam and collaborators[81], the OH groups in PVA adsorb metallic cations. The sequence of the nano-Ag formation would be: $[Ag^+\text{-PVA}]^-$ (chelate) $\rightarrow$ Ag + PVA^-. The group of Kumar[142] submitted PVA hydrogels, loaded with $AgNO_3$ solution, to gamma radiation and they observed that the radiation crosslinks the polymer and reduces the silver.

Park and collegues[143] studied PVA-PEG hydrogels (15wt% PVA), submitted to gamma radiation (50 kGy). The swelling degree in water was high with the increase of PEG content, since the PEG acts as a plasticizer decreasing the interactions between the PVA chains. When irradiating the samples, the silver reduction would compete with the polymer crosslinking. The presence of Ag could act as a plasticizer and it could also interfere with the PVA capacity of crystallization, leading to a material with high amorphous phase, justifying their high swelling capacity.

Regarding the type of medium, there is a difference between Solution pH 4.0 and the other media. The samples presented low swelling in Solution pH 4.0 at 37°C. The samples presented lower affinity for acid pH.

The immersion of the samples in the different media, alters the colour of the PVA-Ag hydrogels. PVA remains transparent, but the PVA-Ag samples become transparent after swelling (not shown), indicating silver delivery or silver speciation due to interactions with the media.

According to Liu and collaborators[144], silver speciation ($Ag^0 \rightarrow Ag^+$; $Ag^0 \rightarrow Ag^+$, $Ag^+ + Cl^- \rightarrow AgCl_{(s)}$; etc) depends on the composition of the medium. Silver binds to anionic species, such as Cl^-, S^{2-}, thiols (-SH)[145]. Chernousova and Epple[146] studied silver in different media (freshwater, saltwater, blood) and they observed that Cl^- reacts with silver forming AgCl (transparent/white), that could be in colloidal dispersed form or precipitated.

Gemeinhart and collaborators[147] studied the swelling of poly (acrylamid-co-acrylic acid) stimuli-sensitive hydrogels and they found out that the increase in ionic

strength of the media resulted in the decrease of the swelling capacity. The same trend was found by Ganji and colleagues[148] when they studied hydrogels swelling in solutions with different ionic strength, lower swelling with increasing ionic strength.

The PVA samples swelling capacity followed the progression: saline < PBS < PBS pH 4.0. PVA samples presented higher swelling in PBS than in saline. The ionic strength of saline is 154 mM and the ionic strength of PBS is 279.7 mM. Apparently, high ionic strength and low pH meant high PVA swelling.

The medium type was significant to the swelling behavior of the PVA-Ag samples, but there was a difference only between PBS and the other media. The swelling is higher in PBS for all cases. The higher swelling in PBS than in saline would probably be related to the ionic strength of each solution, saline - 154 mM and PBS - 279.7 mM.

So far, it can be inferred that the ionic strength affects the swelling capacity of the samples.

b. Weight loss

After drying the samples immersed in saline, in PBS and in Solution pH 4.0 at 37°C for 4 days, the weight loss was obtained and is shown in Figure 5.1.6.

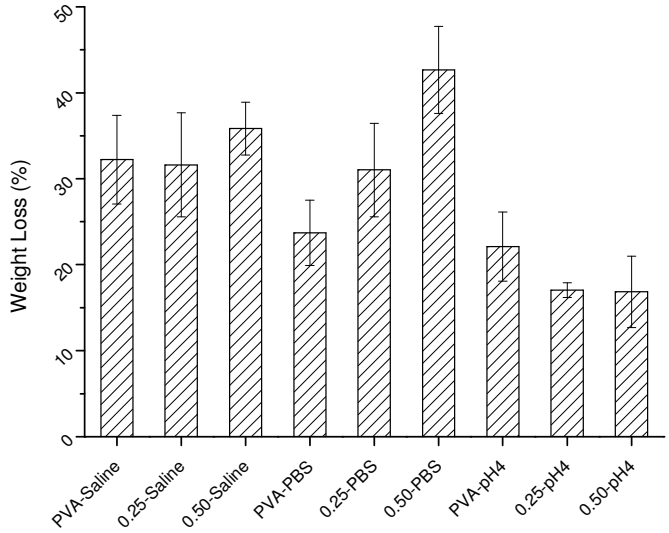


Figure 5.1.6 - Weight loss occurred after 4 days of samples immersion in saline, PBS and Solution pH 4.0 at 37°C

The ANOVA analysis, Annex IV, Table IV.2, showed that at the 0.05 level, the type of medium, the amount of Ag, as well as their interaction, was significant to the weight loss results. The weight loss in pH 4.0 was considerably lower than in the other media and the weight loss of sample 0.50 was significantly higher than the weight loss of the other samples.

c. Silver delivery tests

After 4 days of swelling at 37°C, the media were analyzed by UV-Vis and their profile were similar to the profile showed in Figure 5.1.7. It could be observed a band around 450 nm in all samples and their transmittance results are displayed in Figure 5.1.8.

According to Chahal and collaborators[113], samples of PVA-Ag present a band around 425 nm that is related to the surface Plasmon resonance (SRP) of silver. The SPR occurs when the electrons in the conduction band of the nano-silver interact with the electromagnetic radiation generating the resonance of the collective oscillations of the nano-Ag conduction bands[149].

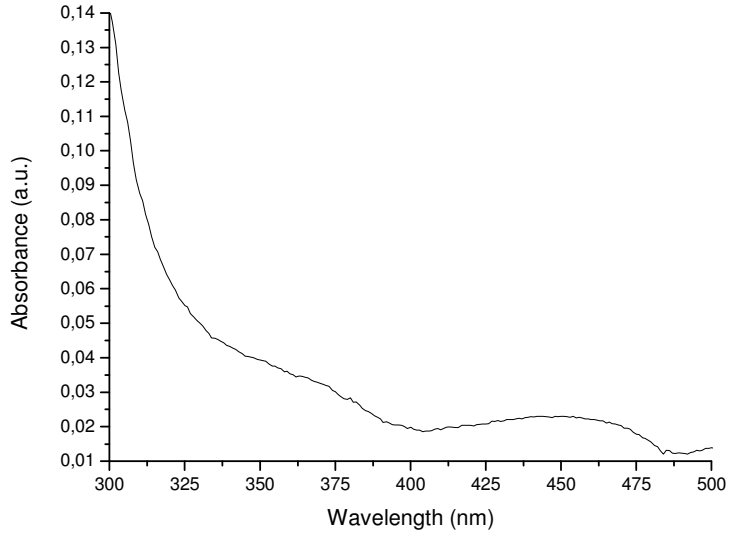


Figure 5.1.7 - Spectrum of the medium of the 0.50 sample in Solution pH 4.0.

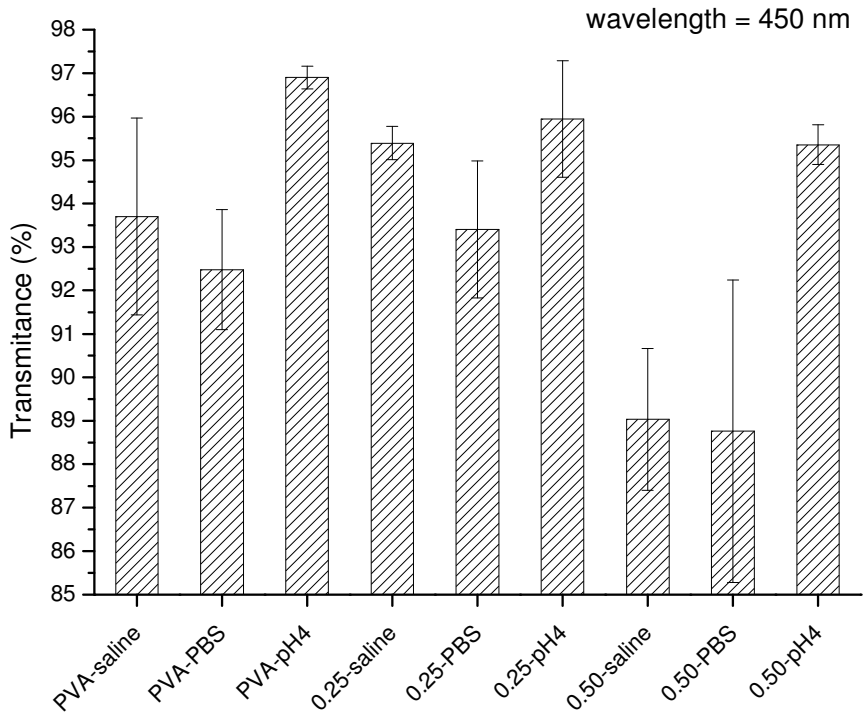


Figure 5.1.8 – Average transmittance results (450 nm) of the swelling media after 4 days.

Ananth and collegues[149], studied PVA-Ag nanocomposites and encountered a band at 420 nm related to Ag particles with average diameter of 20 nm and they also realize that SPR band is shifted to longer wavelengths when the particles are bigger than 20 nm. The present work found the SPR at 450 nm, an indicative of particles larger than 20 nm, fact that corroborates the AFM results.

The lowest values of transmittance were related to the samples with the highest amount of silver, which could mean high silver delivery to the media. The ANOVA analysis showed that, at the 0.05 level, Annex IV, Table IV.3, both the type of media and the amount of silver were significant to the silver delivery results, although their interaction is not. The lowest nano-Ag delivery occurred for pH 4.0 medium, irrespective to the amount of silver in the samples. The highest nano-Ag delivery occurred for the 0.50 samples.

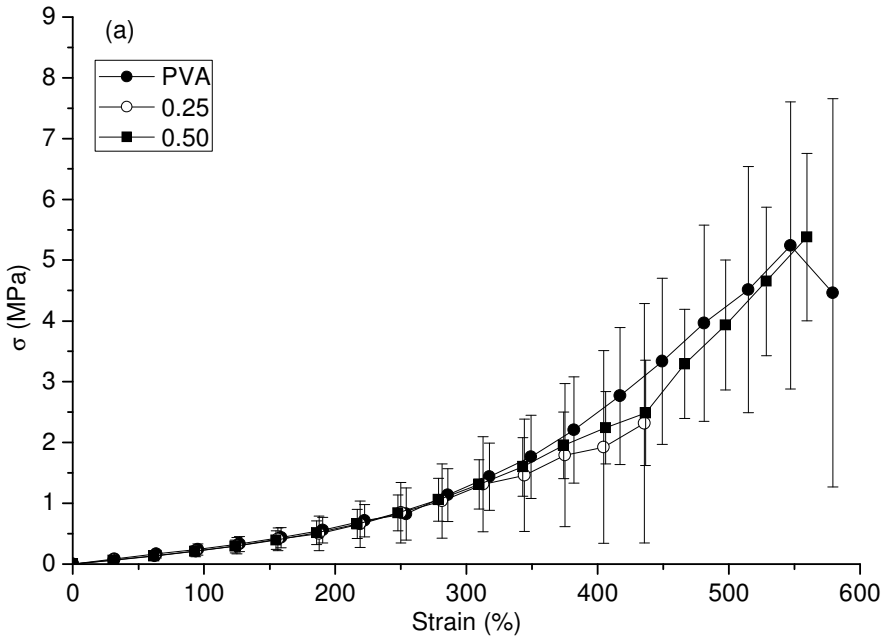
All samples swollen in acid pH presented higher transmittance. The high transmittance could indicate a small delivery of nano-Ag, but it could also means that the silver nanoparticles oxidized in contact with the fluids. Silver oxidation could result in different Ag species[146,150], e.g. Ag^{+}.

Ionic silver presents a band at 200-230 nm[151], which could not be identified since the wavelength's inferior limit of the test was 300 nm. Since the weight loss in pH 4.0 was low, probably the silver remained trapped in the gels, independent of its oxidation state.

The analysis of the transmittance when the parameter in study was the amount of silver, revealed that the 0.50 samples presented the highest swelling degree coupled with the highest weight loss and lowest transmittance (~450 nm). Since the solution uptake was high, the network would be stretched, allowing the high silver delivery, and the high weight loss (related to Ag and to PVA).

5.1.6. Mechanical analysis

The tensile tests of the average curves based on the 10 tested samples of each composition, after 1 day of swelling in the three media used, are displayed in Figure 5.1.9.



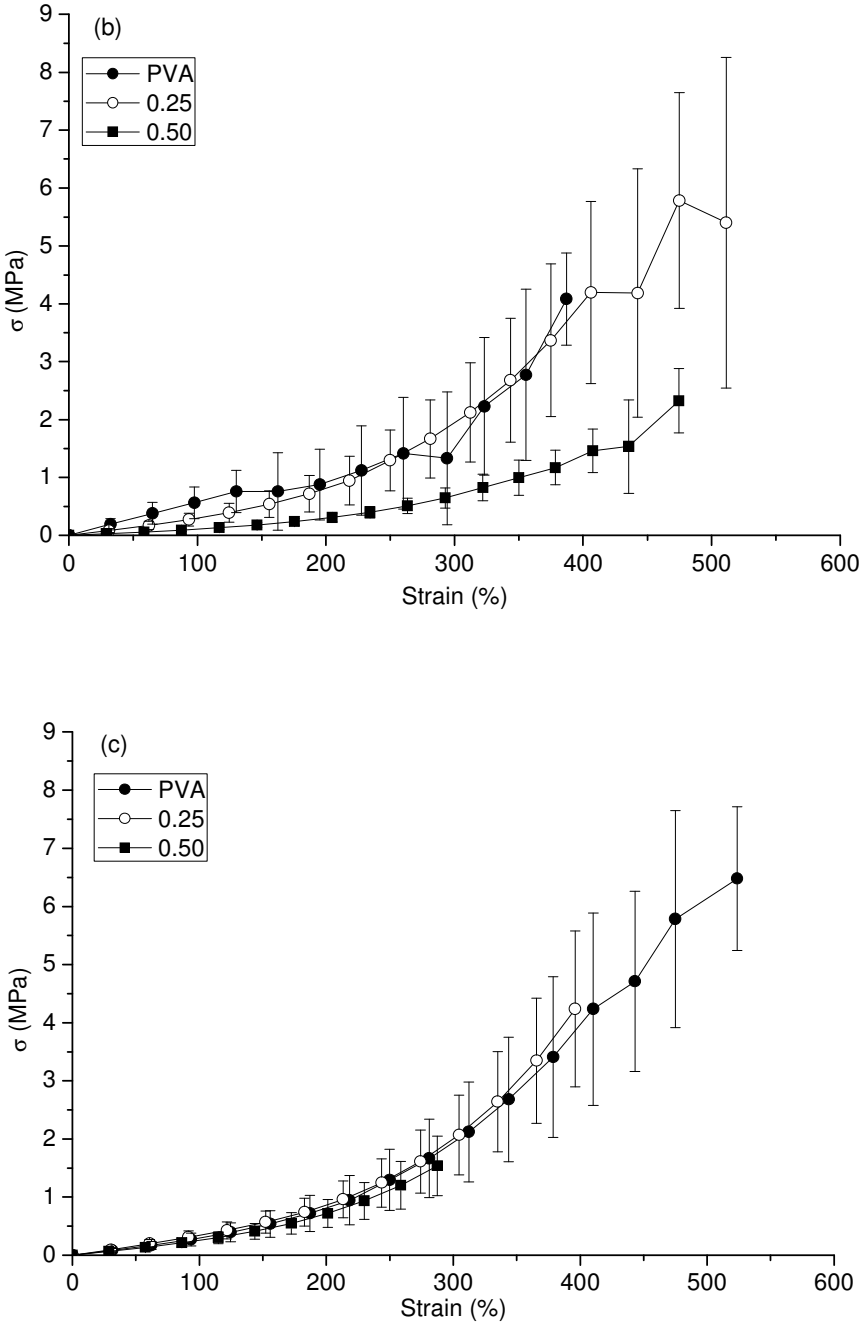
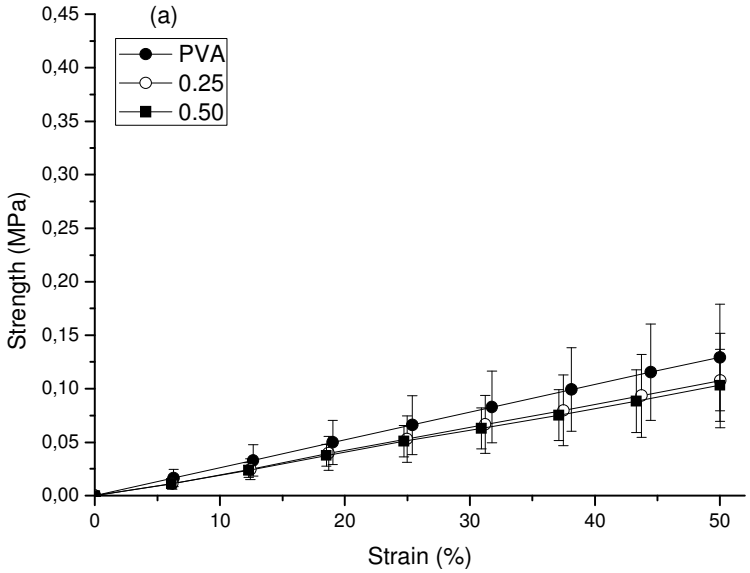
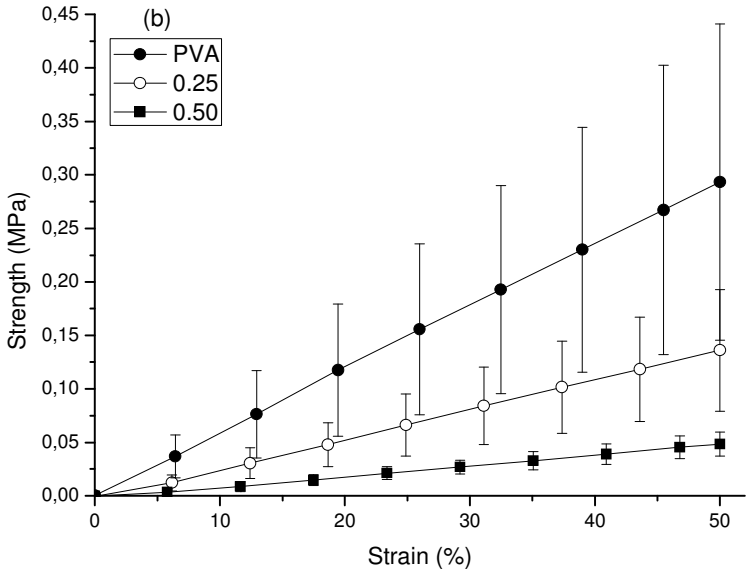


Figure 5.1.9- Profile of the average curves for PVA, 0.25 and 0.50 samples immersed in (a) saline, (b) PBS and (c) solution pH 4.0.

The results relate to the average value of each sample using saline solution, PBS and PBS ph 4 until strain of 50%, used to calculate the Secant modulus, are displayed in Figure 5.1.10 (a) - (c). The behaviour of all compositions followed the same trend.

The Secant modulus average result of each sample, the fracture strength average result and the fracture strain average results, analyzed in each media, are displayed in Table 5.1.1.





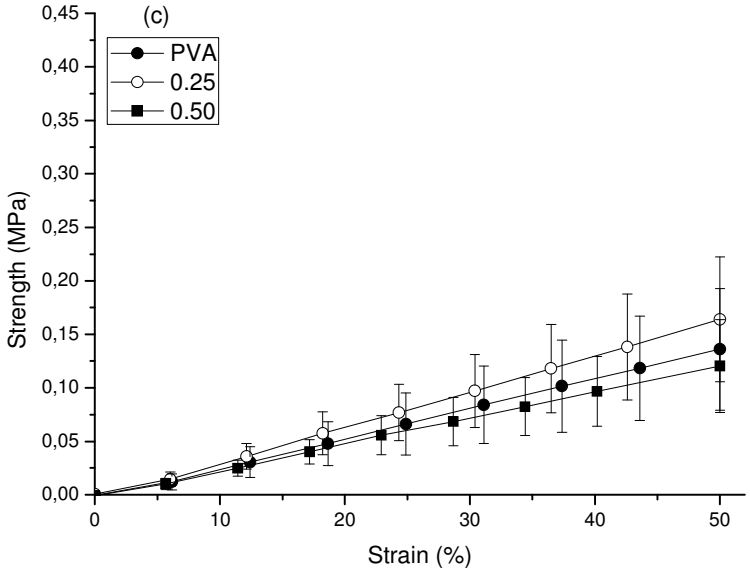


Figure 5.1.10 - Stress-Strain curves, until 50% strain, of the samples swollen in: (a) saline; (b) PBS; and (c) Solution pH 4.0.

Table 5.1.1 - Secant modulus (E), fracture strength (σF) and strain at failure (e) of all samples in different media.

	Saline			**PBS**			**Solution pH 4.0**		
	E (MPa)	**σ_F (MPa)**	**e (%)**	**E (MPa)**	**σ_F (MPa)**	**e (%)**	**E (MPa)**	**σ_F (MPa)**	**e (%)**
PVA	0.24±0.09	4.30±3.20	484±136	0.57±0.29	4.91±4.80	345±183	0.26±0.10	7.09±3.06	486±118
0.25	0.20±0.08	3.93±2.39	481±142	0.26±0.10	7.09±3.07	486±118	0.31±0.10	9.68±4.75	518±76
0.50	0.19±0.07	3.86±2.16	475±107	0.08±0.01	1.84±0.73	427±62	0.22±0.07	3.69±2.17	395±69

It can be observed that the average values of secant modulus of all samples in different media are between 0.2 - 0.3 MPa, although the secant modulus of the samples swollen in PBS are higher. The two-way ANOVA analysis, $\alpha < 0.05$, on the secant modulus, Annex IV, Table IV.4, revealed that the 0.50 samples presented the lowest modulus in all media.

The fracture strength varied considerably within all samples; nonetheless all average values were higher than 1 MPa. The two-way ANOVA analysis, $\alpha < 0.05$, on the fracture strength, Annex IV, Table IV. 5, revealed that for PVA and for 0.25, the

fracture strength in pH 4.0 was higher than in other media. For 0.50: $\sigma_{F(pH4)} > \sigma_{F(PBS)}$ and $\sigma_{F(pH4)} < \sigma_{F(saline)}$. For all media, the fracture strength of the samples with 0.5% Ag was the lowest.

All the samples presented average fracture strain above 300%. The two-way ANOVA analysis, $\alpha < 0.05$, Annex IV, Table IV.6, revealed that only the amount of silver was significant. No significant difference was observed between the levels.

For all the significant results, a trend was observed: the lower the swelling degree, the higher were both the Secant modulus and the fracture strength. Since the media ionic strength interferes with the gels' swelling degree, it could also interfere with the gels' mechanical properties. It could be noticed that the increase of silver amount led to high swelling, low Modulus and low fracture strength compared to the other samples. According to Alcântara and collaborators[152], the higher the crosslinking degree of irradiated PVA membranes, the higher would be the Secant modulus. Probably, the presence of silver diminished the crosslinkage degree, since the ionic silver can be attached to the same sites in PVA that would originate the crosslinkages when irradiated.

Varshney[153] studies on PVA irradiated hydrogels showed that, after 25% of water uptake, the gels presented tensile strength of 4.5 kPa and their mechanical properties were considered insufficient for dressings. Singh and Pal[59] analyzed the tensile properties of sterculia-cl-poly(VA) and of sterculia-cl-poly(VA-co-AAm) films. Their tensile strength and their elongation at break were (0.13 ± 0.002) MPa, 44.30% and (0.35 ± 0.001) MPa, 111.92%, respectively. Singh and Pal[59], 2012, p.19 concluded that "each polymeric film has sufficient mechanical strength which is required in biomedical applications. So, all these polymeric films can be used for covering of wound".

The hydrogels mechanical strength of the present work were higher than the values suggested by Singh and Pal and by Varshney's group, indicating the mechanical properties of the gels of the present work are suitable for the application.

5.1.7. Antimicrobial tests

The antimicrobial tests, Figure 5.1.11, revealed that the samples with silver presented antimicrobial activity against all organisms studied. The one-way ANOVA analysis ($\alpha < 0.05$), Annex IV, Table IV.7, revealed that the amount of silver "%Ag" was significant to the antimicrobial activity results for *E. coli, S. aureus* and *C. albicans*. The antimicrobial properties of silver have long been recognised. Silver nanoparticles are thought to disinfect via a number of mechanisms including causing damage to the cell membrane and the generation of reactive oxygen species (ROS)[154].

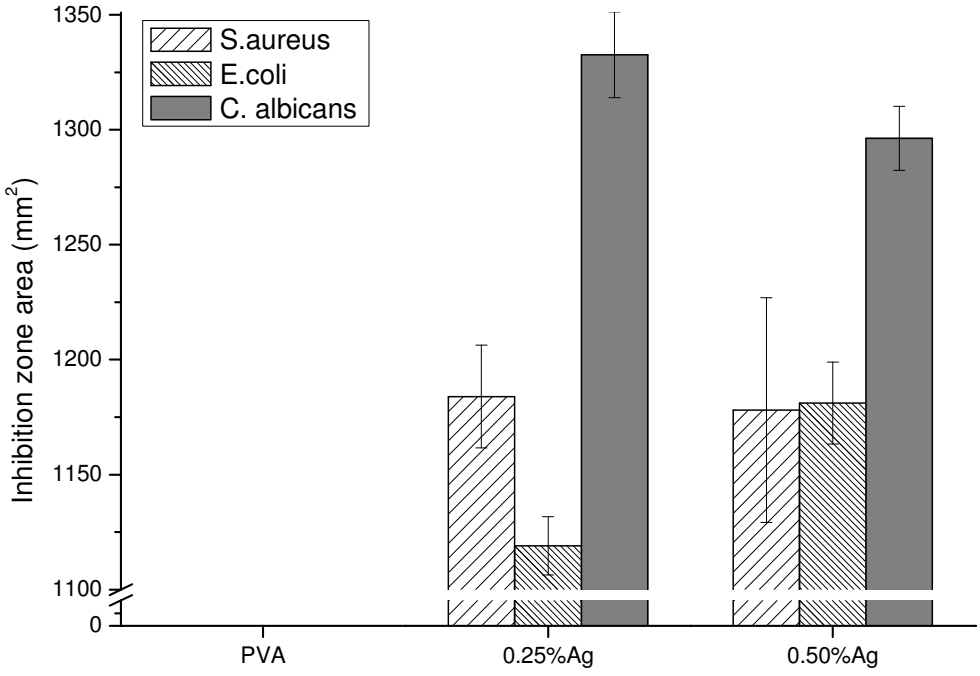


Figure 5.1.11- Antimicrobial results of the samples

The amount of silver had a significant effect on the E. coli inhibition. There was a significant difference between level (PVA, 0.25 and 0.50) for *E. coli* inhibition, where the increase in the silver amount resulted in higher inhibition zone: 0 mm^2 for PVA, ~1120 mm^2 for 0.25, ~1180 mm^2 for 0.50.

For *S. aureus* there is a significant difference between the PVA samples (no inhibition) and the PVA-Ag samples, where 0.25 and 0.50 samples presented ~1180 mm^2 of inhibition zone. The *S. aureus* antimicrobial activity, compared to the *E. coli*

inhibition zones, is high even for the 0.25 samples. According to Sedlarik and collaborators[155], who studied similar materials in similar conditions of the present work, the inhibition zones were similar to the ones of the present work (~1000 mm^2). They also realized that gram positive bacteria (e.g. *S. aureus*) were more resistant to the antimicrobial agent than the gram negative bacteria (e.g. *E coli*), the last ones considered as more complex organisms[155,156]. Based in these conclusions it could be assumed that the amount of silver and its form were appropriate to the bactericide effect against both gram-positive and gram-negative bacteria.

Ag was also effective against *C. albicans* (fungi)[157]. The 0.25 and 0.50 samples' inhibition zones of *C. albicans* were higher than those of the bacteria. For the fungi species, there was a significant difference between PVA samples (no inhibition) and PVA-Ag samples (~1300 mm^2).

The antimicrobial penetration test could determine if the samples are able to protect the wound from a secondary bacterial infection[59], Figure 5.1.12. The test revealed that all samples were barriers to microbial penetration. After one month of test tube coverage, the solution in the tubes covered by all the samples and the solution of the negative control (sealed tube) presented no turbidity, while in the positive control (open tube), high turbidity was observed. No difference was encountered between samples with and without silver. The microbial penetration prevention is probably related to the polymer network that blocks/entraps the microbials[29].

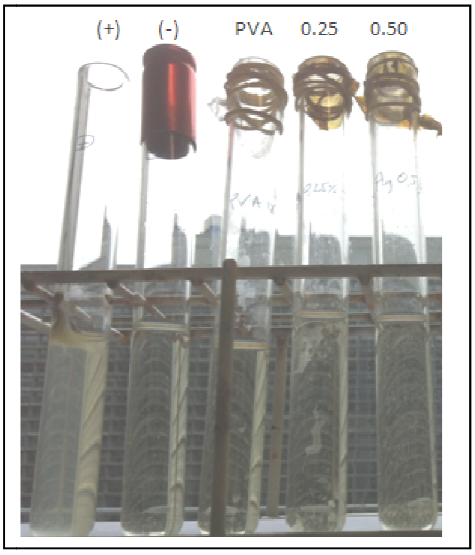


Figure 5.1.12 - Microbial penetration test for PVA, 0.25 and 0.50 samples.

5.1.8. Cytotoxicity tests

The HACAT cells viability in contact with the samples extracts was ~70% for PVA samples, ~84% for 0.25 samples and ~85% for 0.50 samples, Figure 5.1.13. The cells viability was higher than 75% for PVA-Ag samples, indicating that these samples were not cytotoxic for human keratinocytes. Silver nitrate and nanoparticles themselves were expected to be cytotoxic to keratinocytes and fibroblasts when these cells were exposed to silver concentration lethal to bacteria[158,159]. However, studies on silver nanoparticles imbibed in polymeric matrices revealed that these systems can be antimicrobial without being cytotoxic to fibroblasts[160-162].

The ANOVA analysis, Annex IV, Table IV.8, revealed that the amount of Ag was significant to the cells viability and that the cells viability when in contact with the samples was considerably different compared to the positive control. The cells viability in contact with the samples extracts was higher than 70%. PVA-Ag samples were considered to be non cytotoxic to HACAT cells.

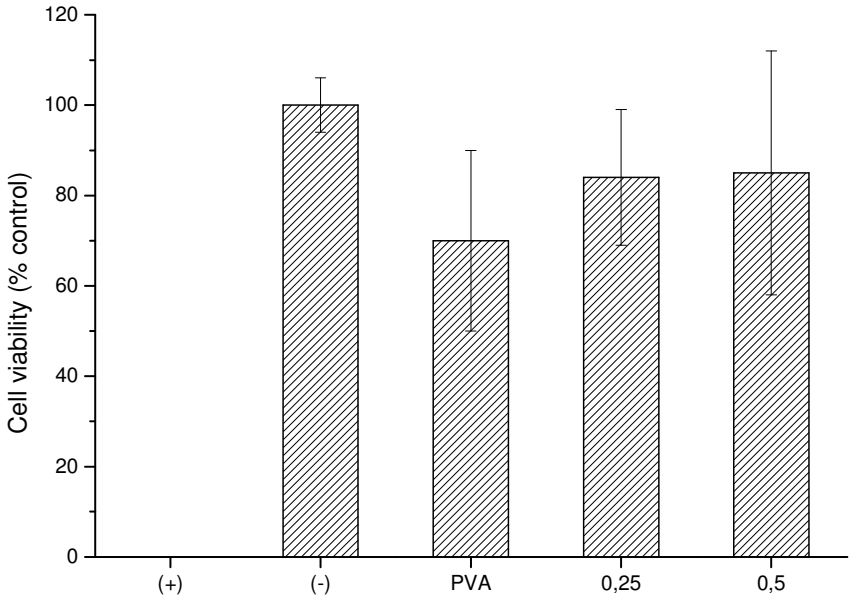


Figure 5.1.13 - Cytotoxicity analysis of the PVA, 0.25 and 0.50 samples on the HACAT cells.

5.2. PVA-Propolis - Results and Discussion

5.2.1. Microstructural analysis

The microstructural analysis involved XRD and FTIR. The XRD profiles of the PVA-propolis samples can be observed in Figure 5.2.1. Propolis interferes with the crystalline phase of PVA, although no linear trend was observed.

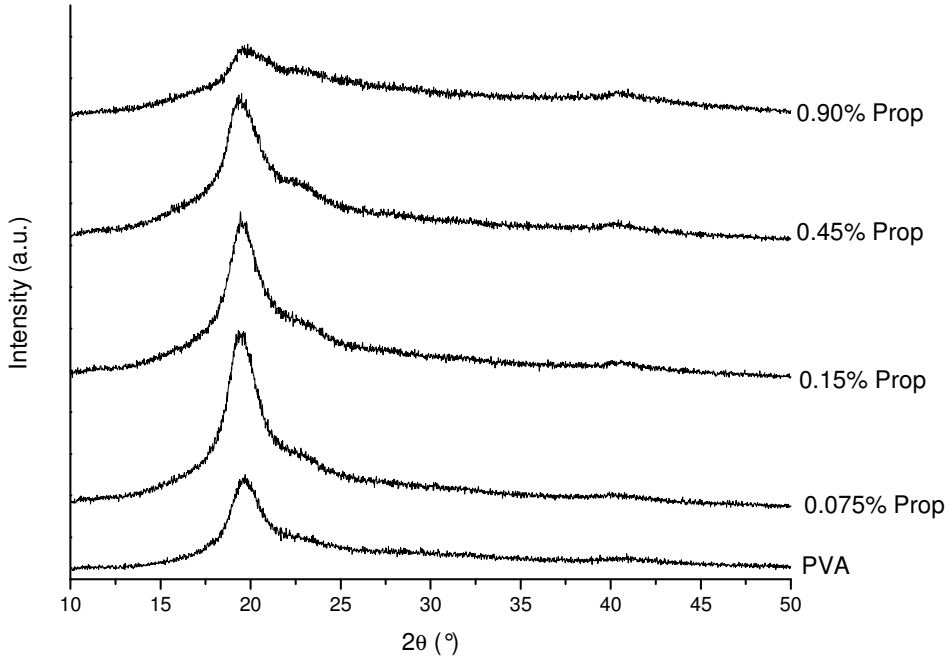


Figure 5.2.1 - XRD of the PVA-propolis samples

The FTIR bands of the samples and the PVA and propolis respective bands are displayed in Figure 5.2.2.

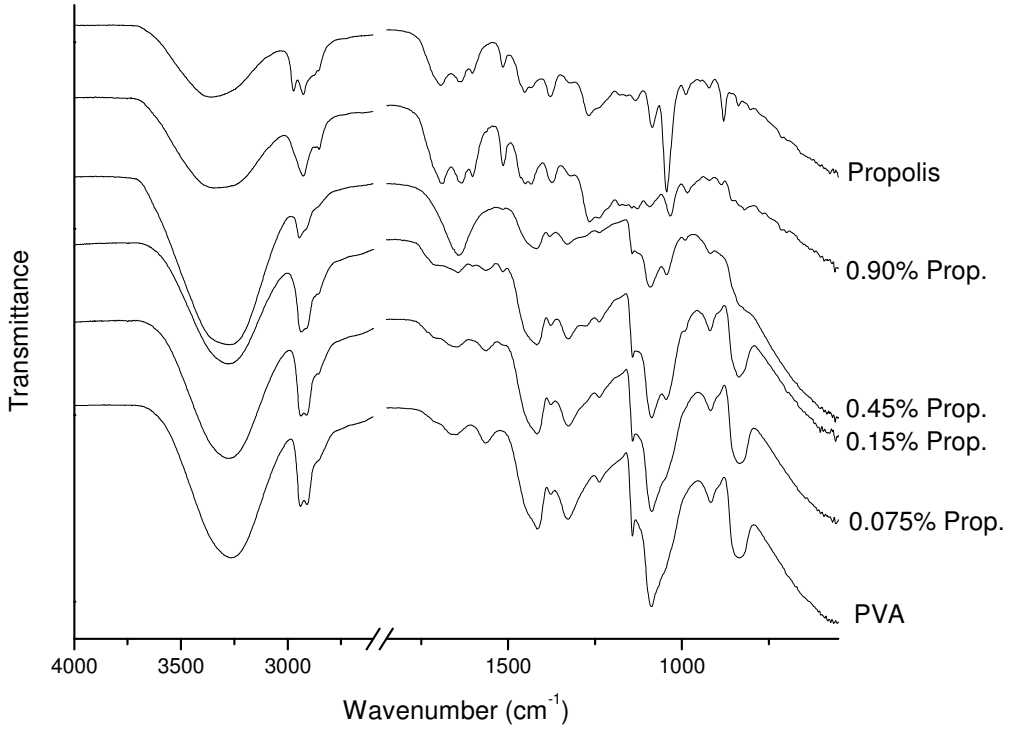


Figure 5.2.2 - FTIR spectra of PVA, 0.075%, 0.25%, 0.45%, 0.90% propolis samples and of propolis.

The FTIR bands of PVA and of propolis, and their experimental bands, as well as the theoretical groups vibration modes related to these bands, are displayed in Table 5.2.1. Depending on the source of the propolis, the type of propolis changes (green, red, brown propolis), as well as its compounds and the amount of the compounds. There are at least 200 different compounds identified in various samples of propolis and the current propolis present at least 180 compounds non-specified[90]. The non-identified (N.I.) bands might be related to some compounds unusual to the previously studied propolis samples.

Table 5.2.1 - PVA, samples 0.075%, 0.15%, 0.45%, 0.90% propolis and propolis bands, as well as PVA and propolis characteristics groups vibration modes.

PVA	*PVA groups' vibration modes*[41,113,114,126-135]	*0.075%*	*0.15%*	*0.45%*	*0.90%*	*Propolis*	*Propolis groups' vibration modes*[102,103,163,164]
(cm^{-1})		*(cm^{-1})*					
3259	ν(O-H)	3280	3278	3284	3326	3353	Wagging (OH) of phenolic compounds; stretching of (OH) groups
-	-	-	-	-	-	2973	Aliphatic ν(CH_2), C-H bands of aromatic compounds
2942	ν(C-H) – alkyl groups	2939	2939	2943	2931	2928	C-H bands of aromatic compounds
2909		2911	2909(o)	2912(o)	-	2873(o)	
2850(o)		2853(o)	2854(o)	2854(o)	2854	2856(o)	
-	-	-	1715(o)	2112	2637	-	-
		-	-	-	1689	1694	Stretching of carboxyl groups
1655	ν(C=O) of unhydrolyzed acetate groups, ν(C=C)	1652	1645	1642	1634	1640	ν(C=O) of CAPE and its derivatives; ν(C=C), Aromatic ring bands
-	-	-	1602	-	1602	1602	Aromatic ring bands
1564	ν(C=C)	1563	1565	-	-	-	
-	-	-	1516	1516	1514	1514	
		-	-	-	1465	-	
		-	-	-	1451	1452	
1415	δ, wagging, in plane(C-H in CH_2	1417	1417	1419	1434	1435	N.I.

	groups); ν(C-O-C) of unhydrolyzed acetate groups, in plane(O-H)						
1378	coupling of in plane(O-H) wagging(C-H)	1378	1378	1380	1374	1378	
1329	bending(CH + OH), fan and twist(-CH_2-)	1326	1327	1330	1318	1320	
-	-	-	1276	1280	1265	1270	C-O groups of polyols, e.g. hydroxyflavonoids
1236	ν(C-C), fan and twist(-CH_2-)	1236	1238	1236	1239	-	-
-	-	-	-	-	1179	1177	N.I.
1142	ν(C-O-C), ν_S(C-C) crystalline sensitive band	1142	1142	1143	1144	1159	
-	-	-	-	-	1128	1131	
1088	ν(C-O), δ(O-H)	1089	1087	1091	1092	1087	ν(C-O) of ester groups
-	-	-	1045	1044	1033	1043	
		-	-	991	984	989	N.I.
917	ν,[δ out of plane](C-H)	917	919	918	921	919	
-	-	-	-	-	887	880	Aromatic ring vibration
836	ν, pendular(C-C)	835	836	-	817	837	

All PVA bands can be found in all samples, although some of them present lower intensity with the increase of propolis amount. No band related to propolis only was encountered in the sample with 0.075% Propolis, although some PVA bands in these samples could overlap some of the propolis bands.

Samples with 0.15% of Propolis or more present both PVA and propolis bands and, in some cases, some shift of the PVA bands that overlap propolis bands toward the propolis bands position. These are indicative of chemical interaction between propolis compounds and the PVA chains[103,164]. More propolis bands were observed in samples with more propolis, indicating that the increase of propolis meant more and different propolis groups attaching to PVA.

In all samples there are the PVA bands and samples with amount of propolis ≥ 0.15% present also the propolis bands. Since both components are present, their bands should be expected.

The spectra of the dried samples after swelling were displayed in Annex II. The main bands of the PVA are present in all PVA samples not only before, but also after swelling. The band at 1564cm^{-1}, related to non-hydrolyzed acetate groups, is absent after swelling, indicating probably some leaching of these groups by the media. In addition, after swelling in Solution pH 4.0, a band at 1713cm^{-1} emerges. This band is related to stretching of C=O of lactic acid, indicating that it interacts with the PVA chains[113,134,136-138].

The main bands of the PVA are present in all 0.075% propolis samples even after swelling. The band at 1564 cm^{-1}, related to non-hydrolyzed acetate groups, was absent after swelling, indicating probably some leaching of these groups by the media. A shoulder at 1713 cm^{-1} emerges after swelling in both media.

Samples with 0.15% of propolis or more are the ones in which the bands of PVA and the bands of propolis can be distinguished. In the 0.15% propolis samples, the band at 1564 cm^{-1}, related to non-hydrolyzed acetate groups, is, again, absent after swelling indicating, probably, some leaching of these groups by the media. In pH 4.0, the PVA band at 2909 cm^{-1} that overlaps the propolis band at 2928 cm^{-1}, is shifted toward the propolis band after immersion. Some bands related to propolis only are also present after immersion in pH 4.0, between 1602 and 1456 cm^{-1}, related to aromatic ring vibration, and the band between 1276 and 1270 cm^{-1}, related to the vibration of C-O

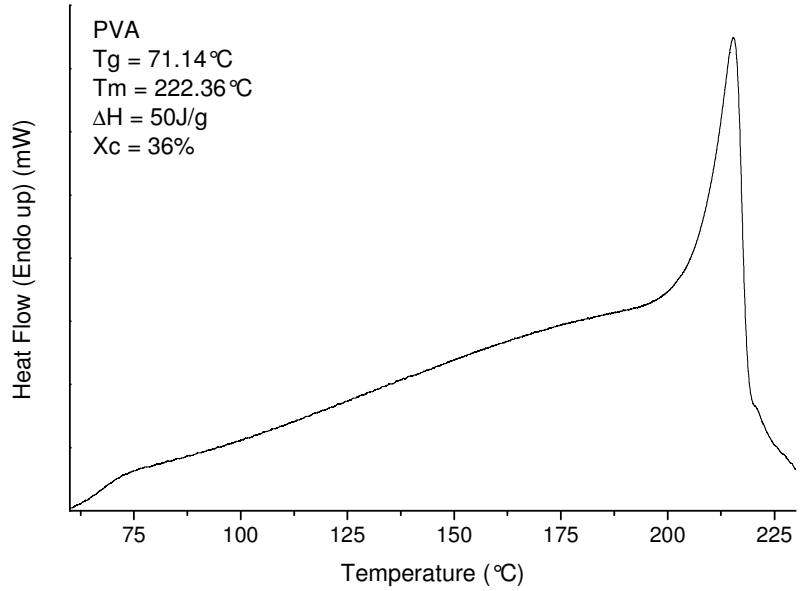
groups of polyols, e.g. hydroxyflavonoids. For samples swollen in PBS, there was the band between 1276 and 1270 cm^{-1}, but the other bands, related to aromatic ring vibration, were absent. This fact could be related to the delivery of these compounds to the media, since propolis delivery is high in PBS.

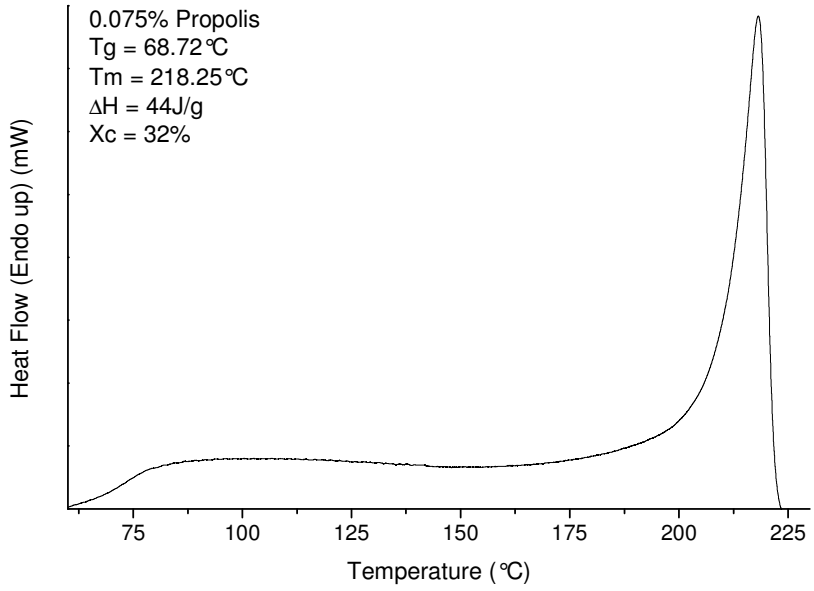
Besides the PVA bands in 0.45% propolis samples after swelling, it can be noticed that there were some bands related to the propolis itself, the bands between 1602 and 1425 cm^{-1}, related to aromatic ring vibration, the vibration of C-O groups of polyols, e.g. hydroxyflavonoids, between 1330 and 1272 cm^{-1}, a non-identified band at ~990 cm^{-1}, bands between 1030 and 1042 cm^{-1}, due to ν(C-O) of ester groups and between 890 and 833 cm^{-1}, related to aromatic ring vibration. Some of these bands can be noticed only after swelling, probably due to PVA degradation that enabled these groups vibrations after swelling. It is worth noticing that the original (pre-swelling) spectra of the 0.45% propolis samples present some peculiar features. It presented the PVA bands present with lower intensity and the propolis bands seemed to be not well-defined or they seemed to be dislocated. This fact could indicate some chemical interactions between the propolis and the PVA can be observed. However, since the propolis bands vibration modes are enabled after swelling, it can be observed that there was not chemical interactions between the PVA and propolis. There was a physical impairment to the propolis vibration modes prior to the swelling.

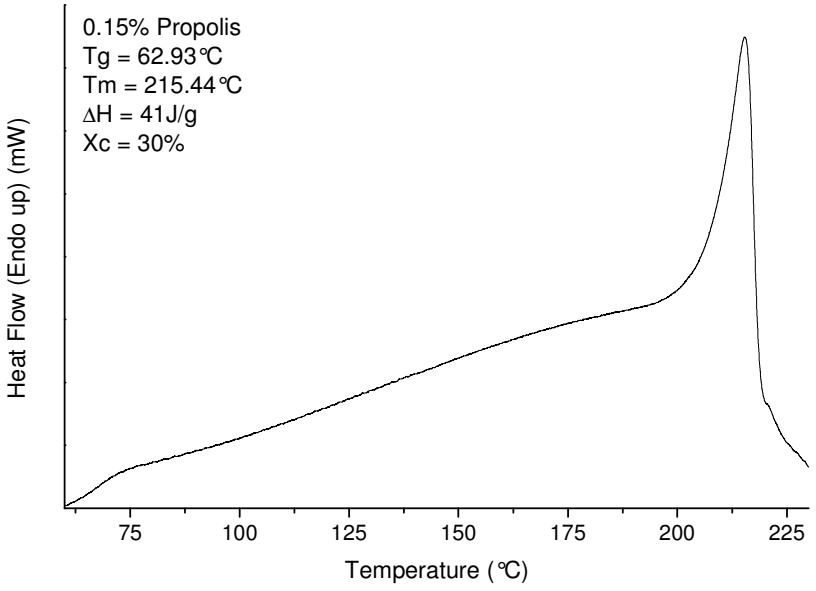
In the 0.90% propolis samples after swelling mainly all the bands of PVA and mainly all the bands of propolis can be distinguished. Some of the original PVA bands that could be overlapped to the propolis ones are shifted toward the propolis bands.

5.2.2. Thermal analysis

Based on the scanning differential calorimetry, Figure 5.2.3, the gels glass transition temperature (Tg) and melting temperature (Tm) were obtained and a trend was observed: the Tg increases with the increase of propolis amount and the Tm decreases with the increase of propolis.


PVA
Tg = 71.14℃
Tm = 222.36℃
ΔH = 50J/g
Xc = 36%
Heat Flow (Endo up) (mW)
75
100
125
150
175
200
225
Temperature (℃)


0.075% Propolis
Tg = 68.72℃
Tm = 218.25℃
ΔH = 44J/g
Xc = 32%
Heat Flow (Endo up) (mW)
75
100
125
150
175
200
225
Temperature (℃)


0.15% Propolis
Tg = 62.93℃
Tm = 215.44℃
ΔH = 41J/g
Xc = 30%
Heat Flow (Endo up) (mW)
75
100
125
150
175
200
225
Temperature (℃)

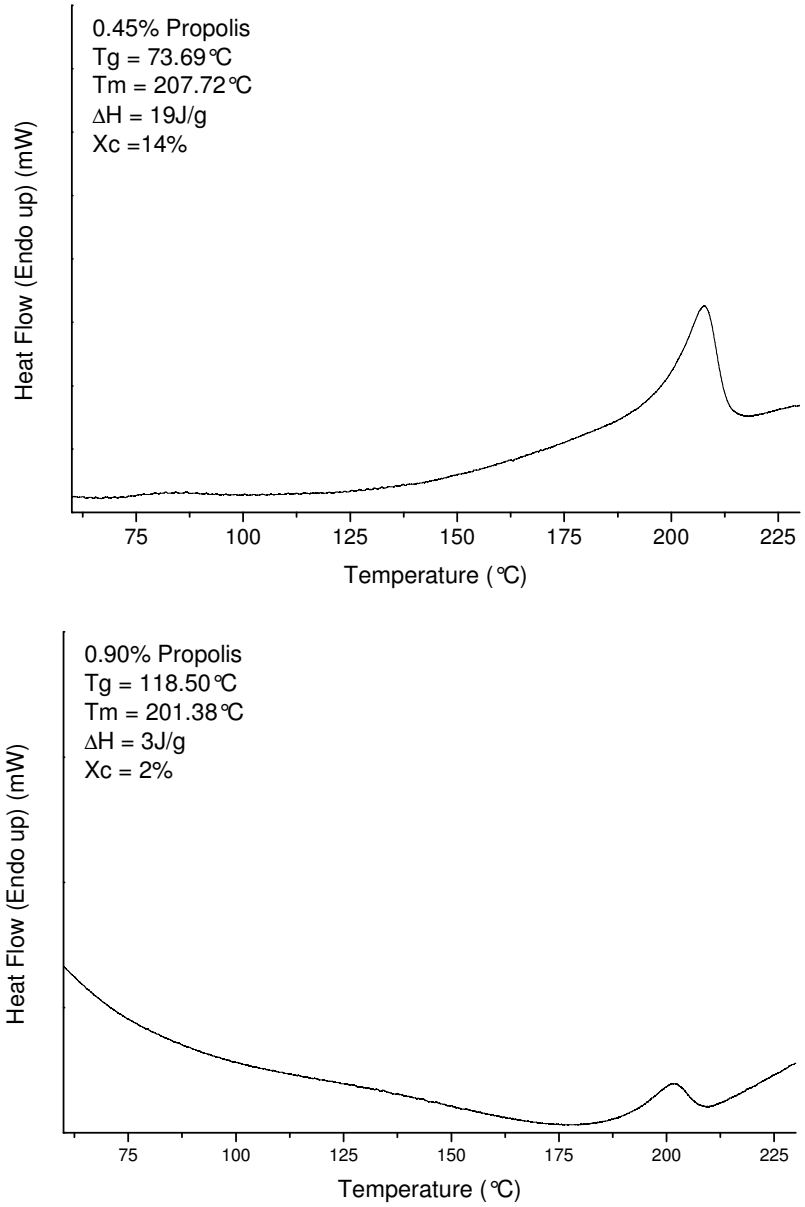


Figure 5.2.3 - DSC results for PVA-propolis samples, PVA, 0.075% propolis, 0.15% propolis, 0.45% propolis and 0.90% propolis, where the Tg is the glass transition temperature, the Tm is the melting temperature, ΔH is the enthalpy and the Xc is the degree of crystallinity.

The Tg represents the temperature above which the chains of the amorphous phase gain mobility[165]. There is a decrease in the Tg with the increase of propolis until amount of propolis ≤ 0.15%. For higher amounts of propolis, there is a Tg rise indicating that the presence of propolis (≥ 0.45%) diminished the chains mobility of the PVA amorphous phase. High amounts of propolis (≥ 0.45%) could mean enough

propolis to limit the PVA chains mobility / to anchor the PVA chains of the PVA amorphous phase.

Regarding the PVA crystalline phase, the melting temperature (Tm) and the degree of crystallinity (Xc) decreased with the increase of the propolis amount in the samples. The propolis could be considered a physical impairment to the chains' movement, as a consequence of increased amounts of propolis in the samples, making chains packing more difficult, diminishing Tm and Xc and increasing the amount of PVA amorphous phase.

Drugs can interact with polymers and alter their behaviour. Mc Gann and collaborators[46] studied PVA/PAA hydrogels loaded with aspirin (hydrogels freeze-thawed) and they realized that in PVA/PAA gels only, there is an increase in the Tg compared to PVA, due to interactions between PVA and PAA. The presence of aspirin changed (lowered) the Tg of the PVA/PAA gels due to chemical interactions between aspirin the PAA, diminishing the interactions between PVA and PAA. The aspirin also decreased the Tm of PVA/PAA gels, probably related to changes in the morphology of the polymer matrix.

Suri and collaborators[166] studied PVA hydrogels freeze-thawed loaded with liposomes and they also observed some changes in the PVA crystalline phase, the presence of the liposomes alter the PVA gels crystallinity. The PVA's Tm is absent when the liposomes are incorporated. It can be noticed that PVA-drug delivery systems can have different characteristics from PVA systems only due to interactions between PVA and the drug.

5.2.3. Swelling, degradation and drug delivery tests

a. Swelling tests

The swelling tests were done in PBS and in Solution pH 4.0 for 4 days, Figure 5.2.4. It can be observed that all samples swelled at least ~300% and the range of swelling for all (in all media) would be between 300-600%. PVA hydrogels immersed

in saline solution usually present hundreds percent of equilibrium of the swelling degree[50].

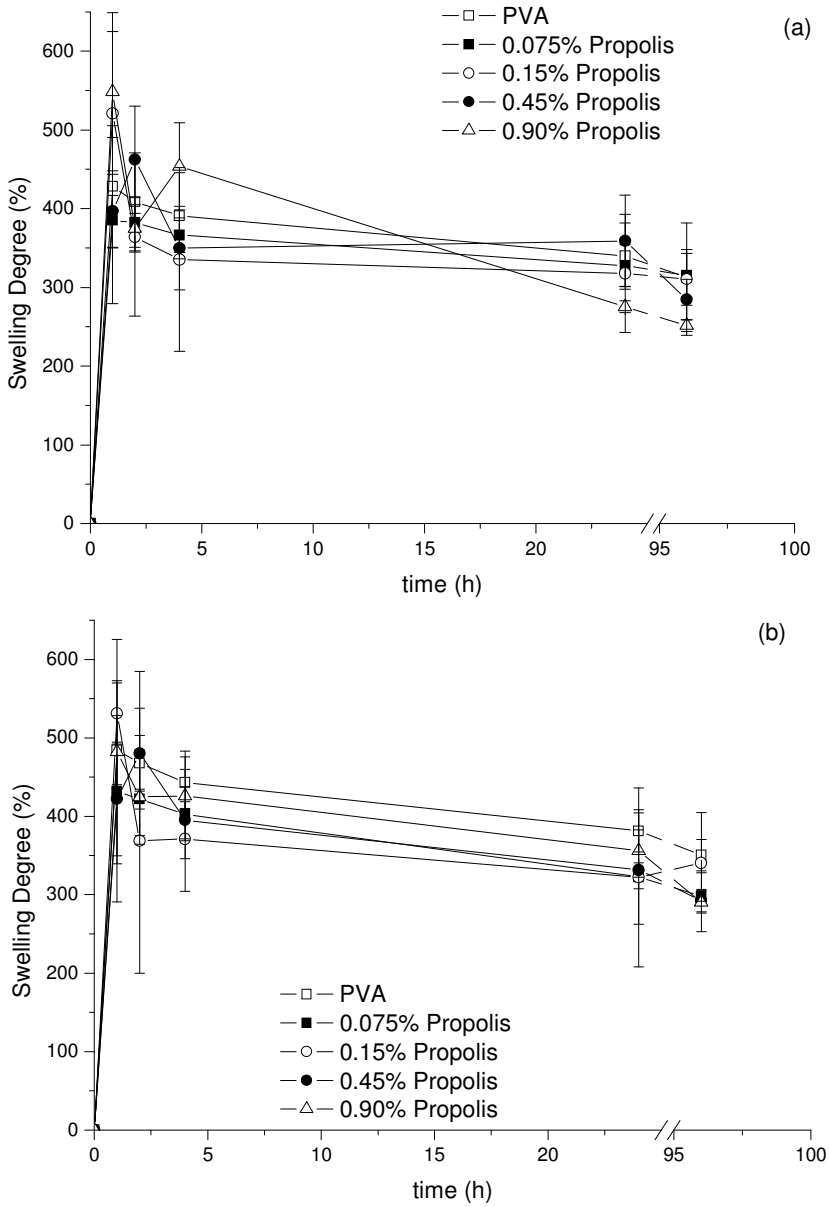


Figure 5.2.4- Swelling profile of the PVA-propolis samples in (a) PBS and in (b) Solution pH 4.0

A peak of media uptake can be observed at the beginning of the immersion in all curves. The equilibrium of the swelling degree (ESD) occurs when the hydration forces (the network stretching by the initial fluid uptake) and the elastic force of the crosslinkages reach the equilibrium[167]. The Equilibrium of the swelling degree (ESD)

was achieved when the swelling became constant, after one day of swelling, Table 5.2.2.

The ANOVA analysis, $\alpha = 0.05$, on the ESD showed that no factor was significant to the equilibrium of the swelling degree, Annex IV, Table IV.9. It means that the fluids uptake by all samples in the different media was approximately the same.

Table 5.2.2 - Equilibrium of the Swelling Degree (ESD) of the samples in PBS and in Solution pH 4.0 after 1 day of immersion.

Amount of Propolis	ESD (%)	
	PBS	Solution pH 4.0
0% (PVA)	339 ± 41	381 ± 26
0.075%	327 ± 26	322 ± 60
0.15%	317 ± 75	322 ± 114
0.45%	359 ± 58	331 ± 9
0.90%	275 ± 7	355 ± 48

In addition, after the 4 days of immersion, some propolis delivery could have happened as well as some polymer degradation, resulting in weight loss and drug delivery.

b. Drug delivery tests

The propolis delivery profiles can be observed in Figure 5.2.5. It can be noticed an increase of the delivery in the first hours, followed by a plateau. A study of propolis delivery by polymeric systems loaded with propolis recognizes the delivery of certain amounts of propolis in the first day of swelling, as well as a prolonged delivery in some cases[168]. A trend can be observed in all curves after 4 days of immersion: there is a high propolis delivery in the first hours and the delivery reaches constant values after 1 day of immersion, indicating that the total delivery ends in the first day. A better description of the propolis delivery can be found in the Annex III.

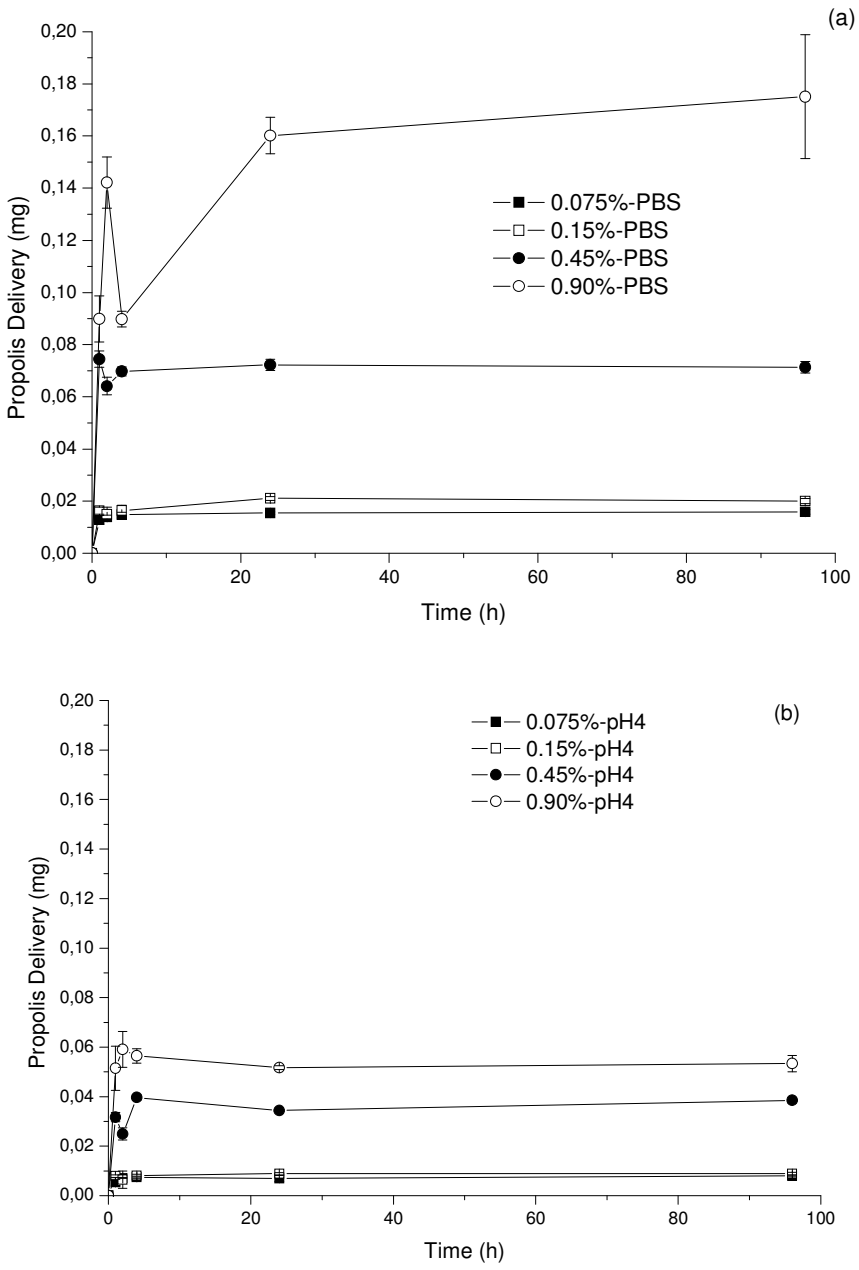


Figure 5.2.5 - Propolis delivery profile of the PVA-propolis samples in (a) PBS and in (b) Solution pH 4.0

The ANOVA analysis on the total propolis delivered by the samples in 4 days of immersion, Annex III, revealed that the type of media and the amount of propolis in the original samples were significant to the total amount of propolis delivered. In addition, there were differences between the levels except between 0.075% and 0.15% propolis.

The results mean that the higher the propolis amount in the samples, the higher was the delivery, but for the pair of levels 0.075% – 0.15% propolis, in which the

delivery could be considered equal for them both. The samples delivered more propolis to PBS than to Solution pH 4.0.

c. Weight loss

The samples weight loss was higher for samples with more propolis, Figure 5.2.6. Since the propolis delivery was also higher for samples with more propolis, the higher weight loss could be related to the propolis delivery as well as to the polymer degradation.

Kamoun and collaborators[169] studied PVA-alginate hydrogels for wound dressings and they analyzed the gels weight loss after immersion in PBS. The higher the amount of sodium alginate (SA), the higher was the gels weight loss. The alginate was blended to PVA, so it was not crosslinked, presenting high ability to be soluble in the media. According to Kamoun and collaborators[169], 2013, p. 7 "the degradation of PVA-SA hydrogel membranes are predominantly the cleavage of entanglement segments of PVA and is consistent with the fact that the degradation of PVA is quite limited, whereas the degradation of PVA-SA is quite high".

High amounts of propolis led to low degree of crystallinity and low percentage of crystalline phase. Since the PVA crystallites act as physical crosslinkages, the crosslinkage degree of the gels would be lower with high amounts of propolis. When the samples swell, the amorphous chains have more freedom to move and, if they are not crosslinked (or in a crystallite), with the help of the fluidic media, they are able to detach from the network to the media (cleavage of entanglement segments of PVA), increasing the weight loss.

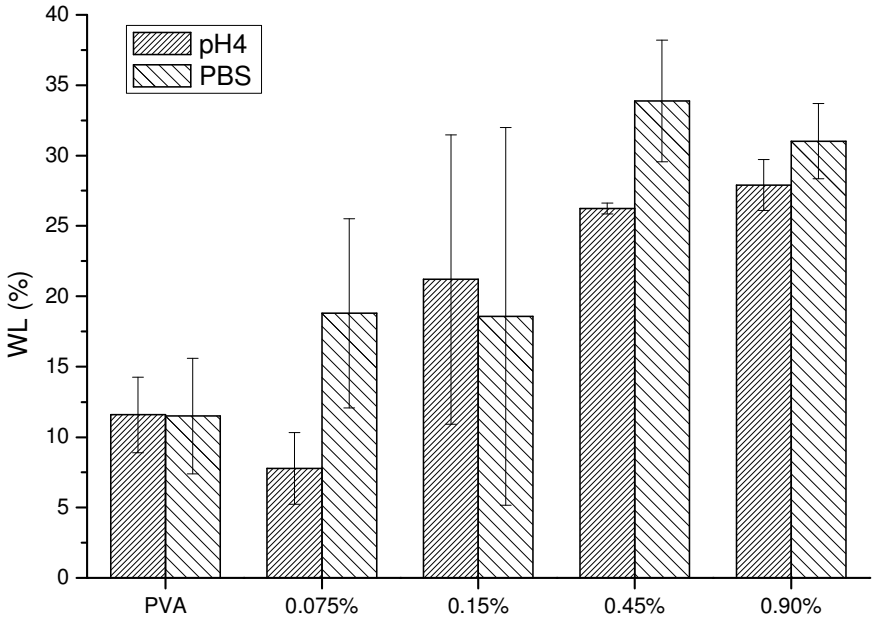


Figure 5.2.6 - Weight loss (WL) of the PVA-propolis samples after 4 days of immersion in PBS and in Solution pH 4.0

The two-way ANOVA analysis, $\alpha < 0.05$, on the weight revealed that there is a significant difference between the 0.15% propolis samples weight loss and the others: $WL_{0.15\%} > WL_{PVA,\ 0.075\%}$ and $WL_{0.15\%} < WL_{0.45\%,\ 0.90\%}$. The samples weight loss could be related to the propolis deliveryas well as to the PVA degradation, since more propolis in the samples meant more propolis delivered as well as more PVA chains in the amorphous phase.

5.2.4. Tensile tests

The tensile curves of 10 samples of each condition gave rise to plot the average curves. The average curves of the samples swelled in PBS and swelled in Solution pH 4.0 can be observed in Figure 5.2.7.

Based in the normalized curves, the secant modulus (E) was calculated (at strain of 50%) and the fracture strength (σ_F) was also measured, Table 5.2.3.

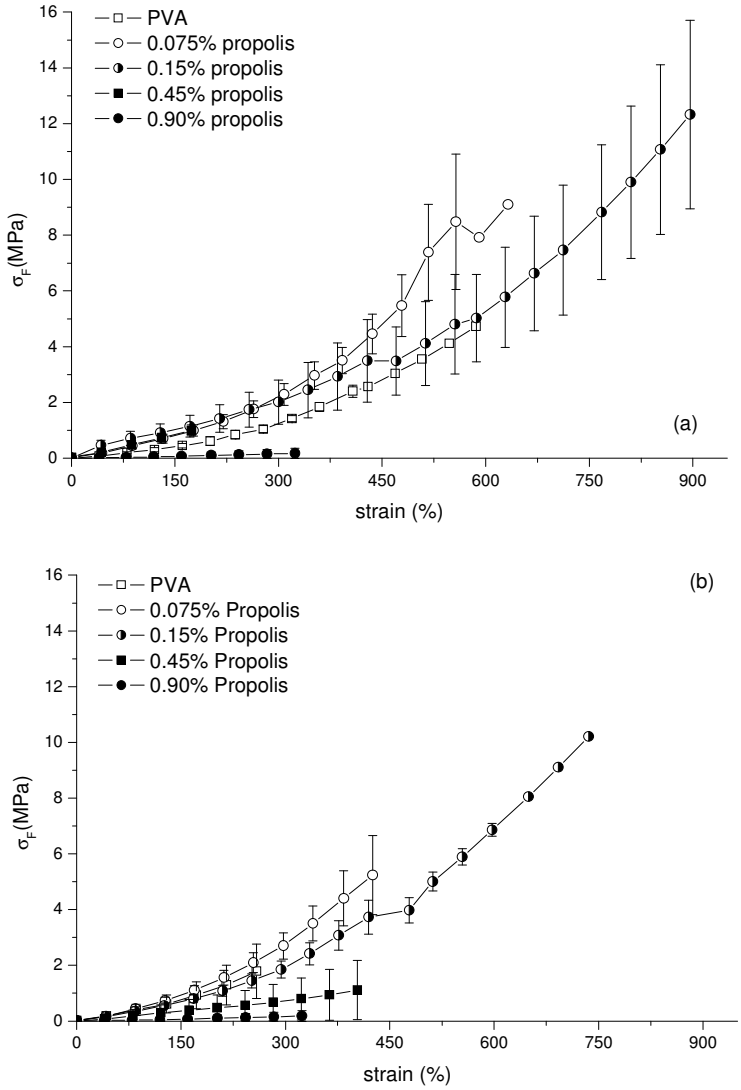


Figure 5.2.7 - Average tensile curves of all samples immersed for 1 day in (a) PBS and in (b) Solution pH 4.0.

It can be observed that, in PBS, the E and the σ_F values increase with the content of propolis until 0.45% of propolis, after which there is a considerable decrease. In Solution pH 4.0, the E value decreases with the content of propolis from 0.15% propolis on and the σ_F decreases with the increase of propolis amount.

Statistical analysis, two-way ANOVA, $\alpha < 0.05$, Annex IV, Table IV.11, on the E values showed that, in PBS: $E_{0.15\%propolis} > E_{0.075\%propolis}$, $E_{PVA} > E_{0.90\%propolis}$ and $E_{0.45\%propolis} > E_{0.15\%propolis}$, $E_{0.075\%propolis}$. The highest modulus was encountered for the samples 0.45% propolis and the lowest modulus was the ones of the 0.90% propolis. In

pH 4.0: $E_{0.15\%propolis} < E_{0.075\%propolis}$, $E_{0.90\%propolis} < E_{PVA}$ and $E_{0.45\%propolis} < E_{0.15\%propolis}$, $E_{0.075\%propolis}$.

Table 5.2.3 - E and σ_F values of the samples

Media	*PBS*		*Solution pH 4.0*	
Samples	E (MPa)	σ_F (MPa)	E (MPa)	σ_F (MPa)
PVA	0.19 ± 0.06	1.85 ± 1.45	0.41 ± 0.21	7.21 ± 5.57
0.075% Propolis	0.43 ± 0.08	6.69 ± 3.42	0.43 ± 0.11	5.77 ± 1.44
0.15% Propolis	0.50 ± 0.11	5.75 ± 3.04	0.37 ± 0.10	4.71 ± 2.79
0.45% Propolis	1.17 ± 0.49	8.42 ± 5.23	0.24 ± 0.26	2.10 ± 2.32
0.90% Propolis	0.03 ± 0.03	0.41 ± 0.65	0.03 ± 0.03	0.37 ± 0.63

According to Hago and Li[170], who studied PVA hydrogels loaded or not with gelatine to be used in wound healing, dried PVA hydrogels submitted to three cycles of freeze-thawing presented Young Modulus of ~0.45 MPa. The secant modulus values encountered in this work varied from ~0.03 to ~1.17 MPa for different samples in different media. Since the samples of the present work were swollen, the presence of fluids could act as a plasticizer diminishing the E values. The 0.15 and the 0.45% propolis samples presented modulus higher than 0.44 MPa and they could be successfully applied even in the swollen state.

The ANOVA analysis, $\alpha = 0.05$, on the fracture strength, Annex IV, Table IV. 12, the amount of propolis is significant to the fracture strength as well as the interaction between media and amount of propolis. In pH 4.0, higher the amount of propolis, lower the fracture strength. In both media, the lowest fracture strength was the one of 0.90% propolis samples.

In PBS, $\sigma_{F(0.90\%propolis)} < \sigma_{F(0.15\%propolis)}$, $\sigma_{F(0.075\%propolis)}$, $\sigma_{F(PVA)}$ and $\sigma_{F(0.15\%propolis)} < \sigma_{F(0.45\%propolis)}$ The highest σ_F value was that of 0.45% propolis samples and the lowest one is that of 0.90% propolis. In pH 4.0, higher the amount of propolis, lower the fracture strength.

The presence of an additive to PVA hydrogels or the production of PVA blends / IPNs usually alters the PVA gels mechanical properties. The blend of PVA with gelatine increased the mechanical properties values[170] weather the blend of PVA and alginate[169], of PVA and dextran[58] and of PVA and heparin[171] decreased the PVA

hydrogels mechanical properties. If the added components bind to PVA chains, the crosslinking / chains packing are hampered, and the mechanical properties deteriorate[43].

In PBS, the propolis delivery was higher than in pH 4.0. High propolis delivery would mean less propolis to interfere with the chains movement remained in the network. Under tensile strain, the chains could pack, leading to high modulus and fracture strength. In pH 4.0 less propolis was delivered, and more propolis remained in the network hampering the possible crystallization during tensile tests. The samples with 0.90% propolis presented the lowest values of the mechanical tests, almost independent of the media. Nonetheless, the amount of propolis in these samples was so high that, even delivering propolis, a considerable amount of propolis could still remain trapped in the network, being responsible for the poor mechanical properties.

Some authors studied different hydrogels for the same application. Sterculia hydrogels, presenting ~0.13 MPa of fracture strength, were considered adequate for wound coverage[59]. In addition, freeze-thawed PVA hydrogels, when dried and under tensile, presented ~0.12-0.40 MPa of fracture strength[171,172]. Based on these results, the PVA hydrogels loaded with propolis, swollen in all media studied, would have enough mechanical strength for the application.

5.2.5. Antimicrobial tests

The antimicrobial tests, Figure 5.2.8, revealed that the samples with amount of propolis equal or superior to 0.15% of propolis presented antimicrobial activity against *S. aureus*. It can be noticed that most of the samples with propolis were effective againt gram-positive bacteria (*S. Aureus*), but no activity was observed against gram-negative bacteria (*E. Coli*) or against fungi (*C. Albicans*).

Although some propolis can present activity against *C. Albicans*[102], the propolis used in the present work did not. A possible explanation would be the propolis origin, which can alter its composition and, consequently, its antimicrobial activity.

Propolis was not active against gram-negative bacteria and against fungi, however it was active against *S.aureus*, one of the most common bacteria in burn

wounds[94]. Since the samples were active against the gram-positive bacteria, the statistical analysis was performed on the *S. aureus* results.

Barud and collaborators[103] studied cellulose membranes loaded with different amounts of propolis against *S. aureus* and for the membranes with the highest concentration of propolis studied, the inhibition zone was ~300 mm^2. According to them, the membranes were effective to be used as bactericide dressings, since the gram-positive bacteria are the responsible for the initial colonization in infected burns. They also highlighted that the membranes activity was dose dependent and their application and residence time on the skin should be considered in order to define the concentration of the membranes. The inhibition zones of the membranes of the present work can be considered effective.

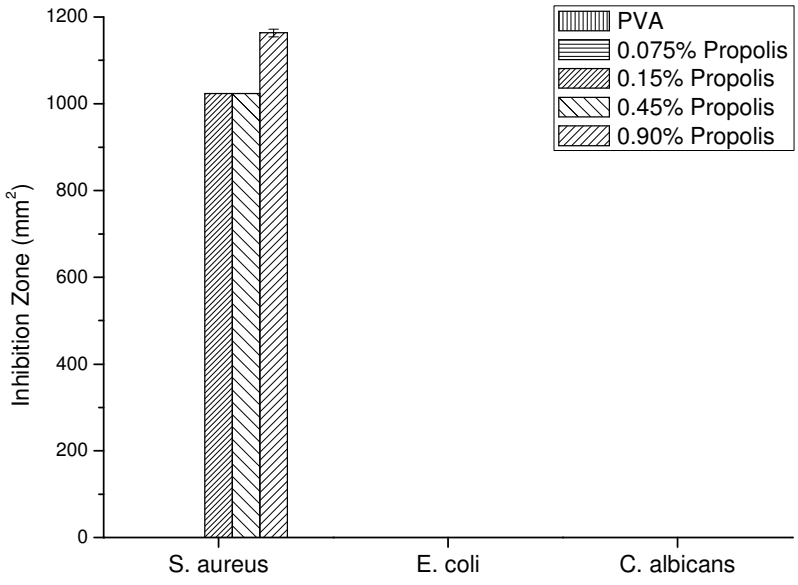


Figure 5.2.8 - Antimicrobial activity of the samples against three different organisms (*S. aureus, E. coli* and *C. albicans*)

The tests were carried out in PBS and an one-way ANOVA analysis, $\alpha < 0.05$, Annex IV, Table IV.13, revealed the amount of propolis was significant to the *S. aureus* inhibition. Both samples PVA and 0.075% propolis were not active against *S. aureus*. The other samples were active against *S. aureus*, where the 0.90 samples presented higher inhibition zone than the others. Higher amount of propolis led to higher inhibition of *S. aureus*.

The microbial penetration test revealed that all samples were barrier to microorganisms' penetration. After one month of environment exposition, the nutrient broth of the test tubes covered by the samples presented turbidity close to the negative control (sealed tube, not shown), indicating no microorganisms growth. The PVA network was responsible for acting as microorganism's barrier[29].

5.2.6. Cytotoxicity analysis

The HACAT cells viability in contact with the samples extracts was ~78% for PVA samples, ~5% for 0.075% samples and 0% for the other samples, Figure 5.2.9. The cells viability was lower than 70% for PVA-propolis samples, indicating that these samples were cytotoxic for human keratinocytes.

The one-way ANOVA analysis, $\alpha < 0.05$, Annex IV, Table IV. 14, revealed that there was a difference between the negative control and the the wells with the samples extracts, where the cells viability was higher in the negative control. In addition, there was a difference between the PVA samples and the controls, where the cells viability (CV) followed the trend: $CV_{(+)} > CV_{PVA} > CV_{(-)}$. There was a difference between the PVA samples and the PVA-propolis samples (the wells containing the samples extracts presented cells viability < 70%,), where the PVA samples were non-cytotoxic to the HACAT cells and the PVA-propolis samples were considered cytotoxic to the human keratinocytes (HACAT cells).

According to Pessolato and collaborators[173], several studies on the effect of propolis on burns healing proved that it is an effective antimicrobial agent and it also had regenerative affects (Pessolato and collaborators[173], 2011, p. 1193 found out 15% propolis oitment on 820 patients' burns had "anaesthetic, antibacterial and regenerative effects as well as limiting the wound-healed surface and proved that the roofs of propolis glued to the injury did not cause trauma to the granulation"; 3% propolis oitment on rats' burns healed faster than the ones treated with silver sulfadiazine). The rats' burns treated with a 5%-propolis-oitment led Pessolato and collaborators[173], 2011, p. 1199 to conclude that "propolis ointment accelerated the process of tissue repair and decreased local inflammation".

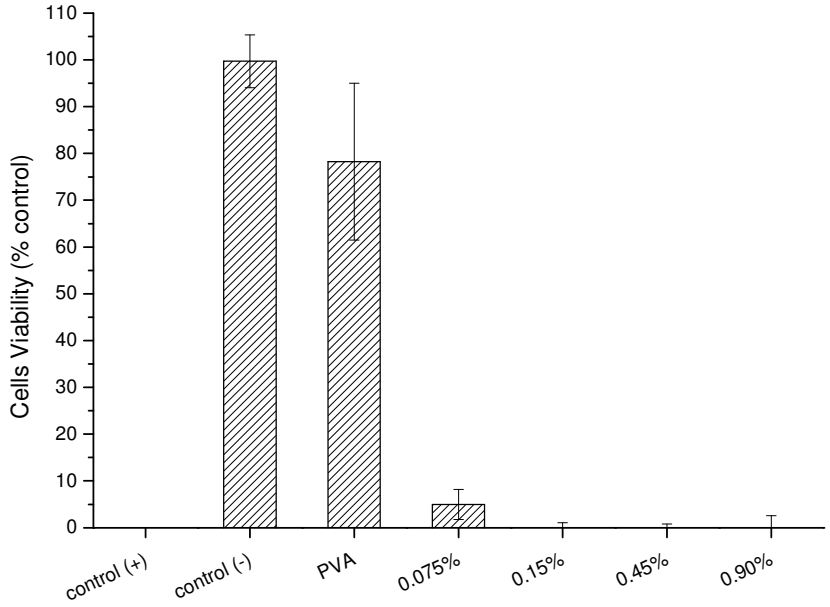


Figure 5.2.9 - Cytotoxicity analysis on the PVA-propolis samples

However, some *in-vitro* studies proved that propolis can be antimicrobial, but also toxic to fibroblasts. Funari et. al.[174] analyzed the effect of propolis from the southwest of Brazil on mouse NIH-3T3 fibroblasts, related to the cicatrization process. Their results showed that 125 and 62.5 µg/ml of propolis were cytotoxic to these cells.

Based on these studies it can be considered that even cytotoxical levels of propolis could still stimulates the reepithalization, which could improve healing. Regarding the present study, *in-vivo* tests would be necessary in order to attest the propolis effects on the reepithalization.

6. Conclusions

- ✓ The silver in PVA-Ag gels was in nanoparticle form, useful in wound healing since it can act as an antimicrobial – the gels studied were active against the microorganisms studied (bacteria and fungi);
- ✓ The presence of nano-silver altered the polymer crystallization profile which alters the gels strength, decreasing it. However the mechanical properties of the swollen gels (in saline solution, in PBS and in Solution pH 4.0) were sufficient for the application;
- ✓ No chemical interaction of the nano-Ag with the polymer was observed;
- ✓ Regarding the media effect on the gels, there was some leaching of the PVA non-hydrolyzed acetyl groups after swelling and there was some chemical interaction of the PVA with the lactic acid of the Solution pH 4.0 after swelling;
- ✓ The gels presented high fluid uptake (at least 300%) and they were not cytotoxic to human keratinocytes, indicating that the amount of silver used was in the range of the therapeutical window of silver;
- ✓ The PVA-propolis gels presented high fluid uptake (~400% in all media);
- ✓ The propolis can also be partially delivered to the media in the first day;
- ✓ No chemical interaction between the propolis and the PVA was observed;
- ✓ The propolis interferes with the polymer crystallization, diminishing it and altering the gels' mechanical properties. Nonetheless, all swollen PVA-propolis gels presented adequate mechanical properties (in all media) for the application ;
- ✓ The propolis samples (amount of propolis > 0.15%) were active against the gram-positive bacteria;
- ✓ The propolis samples were cytotoxic to the human keratinocytes.

6.1 Suggestions for future work

Regarding the characteristics studied, the PVA-silver gels seemed more adequate than the PVA-propolis gels for the burns healing application. However, some challenges remain:

- ✓ To study the toxicity of nano-Ag *in-vivo*;

- ✓ The study of nano-Ag bioaccumulation *in-vivo*;
- ✓ The use of propolis from different sources;
- ✓ The preparation of gamma radiation PVA-propolis gels;
- ✓ PVA-propolis *in-vitro* (cytotoxicity, mutagenicity, etc) analysis;
- ✓ PVA-propolis *in-vivo* studies to analyse the tissue regeneration.

7. References

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Annex I - FTIR of the PVA-Ag dried samples after 4 days of swelling

The FTIR profiles of PVA samples after immersion in saline solution, in PBS and in Solution pH 4.0 for 4 days, Figure I.2, revealed that the band at 1566 cm^{-1}, C=C stretching vibration, related to the acetate groups non-hydrolyzed, disappears after swelling, indicating some leaching of the acetate groups[113,126-128]. The PVA bands presented lower intensity after immersion in acidic media (Solution pH 4.0). Besides that, it can be observed that the shoulder at ~2850 cm^{-1}, C-H stretching vibration from alkyl groups, becomes a band after the immersion in Solution pH 4.0, probably due to a contribution of the vibration of alkyl groups from remaining lactic acid in the polymer network[129,132].

The band at ~1650 cm^{-1} splits in two bands, at 1712 cm^{-1} and at 1648 cm^{-1} with low intensity in PVA after immersion in acidic media. The band at 1650cm^{-1} would be related to the C=C stretching, the band at ~1712 cm^{-1} would be related to the C=O stretching band of lactic acid[113,134,136-138].

The FTIR profiles of 0.25 and of 0.50 samples after immersion in saline solution, in PBS and in Solution pH 4.0 for 4 days, Figure I.3 and Figure I.1 respectively, revealed that the band at 1566 cm^{-1} in the original samples disappears after swelling, indicating some leaching of acetate groups. The PVA bands presented low intensity after immersion in Solution pH 4.0. Also, in Solution pH 4.0, the band at ~1650 cm^{-1} splits in two bands, at 1648 cm^{-1} and at 1712 cm^{-1}, the last one related to lactic acid.

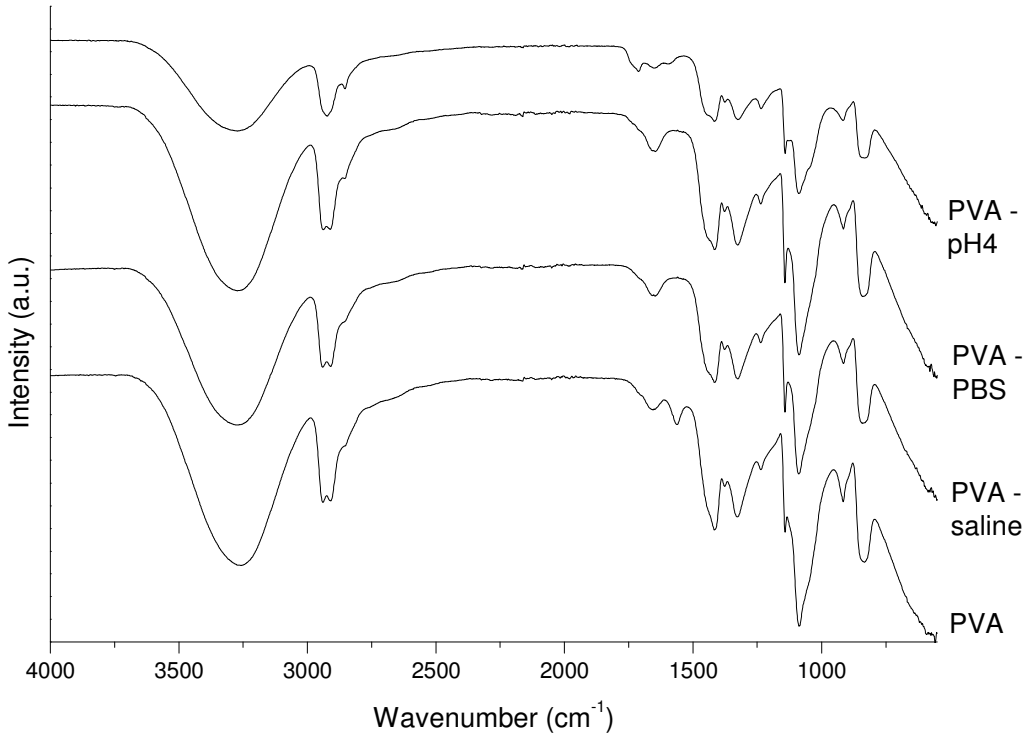

Figure I.2 - FTIR profiles of PVA samples after 4 days of immersion in the 3 different media.

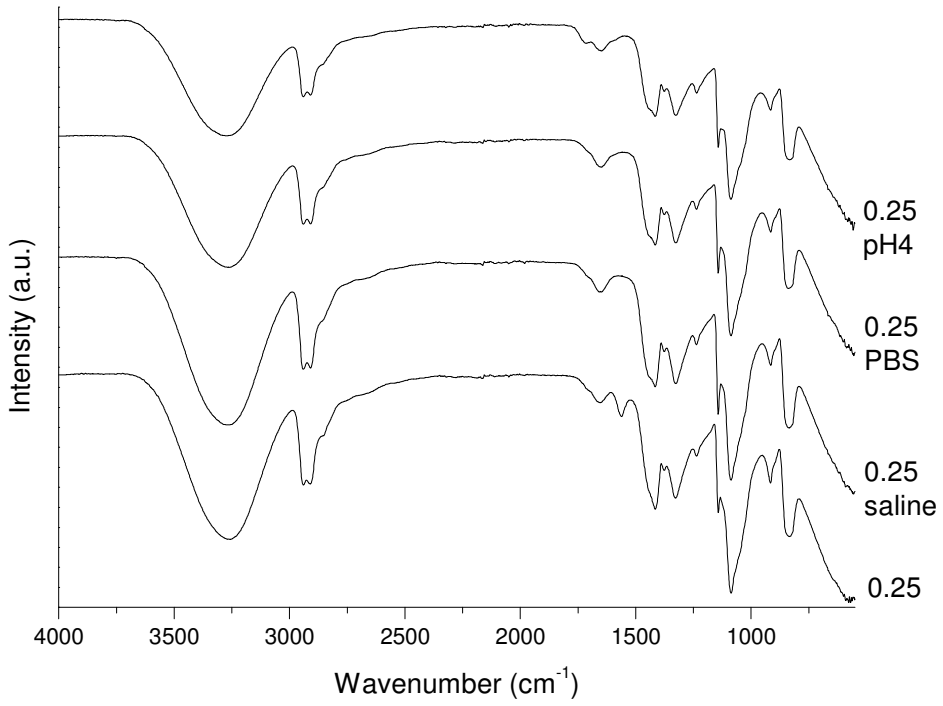

Figure I.3 - FTIR profiles of 0.25 samples after 4 days of immersion in the 3 different media.

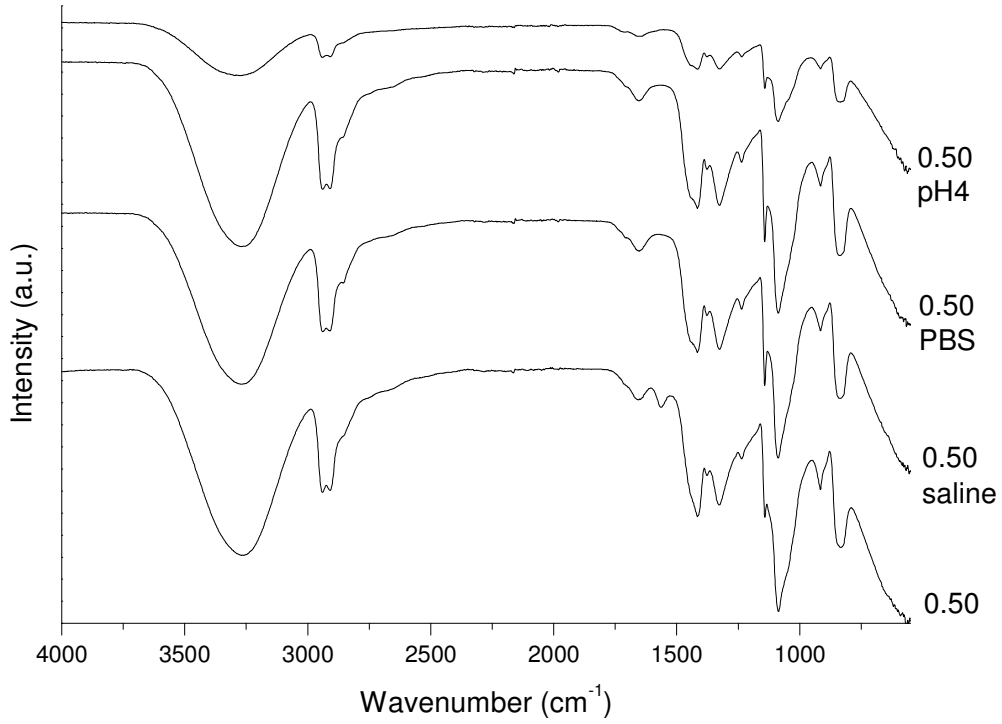

Figure I.4 - FTIR profiles of 0.50 samples after 4 days of immersion in the 3 different media.

Annex II – FTIR of the PVA-Propolis dried samples after 4 days of swelling

II.a. PVA samples

The main bands of the PVA are present in all PVA samples even after swelling, Figure II.1. The band at 1564cm^{-1}, related to non-hydrolyzed acetate groups, is absent after swelling, indicating probably some leaching of these groups by the media, Table II.1. In addition, after swelling in Solution pH 4.0, a band at 1713cm^{-1} emerges. This band is related to stretching of C=O of lactic acid, indicating that it interacts with the PVA chains.

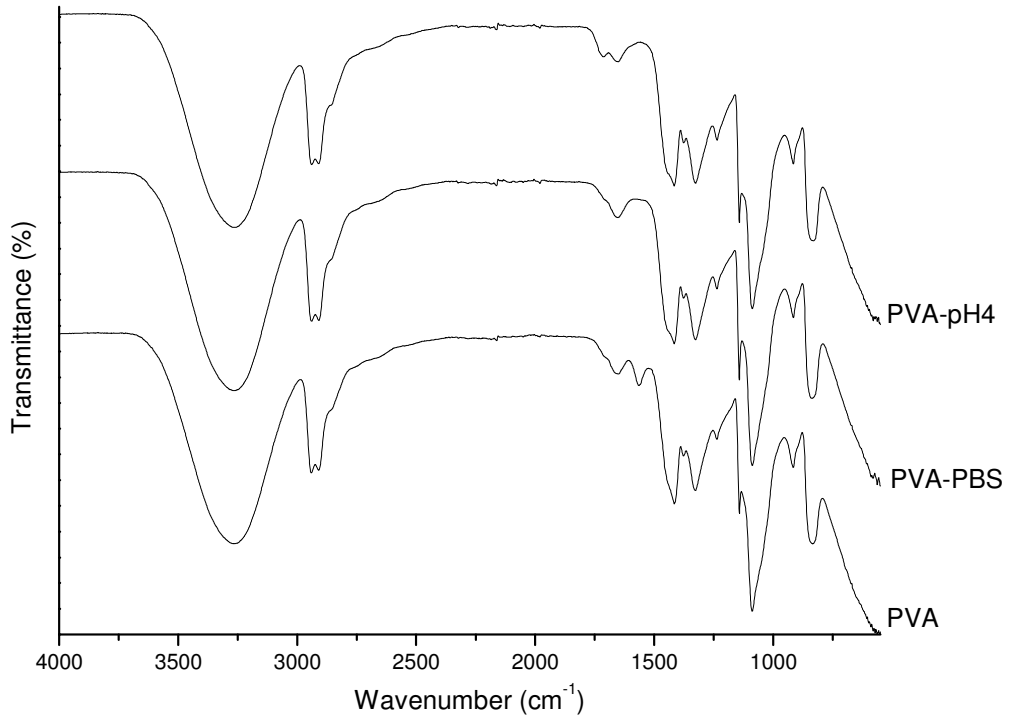

Figure II.1 - PVA samples spectra before and after swelling

Table II. 2 - PVA samples bands before and after swelling

Bands (cm^{-1})			*PVA - Groups' vibration modes*[41,113,114,126-138]
PVA	*PVA-PBS*	*PVA-pH4*	
3259	3263	3263	ν(O-H)
2942	2941	2940	ν(C-H) – alkyl groups
2909	2909	2909	
2850(s)	2853(s)	2853(s)	
		1713	ν(C=O) of lactic acid
1655	1655	1657	ν(C=O) of unhydrolyzed acetate groups, ν(C=C)
1564			ν(C=C)
1415	1416	1417	δ, wagging, in plane(C-H in CH_2 groups); ν(C-O-C) of unhydrolyzed acetate groups, in plane(O-H)
1378	1378	1377	coupling of in plane(O-H) wagging(C-H)
1329	1327	1327	bending(CH + OH), fan and twist(-CH_2-)
1236	1237	1238	ν(C-C), fan and twist(-CH_2-)
1142	1143	1143	ν(C-O-C), ν_S(C-C) crystalline sensitive band
1088	1088	1088	ν(C-O), δ(O-H)
917	916	917	ν,[δ out of plane](C-H)
836	836	836	ν, pendular(C-C)

II.b. 0.075% propolis samples

The main bands of the PVA are present in all 0.075% propolis samples even after swelling, Figure II.2. The band at 1564 cm^{-1}, related to non-hydrolyzed acetate groups, is absent after swelling, indicating probably some leaching of these groups by the media, Table II.3. A shoulder at 1713 cm^{-1} emerges after swelling in both media.

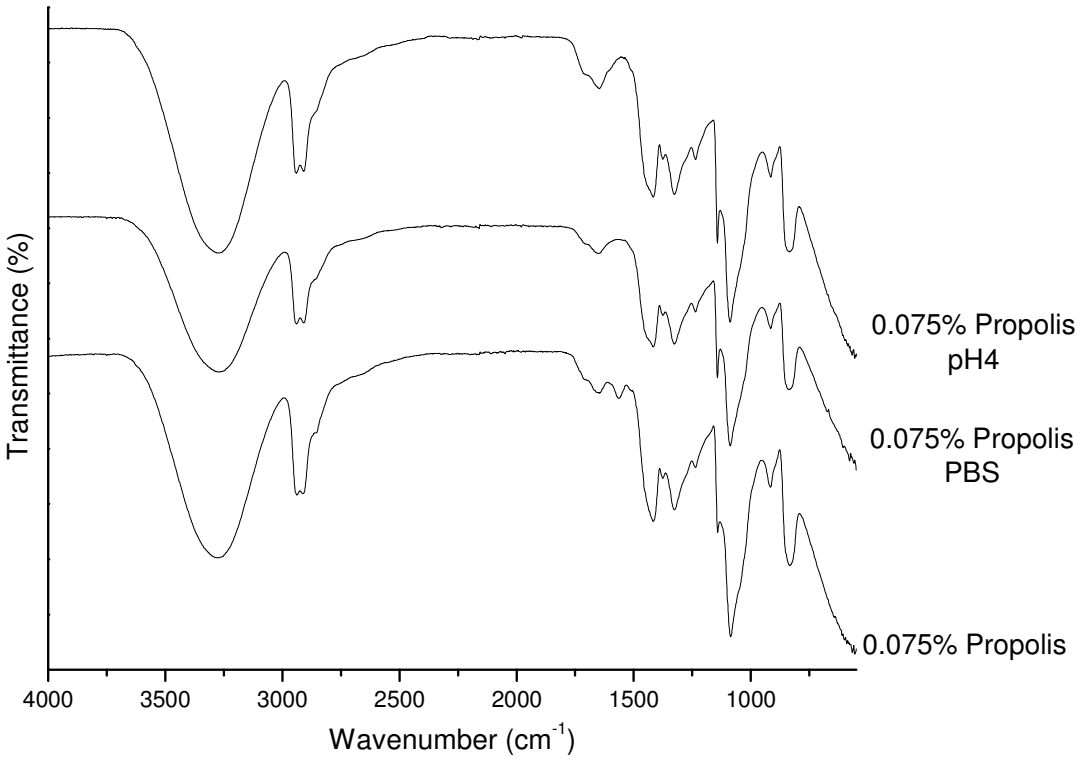

Figure II.2 - 0.075% Propolis samples spectra before and after swelling

Table II.3 - 0.075% Propolis bands before and after swelling.

Bands (cm^{-1})			*PVA - Groups' vibration modes*[41,113,114,126-138]
0.075	*0.075-PBS*	*0.075-pH4*	
3280	3272	3273	ν(O-H)
2939	2940	2941	ν(C-H) – alkyl groups
2911	2910	2909	
2853(s)	2855(s)	2854(s)	
	1713(s)	1708(s)	ν(C=O) of lactic acid
1652	1653	1650	ν(C=O) of unhydrolyzed acetate groups, ν(C=C)
1563			ν(C=C)
1417	1417	1416	δ, wagging, in plane(C-H in CH_2 groups); ν(C-O-C) of unhydrolyzed acetate groups, in plane(O-H)
1378	1376	1376	coupling of in plane(O-H) wagging(C-H)
1326	1327	1326	bending(CH + OH), fan and twist(-CH_2-)
1236	1237	1235	ν(C-C), fan and twist(-CH_2-)
1142	1143	1143	ν(C-O-C), ν_S(C-C) crystalline sensitive band
1089	1089	1089	ν(C-O), δ(O-H)
917	917	917	ν,[δ out of plane](C-H)
835	836	835	ν, pendular(C-C)

II.c. 0.15% propolis

Samples with 0.15% of propolis or more are the ones in which the bands of PVA and the bands of propolis can be distinguished, Figure II.3. The band at 1564cm^{-1}, related to non-hydrolyzed acetate groups, is, again, absent after swelling indicating, probably, some leaching of these groups by the media, Table II.4.

The bands related to PVA are present. In addition, the band at 2909cm^{-1} of PVA that overlaps the propolis band at 2928cm^{-1}, is shifted toward the propolis band after immersion in pH 4.0. Some bands related to propolis only are also present, between 1602 and 1456 cm^{-1}, related to aromatic ring vibration, and the band between 1276 and 1270 cm^{-1}, C-O groups of polyols vibration, e.g. hydroxyflavonoids. The sample swollen in PBS presents only the band between 1276 and 1270 cm^{-1}, the other bands, related to aromatic ring vibration, are absent. This fact could be related to the delivery of these compounds to the media, since propolis delivery is high in PBS.

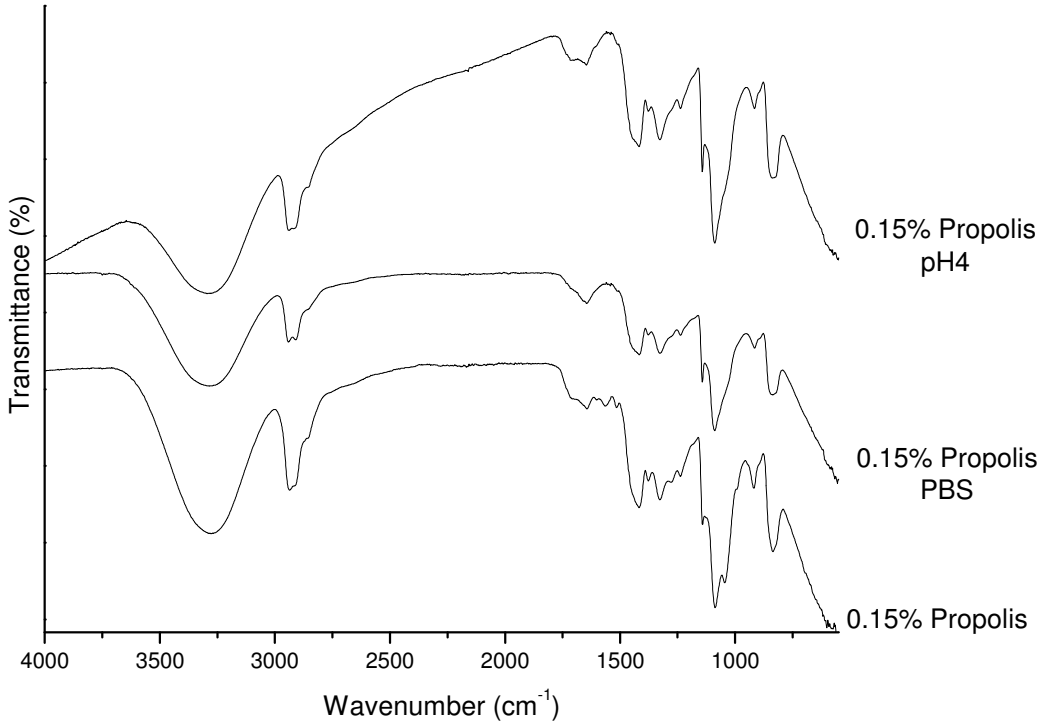

Figure II.3 - FTIR spectra of original and swollen 0.15% propolis samples

Table II.4 - FTIR bands of the 0.15% propolis samples

Bands (cm^{-1})			*Groups' vibration modes*	
0.15	*0.15-PBS*	*0.15-pH4*	*PVA*[41,113,114,126-138]	*Propolis*[102,103,163,164]
3278	3285	3291	ν(O-H)	Wagging (OH) of phenolic compounds; stretching of (OH) groups
2939	2941	2940	ν(C-H) – alkyl groups	-
2909(s)	2909	2919♦	ν(C-H) – alkyl groups	♦shift toward the position of C-H bands of aromatic compounds
2854(s)	2854(s)	2851(s)		C-H bands of aromatic compounds
1715(s)	1714(s)	1716(s)	-	-
1645	1643	1646	ν(C=O) of unhydrolyzed acetate groups, ν(C=C)	C-H bands of aromatic compounds
1602			-	Aromatic ring bands
1565			ν(C=C)	-
1516			-	Aromatic ring bands
		1456		
1417	1416	1418	δ, wagging, in plane(C-H in CH_2 groups); ν(C-O-C) of unhydrolyzed acetate groups, in plane(O-H)	-
1378	1377	1377	coupling of in plane(O-H) wagging(C-H)	N.I.
1327	1325	1328	bending(CH + OH), fan and twist(-CH_2-)	
1276	1276	1270	-	C-O groups of polyols, e.g. hydroxyflavonoids
1238	1237	1238	ν(C-C), fan and twist (-CH_2-)	-
1142	1142	1143	ν(C-O-C), ν_S(C-C) crystalline sensitive band	-
1087	1088	1089	ν(C-O), δ(O-H)	ν(C-O) of ester groups
1045		1037(s)	-	
919	916	917	ν,[δ out of plane](C-H)	N.I.
		889	-	Aromatic ring vibration
836	837	834	ν, pendular(C-C)	

II.d. 0.45% propolis

Besides the PVA bands, it can be noticed that there are some bands related to the propolis itself, Figure II. 4. They are the bands between 1602 and 1425 cm^{-1} (related to aromatic ring vibration band, non-identified (N.I.) bands and C-O groups of polyols bands), the bands between 1330 and 1272 cm^{-1}, a non-identified band at ~990 cm^{-1}, the bands between 1030 and 1042 cm^{-1}, due to ν(C-O) of ester groups and the bands between 890 and 833 cm^{-1}, related to aromatic ring vibration, Table II.5. Some of these bands can be noticed only after swelling, probably due to interactions between PVA

and/or propolis and the media, which allow these groups' bonds vibration after swelling.

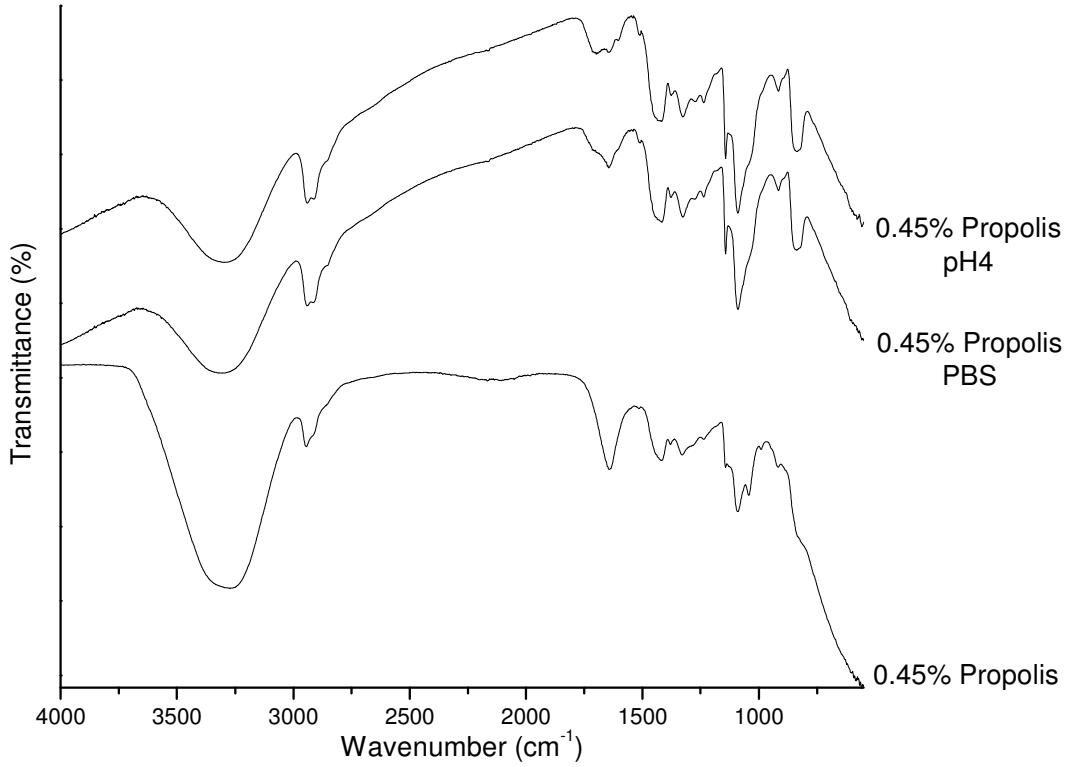

Figure II. 4 - FTIR spectra of the originals and swollen 0.45% Propolis samples

Table II.5 - FTIR bands of the 0.45% propolis samples

Bands (cm^{-1})			*Groups' vibration modes**	
0.45	*0.45-PBS*	*0.45-pH4*	*PVA*[41,113,114,126-138]	*Propolis*[102,103,163,164]
3284	3308	3291	ν(O-H)	Wagging (OH) of phenolic compounds, ν(O-H)
2943	2941	2941	ν(C-H) – alkyl groups	-
2912(o)	2914	2911	ν(C-H) – alkyl groups	C-H bands of aromatic compounds
2854(o)	2850(o)	2851(o)		
2112			-	-
	1716(o)	1696♦		♦Stretching of carboxyl groups
1642	1646	1644	ν(C=O) of unhydrolyzed acetate groups, ν(C=C)	ν(C=O) of CAPE and its derivatives; ν(C=C), Aromatic ring bands
	1605(o)	1604	-	Aromatic ring bands
1516	1513	1510		
	1447			
1419	1416	1425(o)	δ, wagging, in plane(C-H in CH_2 groups); ν(C-O-C) of unhydrolyzed acetate groups, in plane(O-H)	-
1380	1378	1377	coupling of in plane(O-H)	N.I.

			wagging(C-H)	
1330	1326	1327	-	
1280	1276	1272		C-O groups of polyols, e.g. hydroxyflavonoids
1236	1239	1237	ν(C-C), fan and twist(-CH_2-)	-
1143	1143	1143	ν(C-O-C), ν_S(C-C) crystalline sensitive band	
1091	1089	1090	ν(C-O), δ(O-H)	ν(C-O) of ester groups
1044	1030	1042(o)	-	
991	990(o)	992(o)		N.I.
918	916	916	ν,[δ out of plane](C-H)	
	890(o)	890(o)	-	Aromatic ring vibration
	833	835		

II.e. 0.90% propolis

In these samples mainly all the bands of PVA and of propolis can be distinguished, Figure II.5. Some of the original PVA bands that could be overlapped to the propolis ones are shifted toward the propolis bands, Table II.6.

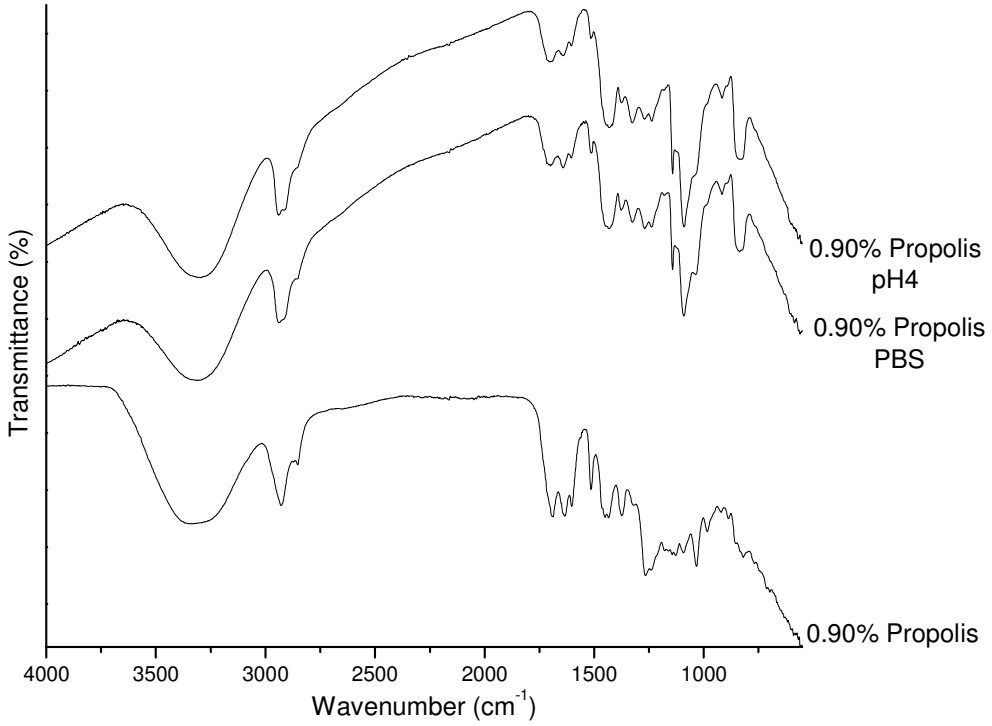

Figure II.5 - FTIR spectra of the original and swollen 0.90% propolis samples

Table II.6 - FTIR bands of the 0.90% propolis

Bands (cm^{-1})			*Bands (cm^{-1})*	
0.90	*0.90-PBS*	*0.90-pH4*	*PVA*[41,113,114,126-138]	*Propolis*[102,103,163,164]
3326	3312	3297	ν(O-H)	ν(O-H), Wagging (OH) of phenolic compounds
2931♦	2937	2939	ν(C-H) – alkyl groups	♦Shift towards C-H bands of aromatic compounds
	2915(o)♦	2915(o)♦	ν(C-H) – alkyl groups	
2854	2855(o)	2852(o)		C-H bands of aromatic compounds
2637			-	-
1689	1697	1700		Stretching of carboxyl groups
1634	1641	1643	ν(C=O) of unhydrolyzed acetate groups, ν(C=C)	ν(C=O) of CAPE and its derivatives; ν(C=C), Aromatic ring bands
1602	1605	1604	-	Aromatic ring bands
1514	1514	1514		
1465				
1451				
1434♦	1432♦	1431♦	δ, wagging, in plane(C-H in CH_2 groups); ν(C-O-C) of unhydrolyzed acetate groups, in plane(O-H)	♦shift towards this band - N.I.
1374	1375	1373	coupling of in plane(O-H) wagging(C-H)	N.I.
1318	1324	1325	bending(CH + OH), fan and twist(-CH_2-)	
1265	1268	1270	-	C-O groups of polyols, e.g. hydroxyflavonoids
1239	1239	1238	ν(C-C), fan and twist(-CH_2-)	N.I.
1179	1179	1181	-	
1144	1143	1143	ν(C-O-C), ν_S(C-C) crystalline sensitive band	
1128			-	
1092	1091	1090	ν(C-O), δ(O-H)	ν(C-O) of ester groups
1033	1034	1038	-	
984	987	984		N.I.
921	918	916	ν,[δ out of plane](C-H)	
887	889(o)	892	-	Aromatic ring vibration
817	836	833	ν, pendular(C-C)	

In order to quantify the amount of propolis delivered, a standard curve was established. The volume analyzed was normalized for the whole propolis delivery test (2 ml). The propolis extract was diluted and the dilutions were analyzed via UV-Vis spectroscopy. The polystyrene cuvettes used in the experiment limited the analysis' range and the propolis peak position would be close to the inferior limit, Figure III.1.

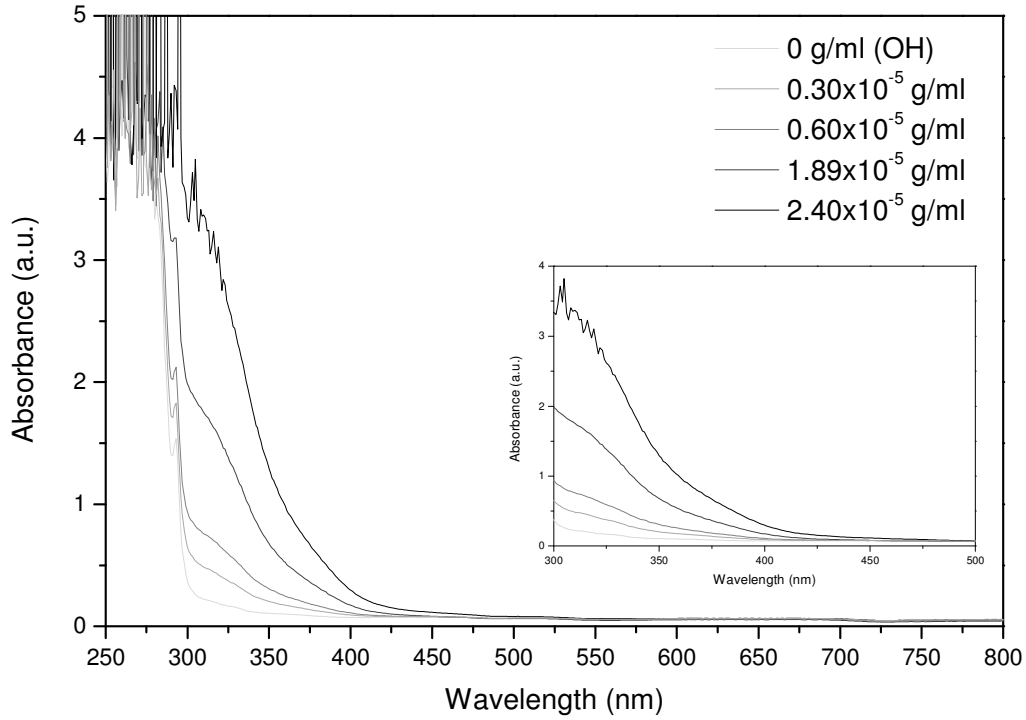

Figure III.1 - spectra of propolis dilutions. (OH) is the curve of the isopropyl alcohol used to prepare the dilutions.

The propolis peaks' intensity (peaks' maximum height) could not be correlated to the amount of propolis in each dilution. However, since the slope of the curves raised with the increase of the propolis amount in the dilutions, the area of the curves in a fixed range (350-500 nm) was calculated and correlated to the amount of propolis in each dilution. The area of the OH (isopropyl alcohol, used in the dilutions) curve in the same range was calculated and discounted from the dilution areas to plot the standard curve, Figure III.2, $R^2 = 0.91$.

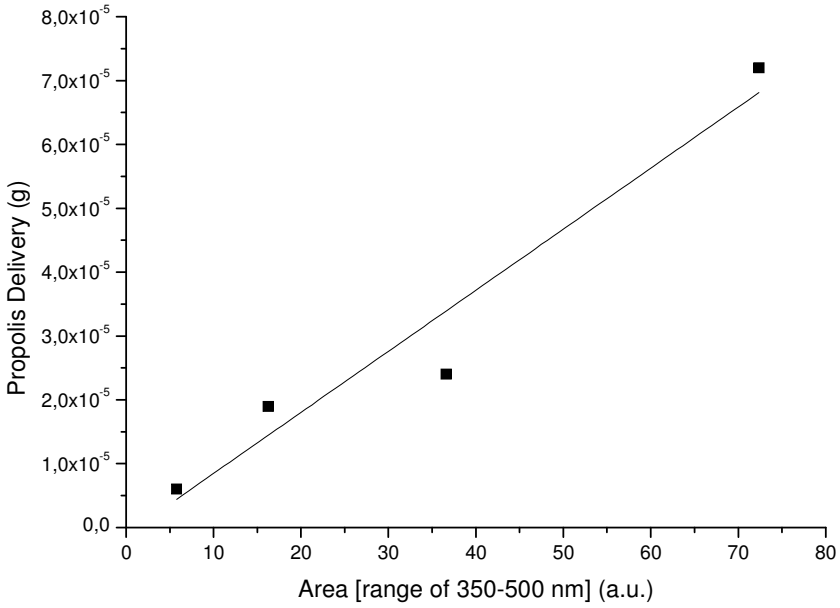

Figure III.2- Standard curve of propolis.

To quantify the amount of propolis delivered, samples of approximately 2 cm^2, weight normalized, were immersed in 2 ml of each media (PBS and Solution pH 4.0). After regular intervals of time (triplicates were used to each time interval), the media was removed and analyzed via UV-Vis spectroscopy.

The PBS used as swelling media was analyzed via UV-Vis spectroscopy and its area in the same range (350-500 nm) was subtracted from the propolis delivery in PBS for all samples curves. The plot of the delivery at all time intervals for all samples immersed in PBS, compared to the extrapolated standard curve, $R^2 = 0.91$, can be observed in Figure III.3. It can be noticed that the propolis delivery increased with the increase of the amount of propolis in the samples.

Based on these results, it was possible to display the amount of propolis delivered by each samples composition with time in 2 ml of PBS, Figure III.4. A trend can be observed in all curves after 4 days of immersion: there is a high propolis delivery in the first hours and the delivery reaches constant values after 1 day of immersion. It can be noticed some “instability” in the propolis delivery for the first hours, probably related to the samples inherent variability, nonetheless, the whole profiles were not considerably affected by this instability. In addition, higher the amount of propolis in the samples, higher the propolis delivered.

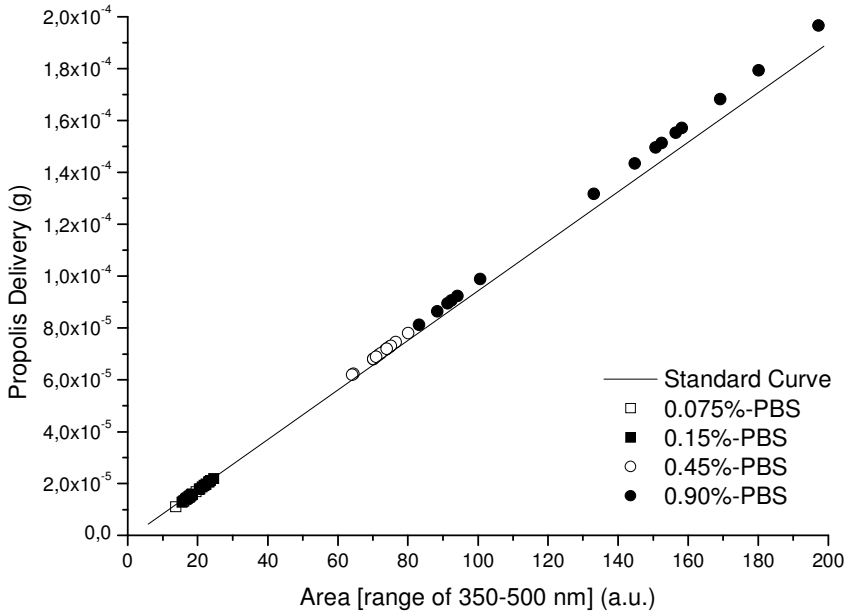

Figure III.3 - Propolis delivered by all samples in PBS compared to the standard curve.

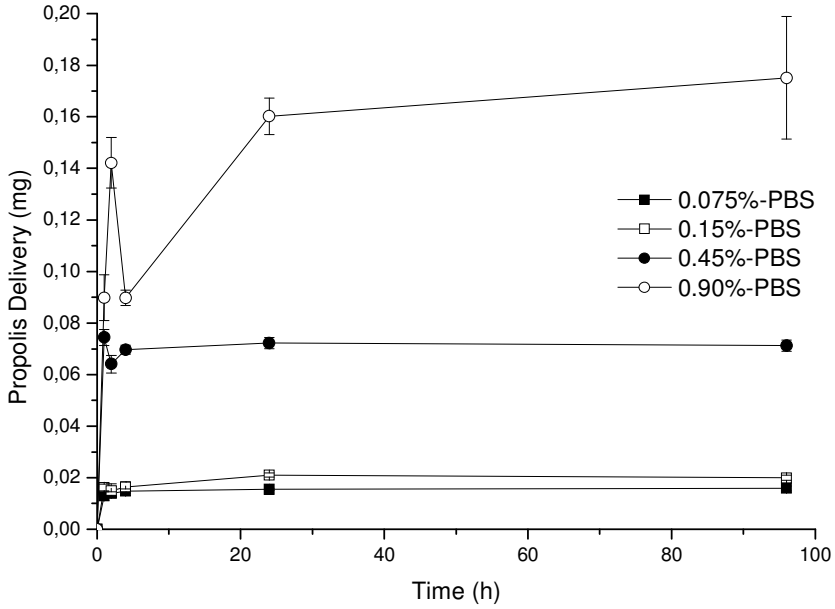

Figure III.4 - Amount of propolis delivery per time interval to PBS

The Solution pH 4.0 used as swelling media was analyzed via UV-Vis spectroscopy and its area in the same range (350-500 nm) was subtracted from the propolis delivery in Solution pH 4.0 for all samples curves. The plot of the delivery at all time intervals for all samples immersed in Solution pH 4.0, compared to the

extrapolated standard curve, $R^2 = 0.91$, can be observed in Figure III.5. It can be noticed that the propolis delivery increased with the increase of the amount of propolis in the samples.

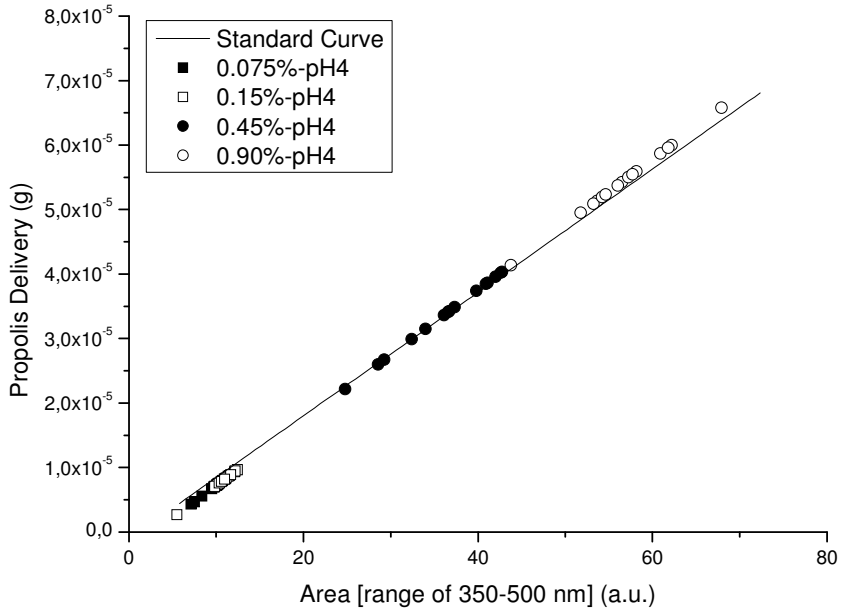

Figure III.5 - Propolis delivered by all samples in Solution pH 4.0 compared to the standard curve

Based on these results, it was possible to display the amount of propolis delivered by each samples composition to Solution pH 4.0 with time in 2 ml of media, Figure III.6. It can be noticed some "instability" in the propolis delivery for the first hours, probably related to the samples inherent variability, nonetheless, the whole profiles are not considerably affected by this instability. A trend can be observed in all curves after 4 days of immersion: there is a high propolis delivery in the first hours and the delivery reaches constant values after 1 day of immersion. In addition, higher the amount of propolis in the samples, higher the delivery.

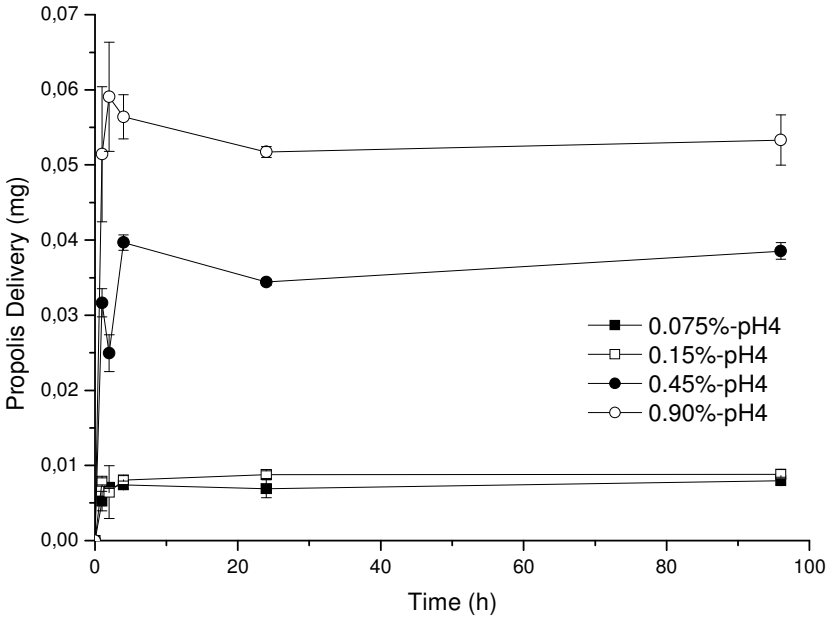

Figure III.6 - Amount of propolis delivery per time interval to Solution pH 4.0

The average values of propolis delivery after 4 days of immersion were displayed in Table III.1. The ANOVA analysis on the amount (mg) of propolis delivered after 4 days of immersion revealed that both factors (type of media and amount of propolis), as well as their interaction, were significant to the total propolis delivery, Table III.2.

Table III.1 - Propolis delivered by the samples to both media after 4 days of immersion.

Samples	Propolis delivery (mg)	
	PBS	Solution pH 4.0
0.075%	0.0158 ± 0.0009	0.0079 ± 0.0004
0.15%	0.0199 ± 0.0009	0.0088 ± 0.0006
0.45%	0.0712 ± 0.0021	0.0385 ± 0.0011
0.90%	0.1751 ± 0.0237	0.0533 ± 0.0033

It can be noticed that there was a significant difference between the amounts of propolis delivered to each media by each samples composition. For each samples composition, there was a high delivery to PBS compared to the same samples in Solution pH 4.0.

Table III.2 - two-way ANOVA analysis on the total propolis delivery. Two factors were used, type of media and amount of propolis. For type of media, 2 levels were used, PBS and Solution pH 4.0. For amount of propolis, 4 levels were used, 0.075, 0.15, 0.45 and 0.90% propolis.

	DF	Sum of squares	Mean Square	F value	P value
Media	1	1.13 E-8	1.13 E-8	154.91	1.20 E-9
% Propolis	3	4.11 E-8	1.37 E-8	188.06	1.10 E-12
Interaction	3	1.28 E-8	4.28 E-9	58.69	7.44 E-9
Model	7	6.53 E-8	9.32 E-9	127.88	7.71 E-13
Error	16	1.16 E-9	7.29 E-11	--	--
Total	23	6.64 E-8	--	--	--

Tukey Test	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
PBS-pH4	-4.34 E-5	3.48 E-6	17.60	6.18 E-8	0.05	1	-5.07 E-5	-3.60 E-5
0.075-0.15	2.51 E-6	4.93 E-6	0.72	0.95	0.05	0	-1.15 E-5	1.66 E-5
0.075-0.45	4.30 E-5	4.93 E-6	12.33	1.12 E-6	0.05	1	2.88 E-5	5.71 E-5
0.075-0.90	4.04 E-5	4.93 E-6	11.61	2.14 E-6	0.05	1	2.63 E-5	5.46 E-5
0.15-0.45	1.02 E-4	4.93 E-6	29.34	0	0.05	1	8.82 E-5	1.16 E-4
0.15-0.90	9.97 E-5	4.93 E-6	28.62	0	0.05	1	8.56 E-5	1.13 E-4
0.45-0.90	5.93 E-5	4.93 E-6	17.00	0	0.05	1	4.51 E-5	7.34 E-5

Regarding the factor “amount of propolis”, there was a significant difference between the levels, but between the levels 0.075% and 0.15% propolis. A trend can be observed: there was a higher delivery as the content of propolis in the samples increased, independent of the media.

The propolis delivery kinetics could not be described by the Higuchi equation, nor by the Korsmeyer-Peppas equation, since the linear regressions of the values presented high deviation from the fitted curves, $R^2 < 0.95$, independent of the amount of propolis or the media used (plots not shown).

Annex IV – Statistical Analysis

The tables of the statistical analysis are displayed in this section, first the PVA-Ag data, followed by the PVA-propolis data.

Annex IV.1 – PVA-Ag

a. Swelling degree

Table IV.1 - Two-way ANOVA analysis on the equilibrium of the swelling degree of the PVA-Ag samples, after 1 day of immersion at 37°C. Factors: amount of silver (three levels: 0 (PVA), 0.25 and 0.50) and type of media (saline, PBS, solution pH 4.0).

	DF	Sum of Squares	Mean Square	F Value	P Value
Medium	2	92212.17	46106.08	54.64	9.11 E-13
%Ag	2	30481.15	15240.57	18.06	1.74 E-6
Interaction	4	33894.06	8473.51	10.04	6.7 7E-6
Model	8	156587.39	19573.42	23.19	1.42 E-13
Error	45	37969.85	843.77	--	--
Total	53	194557.24	--	--	--

Tukey Test								
%Ag	**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
PVA-0.25%Ag	15.32	9.68	2.23	0.26	0.05	0	-8.14	38.79
PVA-0.50%Ag	56.28	9.68	8.22	1.71 E-6	0.05	1	32.81	79.75
(0.25-0.50)%Ag	40.95	9.68	5.98	3.27 E-4	0.05	1	17.49	64.42
Medium	**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
Saline-PBS	17.09	9.68	2.49	0.19	0.05	0	-6.36	40.56
Saline-pH4	-77.85	9.68	11.37	0	0.05	1	-101.31	-54.38
PBS-pH4	-94.94	9.68	13.86	0	0.05	1	-118.41	-71.48

b. Weight loss

At the 0.05 level, the type of Medium, the amount of Ag, as well as their interaction, was significant to the weight loss results. The difference between the levels of each factor was studied by a Tukey test post-hoc. There was not a considerable difference of the weight loss between saline and PBS, but the weight loss in pH 4.0 was lower than in the other media. Comparing the weight loss of PVA and of 0.25 samples, the difference was not considerable; however, the weight loss of sample 0.50 was significantly higher than that of PVA and of 0.25 samples.

Table IV.2 - Two-way ANOVA analysis of the PVA-Ag dried samples weight loss after 4 days of immersion. Factors: amount of silver (three levels: 0 (PVA), 0.25 and 0.50) and type of media (saline, PBS, solution pH 4.0).

	DF	Sum of Squares	Mean Square	F Value	P Value
Medium	2	2417.80	1208.90	61.76	1.25 E-13
%Ag	2	364.98	182.49	9.32	4.09 E-4
Interaction	4	899.65	224.91	11.49	1.64 E-6
Model	8	3682.45	460.30	23.51	1.11 E-13
Error	45	880.81	19.57	--	--
Total	53	4563.27	--	--	--

Tukey Test	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
PBS/saline	-0.76	1.47	0.72	0.86	0.05	0	-4.33	2.81
pH4/saline	-14.55	1.47	13.96	0	0.05	1	-18.13	-10.98
pH4/PBS	-13.79	1.47	13.23	0	0.05	1	-17.37	-10.22
0.25/0	0.5408	1.47	0.51	0.92	0.05	0	-3.03	4.11
0.5/0	5.76548	1.47	5.52	8.85 E-4	0.05	1	2.19	9.33
0.5/0.25	5.22468	1.47	5.01	0.01	0.05	1	1.65	8.79

c. Silver delivery

The lowest values of transmittance were related to the samples with the highest amount of silver, which could mean high silver delivery to the media. In addition, the ANOVA analysis showed that, at the 0.05 level, both the type of media and the amount of silver were significant to the silver delivery results, although their interaction is not.

The Tukey post-hoc test revealed that the highest transmittance occurred for pH 4.0 medium, irrespective to the amount of silver in the samples, indicating low silver delivery. For all the media, low transmittance occurred for samples 0.50, indicating high silver delivery by the samples with high amount of Ag.

Table IV.3 - Two-way ANOVA analysis on the UV-Vis transmittance values. Factors: amount of silver (three levels: 0 (PVA), 0.25 and 0.50) and type of media (saline, PBS, solution pH 4.0).

	DF	**Sum of Squares**	**Mean Square**	**F Value**	**P Value**
Medium	2	0.002	0.001	17.81	2.98 E-5
% Ag	2	0.002	9.65 E-4	15.35	7.77 E-5
Interaction	4	6.60 E-4	1.65 E-4	2.62	0.06
Model	8	0.005	6.32 E-4	10.05	1.13 E-5
Error	21	0.001	6.28 E-5	--	--
Total	29	0.006	--	--	--

Tukey Test	**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
PBS/saline	0.004	0.003	1.85	0.40	0.05	0	-0.004	-0.013
pH4/saline	-0.016	0.003	6.38	5.31 E-4	0.05	1	-0.025	-0.007
pH4/PBS	-0.020	0.003	8.01	3.61 E-5	0.05	1	-0.029	-0.011
0.25/0	-0.003	0.003	1.47	0.55	0.05	0	-0.012	0.005
0.5/0	0.015	0.003	6.30	6.15 E-4	0.05	1	0.006	0.024
0.5/0.25	0.019	0.003	7.77	5.34 E-5	0.05	1	0.010	0.028

d. Tensile tests

A two-way ANOVA analysis was performed in the Secant modulus values, Table IV.4. It can be observed that, at the 0.05 level, the type of media, the amount of Ag, as well as their interaction, was significant to the secant modulus values. A Tukey test showed that, maintaining the composition of the samples constant, the values in saline and in PBS were considerably different (Sig = 1). For PVA and 0.25 samples: $E_{saline} < E_{PBS}$, although the opposite was observed for 0.50 samples: $E_{saline} > E_{PBS}$. In saline and in PBS: $E_{PVA} > E_{0.25} > E_{0.50}$, but in pH4: $E_{0.25} > E_{PVA} > E_{0.50}$.

Table IV.4 - ANOVA and Tukey test post-hoc results related to the Secant modulus. Two factors, type of medium (Media) and amount of silver (%Ag), with 3 levels were considered to this analysis.

		DF	Sum of squares	Mean Square	F value	P value			
Medium		2	0.157	0.078	5.141	0.007			
% Ag		2	0.656	0.328	21.385	1.706 E-8			
Interaction		4	0.886	0.221	14.435	2.165 E-9			
Model		8	1.654	0.206	13.470	3.487 E-13			
Error		103	1.581	0.015	--	--			
Total		111	3.235	--	--	--			
Tukey Test		**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
Medium	PBS-saline	0.078	0.028	3.932	0.017	0.05	1	0.011	0.146
	pH4-saline	0.053	0.028	2.646	0.152	0.05	0	-0.014	0.121
	pH4-PBS	-0.025	0.029	1.218	0.665	0.05	0	-0.095	0.044
%Ag	PVA-0.25	-0.100	0.028	4.973	0.001	0.05	1	-0.169	-0.032
	PVA-0.50	-0.188	0.029	9.117	0	0.05	1	-0.257	-0.118
	0.25-0.50	-0.087	0.028	4.378	0.007	0.05	1	-0.154	-0.020

The fracture strength varied considerably within all samples; nonetheless all average values were higher than 1 MPa. The two-way ANOVA, Table IV. 5, with a 95% confidence level, revealed that the type of media, the amount of silver as well as their interaction were significant to the fracture strength. For PVA and for 0.25, the

fracture strength in pH 4.0 was higher than in other media. For 0.50: $\sigma_{F(pH4)} > \sigma_{F(PBS)}$ and $\sigma_{F(pH4)} < \sigma_{F(saline)}$. For all media, the fracture strength of the samples with 0.5% Ag was the lowest.

Table IV. 5 - ANOVA and tukey test for fracture strength. Factors: type of medium (levels: saline, PBS and solution pH 4.0) and amount of silver (levels: 0 (PVA), 0.25 and 0.50).

	DF	Sum of squares	Mean Square	F value	P value
Medium	2	154,784	77,391	7,934	6,33 E-4
% Ag	2	266,745	133,372	13,674	5,62 E-6
Interaction	4	132,848	33,212	3,405	0,01
Model	8	553,057	69,132	7,087	2,17 E-7
Error	100	975,364	9,753	--	--
Total	108	1528,421	--	--	--

Tukey Test		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium	PBS-saline	0.512	0.726	0.998	0.760	0.05	0	-1.215	2.240
	pH4-saline	2.896	0.731	5.597	4.129 E-4	0.05	1	1.155	4.636
	pH4-PBS	2.383	0.741	4.546	0.004	0.05	1	0.619	4.147
%Ag	PVA-0.25	1.191	0.732	2.299	0.239	0.05	0	-0.551	2.934
	PVA-0.50	-2.400	0.746	4.546	0.004	0.05	1	-4.177	-0.624
	0.25-0.50	-3.592	0.721	7.038	8.018 E-6	0.05	1	-5.309	-1.875

All the samples presented average fracture strain above 300%. The analysis of variance (ANOVA - Table IV.6), revealed that, at the level of 0.05, only the amount of silver was significant. The Tukey test revealed that no significant difference was observed between the levels.

Table IV.6 - Analysis of variance for the fracture strain. Factors: type of medium (saline, PBS and solution pH 4.0) and amount of silver (0 (PVA), 0.25 and 0.50).

	DF	Sum of squares	Mean Square	F value	P value
Medium	2	73599.88	36799.94	2.66	0.07
% Ag	2	92142.29	46071.14	3.33	0.03
Interaction	4	131123.96	32780.99	2.37	0.05
Model	8	281569.18	35196.14	2.54	0.01
Error	102	1.40 E6	13802.85	--	--
Total	110	1.68 E6	--	--	--

Tukey Test		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium	PBS-saline	-58.01	27.15	3.02	0.08	0.05	0	-122.58	6.56
	pH4-saline	-9.97	27.15	0.51	0.92	0.05	0	-74.55	54.61
	pH4-PBS	48.04	27.69	2.45	0.19	0.05	0	-17.81	113.90
%Ag	PVA-0.25	54.97	27.40	2.83	0.11	0.05	0	-10.21	120.15
	PVA-0.50	-5.47	27.91	0.27	0.97	0.05	0	-71.85	60.91
	0.5/0.25	-60.44	26.79	3.18	0.06	0,05	0	-124.17	3.29

e. Antimicrobial tests

The ANOVA analysis on the antimicrobial results are displayed in Table IV.7.

Table IV.7 - One-way ANOVA analysis on the antimicrobial results, where the factor analyzed was the amount of silver (levels: 0 (PVA), 0.25 and 0.50).

E. coli	**DF**	**Sum of squares**	**Mean Square**	**F value**	**P value**
Model	2	1.76 E6	883792.56	5548.86	4.44 E-6
Error	3	477.82	159.27		
Total	5	1.76 E6			

	Tukey Test	**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
%Ag	PVA-0.25	1119.04	12.62	125.39	5.84 E-6	0.05	1	1066.31	1171.78
	PVA-0.50	1181.20	12.62	132.36	5.14 E-6	0.05	1	1128.46	1233.94
	0.5/0.25	62.15	12.62	6.96	0.03	0.05	1	9.41	114.89

S. aureus	**DF**	**Sum of squares**	**Mean Square**	**F value**	**P value**
Model	2	1.85 E6	929870.07	966.36	6.10 E-5
Error	3	2886.70	962.23		
Total	5	1.86 E6			

	Tukey Test	**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
%Ag	PVA-0.25	1183.95	31.01	53.97	8.14 E-5	0.05	1	1054.32	1313.57
	PVA-0.50	1178.06	31.01	53.70	8.27 E-5	0.05	1	1048.43	1307.68
	0.5/0.25	-5.89	31.01	0.26	0.98	0.05	0	-135.51	123.73

C. albicans	**DF**	**Sum of squares**	**Mean Square**	**F value**	**P value**
Model	2	2.30 E6	1.15 E6	6355.72	3.62 E-6
Error	3	544.02	181.34		
Total	5	2.30 E6			

	Tukey Test	**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
%Ag	PVA-0.25	1332.62	13.46	139.94	4.56 E-6	0.05	1	1276.35	1388.89
	PVA-0.50	1296.33	13.46	136.13	4.83 E-6	0.05	1	1240.06	1352.61
	0.5/0.25	-36.28	13.46	3.81051	0.14	0.05	0	-92.55	19.98

For E. coli inhibition, there was a significant difference between levels (PVA, 0.25 and 0.50), where the increase in the silver amount resulted in higher inhibition zone. For *S. aureus* there is a significant difference between the PVA samples (no inhibition) and the PVA-Ag samples, where 0.25 and 0.50 samples presented ~1180 mm^2 of inhibition zone. For the fungi species, there was a significant difference between PVA and PVA-Ag samples, where Ag ones inhibited the *C. albicans*.

f. Cytotoxicity results

The ANOVA analysis, Table IV.8, revealed that the tested samples composition (controls, PVA, 0.25 and 0.50) was significant to the cells viability. There is a significant difference between the cells viability (CV) of the positive control (CV = 0%) and of the negative control (CV = 100%) and also between the cells viability of the positive control and of the wells with the samples extracts (CV > 70%).

Table IV.8 - One-way ANOVA analysis of the samples on the cells viability, $\alpha < 0.05$. Factor: amount of Ag. Levels: 0 (PVA), 0.25, 0.50, (-) is the negative control, (+) is the positive control.

	DF	Sum of squares	Mean Square	F value	P value
Model	4	18531.06	4632.76	16.13	2.31 E-4
Error	10	2871.33	287.13		
Total	14	21402.4			

Tukey Test		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
% Ag	(+) - (-)	99.66	13.83	10.18	2.18 E-4	0.05	1	54.13	145.20
	(+) - PVA	70.33	13.83	7.18	0.003	0.05	1	24.79	115.86
	(-) - PVA	-29.33	13.83	2.99	0.28	0.05	0	-74.86	16.20
	(+) - 0.25	84	13.83	8.58	8.81 E-4	0.05	1	38.46	129.53
	(-) - 0.25	-15.66	13.83	1.60	0.78	0.05	0	-61.20	29.86
	PVA - 0.25	13.66	13.83	1.39	0.85	0.05	0	-31.86	59.20
	(+) - 0.50	85	13.83	8.68	8.02 E-4	0.05	1	39.46	130.53
	(-) - 0.50	-14.66	13.83	1.49	0.82	0.05	0	-60.20	30.86
	PVA - 0.50	14.66	13.83	1.49	0.82	0.05	0	-30.86	60.20
	0.25 - 0.50	1	13.83	0.10	0.99	0.05	0	-44.53	46.53

Annex IV.2 – PVA-Propolis

a. Swelling Degree

The ANOVA analysis, $\alpha = 0.05$, on the ESD showed that no factor (type of media and amount of propolis) were significant, nor their interaction, to the equilibrium of the swelling degree, Table IV.9. It means that the fluids uptake by all samples in the different media was approximately the same.

Table IV.9 - two-way ANOVA analysis on the equilibrium of the swelling degree of the samples. Factors: amount of propolis, levels: 0, 0.075, 0.15, 0.45 and 0.90% propolis; type of media, levels: PBS and solution pH 4.0.

ANOVA	DF	Sum of squares	Mean Square	F value	P value
Media	1	1092.59	1092.59	0.36	0.55
% Propolis	4	13858.97	3464.74	1.15	0.35
Interaction	4	9946.01	2486.50	0.83	0.52
Model	9	24897.57	2766.39	0.92	0.52
Error	20	59900.69	2995.03	--	--
Total	29	84798.27	--	--	--

b. Weight loss

The two-way ANOVA analysis (factors: type of media and amount of propolis in the original samples) showed that the amount of propolis was the only factor significant to the samples weight loss, Table IV.10. There is a significant difference between the 0.15% propolis samples weight loss and the others: $WL_{0.15\%} > WL_{PVA,\ 0.075\%}$ and $WL_{0.15\%} < WL_{0.45\%,\ 0.90\%}$. The samples weight loss could be related to the propolis delivery, since samples with high amounts of propolis delivery more propolis to the media and present high weight loss.

Table IV.10 - Two-way ANOVA analysis on the samples weight loss. Factors: amount of propolis, levels: 0.075, 0.15, 0.45 and 0.90% propolis; type of media, levels: PBS and solution pH 4.0.

	DF	**Sum of squares**	**Mean Square**	**F value**	**P value**
Media	1	1289.90	1289.90	3.34	0.07
% Propolis	3	12867.47	4289.15	11.13	1.89 E-5
Interaction	3	10236.96	3412.32	8.86	1.25 E-4
Model	7	24394.34	3484.90	9.05	1.19 E-6
Error	40	15402.18	385.05	--	--
Total	47	39796.52	--	--	--

Tukey Test	**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
PBS-pH4	-10.36	5.66	2.584	0.07	0.05	0	-21.81	1.08
0.075-0.15	-38.28	8.01	6.758	1.36 E-4	0.05	1	-59.75	-16.81
0.075-0.45	-0.73	8.01	0.129	0.99	0.05	0	-22.20	20.74
0.075-0.90	0.70	8.01	0.124	0.99	0.05	0	-20.76	22.17
0.15-0.45	37.55	8.01	6.629	1.80 E-4	0.05	1	16.08	59.02
0.15-0.90	-37.57	8.01	6.634	1.79 E-4	0.05	1	-59.05	-16.10
0.45-0.90	-0.02	8.01	0.004	1	0.05	0	-21.49	21.44

c. Tensile tests

Statistical analysis, two-way ANOVA, level of significance = 95%, was performed using 2 factors, type of media with 2 levels (PBS and Solution pH 4.0) and amount of propolis with 5 levels (PVA, 0.075% propolis, 0.15% propolis, 0.45% propolis and 0.90% propolis) on the secant modulus data and on the fracture strength data, Table IV.11 and Table IV. 12, respectively.

Table IV.11 - two-way ANOVA analysis on the secant modulus (E) of the samples swelled in the different media. Factors: amount of propolis, levels: 0.075, 0.15, 0.45 and 0.90% propolis; type of media, levels: PBS and solution pH 4.0.

	DF	Sum of squares	Mean Square	F value	P value
Media	1	0.78	0.78	19.07	3.07 E-5
% Propolis	4	5.08	1.27	30.80	1.11 E-16
Interaction	4	4.13	1.03	25.04	2.20 E-14
Model	9	10.22	1.13	27.50	0
Error	100	4.12	0.04	--	--
Total	109	14.35	--	--	--

Tukey Test	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
PBS-pH4	0.126	0.059	2.975	0.226	0.05	0	-0.040	0.292
PVA-0.075	0.134	0.060	3.133	0.182	0.05	0	-0.034	0.302
PVA-0.15	0.008	0.060	0.190	0.99	0.05	0	-0.160	0.176
0.075-0.15	0.420	0.061	9.705	0	0.05	1	0.250	0.591
PVA-0.15	0.294	0.061	6.797	5.251 E-5	0.05	1	0.124	0.465
0.075-0.45	0.286	0.061	6.538	1.088 E-4	0.05	1	0.114	0.458
0.15-0.45	-0.277	0.061	6.406	1.567 E-4	0.05	1	-0.448	-0.107
PVA-0.90	-0.403	0.061	9.313	1.527 E-8	0.05	1	-0.574	-0.233
0.075-0.90	-0.412	0.061	9.401	9.452 E-9	0.05	1	-0.584	-0.239
0.15-0.90	-0.698	0.062	15.757	0	0.05	1	-0.872	-0.524
0.45-0.90	0.126	0.059	2.975	0.226	0.05	0	-0.040	0.292

For the E values, it can be noticed that each factor and their interaction were significant to the Secant's modulus. It can be noticed that for PVA, the acidic media, compared to the neutral media (PBS), led to higher secant modulus. In addition, in pH 4.0, for samples with propolis, higher the amounts of propolis led to lower modulus values. On the contrary, in PBS, higher amounts of propolis, higher the modulus, the exception was the 0.90% propolis sample. For the last one the modulus was the lowest one, independent of the media.

There was no relevant difference between the different media. However, there was a significant difference between the modulus values of 0.15% propolis and the other samples, as well as between 0.90% propolis samples and PVA, between 0.90% propolis and 0.075% propolis, and between 0.45% propolis and 0.075% propolis samples.

In PBS, $E_{0.15\%propolis} > E_{0.075\%propolis}$, $E_{PVA} > E_{0.90\%propolis}$ and $E_{0.45\%propolis} > E_{0.15\%propolis}$, $E_{0.075\%propolis}$. The highest modulus was encountered for the samples 0.45% propolis and the lowest modulus was the ones of the 0.90% propolis. In pH 4.0, $E_{0.15\%propolis} < E_{0.075\%propolis}$, $E_{0.90\%propolis} < E_{PVA}$ and $E_{0.45\%propolis} < E_{0.15\%propolis}$, $E_{0.075\%propolis}$.

The ANOVA analysis, $\alpha = 0.05$, on the fracture strength, Table IV. 12, the amount of propolis is significant to the fracture strength as well as the interaction between media and amount of propolis. For PVA only samples, the fracture strength in acidic media is considerably higher than in PBS. In pH 4.0, higher the amount of propolis, lower the fracture strength. In both media, the lowest fracture strength was the one of 0.90% propolis samples.

In PBS, $\sigma_{F(0.90\%propolis)} < \sigma_{F(0.15\%propolis)}$, $\sigma_{F(0.075\%propolis)}$, $\sigma_{F(PVA)}$ and $\sigma_{F(0.15\%propolis)} < \sigma_{F(0.45\%propolis)}$ The highest σ_F value was that of 0.45% propolis samples and the lowest one is that of 0.90% propolis. In pH 4.0, $\sigma_{F(0.90\%propolis)} < \sigma_{F(0.15\%propolis)}$, $\sigma_{F(0.075\%propolis)}$, $\sigma_{F(PVA)}$ and $\sigma_{F(0.45\%propolis)} < \sigma_{F(0.15\%propolis)}$. In acidic pH, higher the amount of propolis, lower the fracture strength.

Table IV. 12 - two-way ANOVA analysis on the fracture strength (σF) of the samples swelled in the different media. Factors: amount of propolis, levels: 0.075, 0.15, 0.45 and 0.90% propolis; type of media, levels: PBS and solution pH 4.0.

	DF	Sum of squares	Mean Square	F value	P value
Media	1	9.59	9.59	0.94	0.33
% Propolis	4	444.55	111.13	10.95	2.07 E-7
Interaction	4	377.49	94.37	9.30	1.95 E-6
Model	9	838.36	93.15	9.18	4.95 E-10
Error	100	1014.06	10.14	--	--
Total	109	1852.43	--	--	--

Tukey Test	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
PBS-pH4	1.605	0.939	2.417	0.432	0.05	0	-1.003	4.214
PVA-0.075	0.585	0.949	0.871	0.972	0.05	0	-2.053	3.223
PVA-0.15	-1.020	0.949	1.519	0.819	0.05	0	-3.658	1.618
0.075-0.15	0.764	0.961	1.124	0.931	0.05	0	-1.906	3.434
PVA-0.15	-0.841	0.961	1.237	0.905	0.05	0	-3.511	1.828
0.075-0.45	0.178	0.971	0.260	0.999	0.05	0	-2.520	2.877
0.15-0.45	-4.254	0.961	6.260	2.334 E-4	0.05	1	-6.924	-1.584
PVA-0.90	-5.859	0.961	8.622	2.009 E-7	0.05	1	-8.530	-3.189
0.075-0.90	-4.839	0.971	7.045	2.576 E-5	0.05	1	-7.538	-2.140
0.15-0.90	-5.018	0.982	7.222	1.535 E-5	0.05	1	-7.748	-2.288
0.45-0.90	1.605	0.939	2.417	0.432	0.05	0	-1.003	4.214

d. Antimicrobial tests

The tests were carried out in PBS, so an one-way ANOVA analysis was used, where the factor was the amount of propolis and 5 levels were used (0 (PVA), 0.075%, 0.15%, 0.45% and 0.90% propolis), Table IV.13. The amount of propolis was significant to the *S. aureus* inhibition. There was a difference between the levels but to the pair PVA and 0.075% propolis and to the pair 0.15% and 0.45% propolis.

Table IV.13 - one-way ANOVA analysis on antimicrobial analysis. Factor: amount of propolis. Levels: 0 (PVA), 0.075%, 0.15%, 0.45% and 0.90% propolis.

S. aureus	**DF**	**Sum of squares**	**Mean Square**	**F value**	**P value**
Model	4	2.77 E6	693516.79	44811.80	1.43 E-11
Error	5	77.38	15.47		
Total	9	2.77 E6			

Tukey Test		**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
% Propolis	PVA-0.075	0	3.93	0	1	0.05	0	-15.78	15.78
	PVA-0.15	1023.50	3.93	367.93	0	0.05	1	1007.71	1039.28
	0.075-0.15	1023.50	3.93	367.93	0	0.05	1	1007.71	1039.28
	PVA-0.45	1023.50	3.93	367.93	0	0.05	1	1007.71	1039.28
	0.075-0.45	1023.50	3.93	367.93	0	0.05	1	1007.71	1039.28
	0.15-0.45	0	3.93	0	1	0.05	0	-15.78	15.78
	PVA-0.90	1163.17	3.93	418.14	0	0.05	1	1147.39	1178.95
	0.075-0.90	1163.17	3.93	418.14	0	0.05	1	1147.39	1178.95
	0.15-0.90	139.67	3.93	50.20	1.69 E-6	0.05	1	123.88	155.45
	0.45-0.90	139.67	3.93	50.20	1.69 E-6	0.05	1	123.88	155.45

e. Cytotoxicity tests

Table IV. 14 - One-way ANOVA analysis on the cytotoxicity results. Factor: amount of propolis. Levels: (-) control – subconfluent wells, (+) control – empty wells, 0 (PVA), 0.075, 0.15, 0.45 and 0.90% propolis samples' extracts.

		DF	Sum of squares	Mean Square		F value		P value
Model		6	36098.22	6016.37		126.77		2.19 E-11
Error		14	664.39	47.45				
Total		20	36762.62					
Tukey Test		**MeanDiff**	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
% Propolis	(+) / (-)	-99.69	5.62	25.06	1.43 E-7	0.05	1	-118.89	-80.48
	(-) / PVA	-21.44	5.62	5.39	0.02	0.05	1	-40.64	-2.23
	(+) / PVA	78.24	5.62	19.67	4.61 E-8	0.05	1	59.04	97.45
	(-) / 0.075	-94.72	5.62	23.81	9.26 E-7	0.05	1	-113.93	-75.51
	(+) / 0.075	4.96	5.62	1.24	0.96	0.05	0	-14.24	24.17
	PVA / 0.075	-73.28	5.62	18.42	5.57 E-8	0.05	1	-92.49	-54.07
	(-) / 0.15	-102.39	5.62	25.74	4.27 E-8	0.05	1	-121.60	-83.19
	(+) / 0.15	-2.70	5.62	0.68	0.99	0.05	0	-21.91	16.49
	PVA / 0.15	-80.95	5.62	20.35	1.19 E-7	0.05	1	-100.16	-61.75
	0.075 / 0.15	-7.67	5.62	1.92	0.81	0.05	0	-26.87	11.53
	(-) / 0.45	-103.37	5.62	25.98	3.90 E-6	0.05	1	-122.57	-84.16
	(+) / 0.45	-3.67	5.62	0.92	0.99	0.05	0	-22.88	15.52
	PVA / 0.45	-81.92	5.62	20.59	1.21 E-7	0.05	1	-101.13	-62.72
	0.075 / 0.45	-8.64	5.62	2.17	0.72	0.05	0	-27.85	10.56
	0.15 / 0.45	-0.97	5.62	0.24	1	0.05	0	-20.17	18.23
	(-) / 0.90	-106.40	5.62	26.75	4.18 E-8	0.05	1	-125.61	-87.19
	(+) / 0.90	-6.71	5.62	1.68	0.88	0.05	0	-25.92	12.49
	PVA / 0.90	-84.96	5.62	21.36	1.27 E-7	0.05	1	-104.16	-65.75
	0.075 / 0.90	-11.67	5.62	2.93	0.41	0.05	0	-30.88	7.52
	0.15 / 0.90	-4.00	5.62	1.00	0.98	0.05	0	-23.21	15.19
	0.45 / 0.90	-3.03	5.62	0.76	0.99	0.05	0	-22.24	16.17