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Preparation and characterization of composite materials for medical applications

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<u>ABSTRACT</u>

This work is focused on the development and characterization of polymeric-ceramic hydrogels with hydroxyapatite, a mineral widely used investigated for dentistry and drug delivery systems applications.

In practice, the project is divided in two phases: firstly, selection of composition of composite matrix based on mechanical studies and, secondly, modification and characterization of the best composite with hydroxyapatite, checking its impact on mechanical properties and sorption capacity of the samples in simulated body fluids.

As a result of these studies, it is possible to conclude than an equal composition of the matrix PVP-histidine presents the best mechanical properties.

Moreover, addition of hydroxyapatite turns the material much less elastic and polymer breaks with almost any effort. At the same time, the additive doesn't exhibit any effect over swelling property, producing elution in inorganic solutions. Conversely, HAp affects to the pH of solution, keep it stable by acting as a buffering agent.

At the end, the main possible application for the polymer would be the tissue engineering scaffolds, being necessary, however, perform more studies for prove its aptitude

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1 Purpose

Unlike another fields, materials engineering has two ways to developing: it's possible to find a material for covering a specific need or looking for applications for a material that was already developed.

This research is focus on the second way, where a polymer-ceramic composite will be created and characterized.

The project is contextualized in the work carried out by Dr hab. inż. Agnieszka Sobczak-Kupiec, whose research focuses on composites containing hydroxyapatite that can be applied in such areas as dentistry. Modification of such materials with active substances also can result in materials used for drug delivery.

So, the purpose is, at the beginning, selection of composition of composite matrix based on mechanical studies and next, modification and characterization of the best composite with hydroxyapatite, checking its impact on mechanical properties and sorption capacity of the samples in simulated body fluids.

2 Introduction

2.1 Biomaterials science

Medicine is a field with a constant developing and requires properties than only materials science can provide sometimes.

That is how biomaterials field was defined around 70 years ago: synthetic and natural materials used in implants and devices than contact biological systems (Ratner 2013).

The magnitude of the biomaterials and healthcare market is huge and growing: for 2013, the world medical devices market forecast was around \$286 billion. It becomes in a very interested and profitable field that has as integral subjects the next topics (Ratner 2013):

- Toxicology
- Biocompatibility
- Inflammation and healing
- Functional tissue structure and pathobiology
- Dependence on specific anatomical sites for implantation
- Mechanical requirements and physical performance requirements
- Industrial involvement
- Risk-benefit and corporate realities
- Ethics

- Regulations

In conclusion, biomaterials science is a complex and new field where there is still a lot of research to do.

2.2 Biomaterials

2.2.1 History of biomaterials

The introduction of non-biological materials into the human body appeared a lot of centuries ago, being one of the first proofs a spear point in the hip of a 9000 years old human remains. This case shown how the body can "tolerate" some estrange materials and continue with its normal activity (Ratner 2013).

Moreover, sutures were used since Neolithic period with a big variety of raw along the time: line, catgut, metals…

Real applications appeared some time ago, in different early civilizations as Mayans (600 AD) or Gauls (200 AD), where dental implants has been discovered made with sea shells or iron.

From there, a lot of small concepts and applications had being developing, like contact lenses, started with the idea of Da Vinci in 1508 and finally developed successfully for first time by Adolf Gaston Eugen Fick roughly 1860 (Dumitriu 2002).

But the first big change of mind about biomaterials arrived with the World War II, when newly developed high performance metal, ceramic, and especially polymeric materials, transitioned from wartime restriction to peacetime availability. These early biomaterials included silicones, polyurethanes, Teflon, nylon methacrylates, titanium and stainless steel (Ratner 2013).

However, the real advance in this field was in the 60's decade, with the designed biomaterials. Where materials were developed specifically for biomaterials applications for first time, becoming engineered materials. Some key classes were: silicones, polyurethanes, Teflon, hydrogels, poly(lactic-glycolic Acid), titanium, hydroxyapatite or bioglass (Ratner 2013).

2.2.2 Slightly review of medical devices materials

As the properties that biomaterials are quite demanding in some aspects, the amount of materials suitable for medical applications is small (Dossett und Totten 2002):

- **Metals:** even metals would be the easier option in some fields like orthopaedics, the inherent corrosion of the most of the metals limits the possibilities to certain stainless steels and heat-resisting cobalt-base alloys. Due to its lower elastic modulus, titanium

alloys have become a good choice, but the mechanical properties (hardness, wear resistance) are lower too.

- **Ceramics:** not too many ceramics have place in this kind of devices. Zirconia, alumina and sometimes vitreous (amorphous) carbon. A few glasses have limited applications. The main ceramic material for medical proposes is hydroxyapatite (bone mineral) which will be explained in detail in another chapter.
- **Polymers:** a major attraction of polymers is that they are a class of materials which have structures and densities similar to that of the tissues of the body. The potentially infinite numbers of structures that can be synthesized and the represent desirable are for development and research.

2.3 Hydrogels

2.3.1 Definition

Hydrogels are three-dimensional polymeric networks that have a solid-like appearance, formed by two or more components, one of which is a liquid present in high quantity. The capability of hydrogels to swell in water is due to the hydrophilic groups present in the polymer chains, while its mechanical resistance is due in part to the physical or chemical network cross-linking (Martínez-Ruvalcaba et al. 2009). Most biomedical hydrogels contain ionisable pendant groups, which can be partially or completely dissociated in solution (Barbucci 2010).

Hydrophilic gels have call the attention of the science community over the last 50 years, thanks to their exceptional promise in wide range of applications (Ahmed 2015). The structure and properties of a specific hydrogel are extremely important in selecting which material is suitable for the specific application. In fact, due to their wonderful properties, hydrogels can be used for microfluidic control, biomimetic biosensor/bioactuator, separation process and artificial muscle (Barbucci 2010; Li 2010).

Another exceptional promise of the hydrogels are their biocompatibility and biostability potentials, by which the hydrogels become excellent substitution for the human body tissues. There are also extensive explorations of the hydrogels in the medical and pharmaceutical applications, such as drug delivery systems, articular cartilage, biomaterial scaffold, dentistry, corneal replacement and tissue engineering (Li 2010).

2.3.2 Classification

Different classifications are possible for hydrogels. The most intuitive one talks about the type source used: if hydrogel is obtained from natural products or synthetics materials (Dumitriu 2002). As synthetic hydrogels have longer service life, they have been replacing naturals during the last two decades (Ahmed 2015).

However, another classifications are intrinsically more interesting and all them are shown in the next table Table 1 from (Dumitriu 2002)

Table 1. Classification of hydrogels (Dumitriu 2002)

Classification	Contents
Component	Homopolymer
	Copolymer
	Multipolymer
	Interpenetrating network
Preparation method	Simoultanious polymerization
	Crosslink of polymer
Electric charge	Nonion
	Anion
	Cation
	Zwitter ion
Physical structure	Amorphous
	Semicrystaline
	Hydrogen bonded
Crosslink	Covalent bond
	Intermolecular force
Functions	Biodegradable
	Stimuli responsive
	Superabsorbent
	Etc.

Depending on the **components**, it's possible to find four kinds (Ratner 2013; Ahmed 2015):

- **Homopolymer hydrogels**, made with one type of polymer.
- **Copolymer hydrogels,** cross-linked with two comonomer units in a random, block or alternating configuration.
- **Multi-polymer hydrogels,** composed with three or more comonomers.
- **Interpenetration network hydrogels**.

Introduction

The crosslinking method is one of the most important classifications because refers to the ways of preparation and the molecular forces involved at the same time. This way, in **chemical crosslinking**, functional groups bonds form insoluble gels due to the use of crosslinking agent or radiation. Conversely, **physical crosslinks** create intermolecular forces bonds such as van der Waals (Dumitriu 2002) .

2.3.3   Synthesis

In the most of situations, water is part of the initial formation of the crosslinked structure. There are three types of synthesis methods: irradiation, chemical reactions and physical association.

The important one for this project is the radiation. Two water-soluble polymers may be cross-linked together by formation of free radicals on both polymer molecules, which combine to form the cross-link. Ionizing radiation methods utilize electron beams, gamma rays or X-rays to excite a polymer and produce a cross-linked structure via free radical reactions on unsaturated compounds. Covalent bonds are generated through the recombination of macro-radicals on different chains, as it is possible to observe in Figure 1 (Caló und Khutoryanskiy 2015).
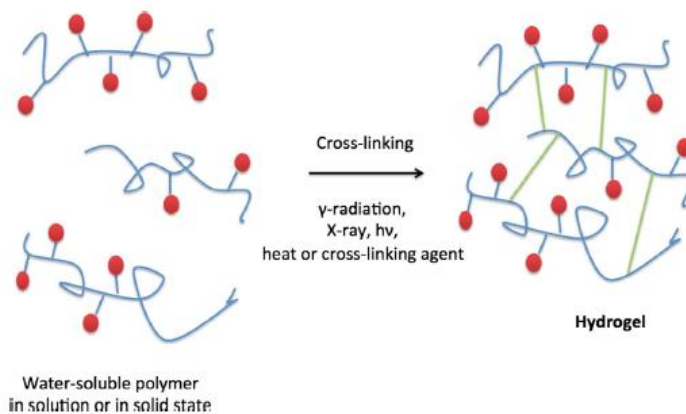
Figure 1. Synthesis of hydrogels by cross-linking of ready-made water-soluble polymers (Caló und Khutoryanskiy 2015)

With this type of initiation, comparing with the chemical initiation, it is possible to obtain a purer and initiator-freer hydrogels. (Ratner 2013; Ahmed 2015). Another advantage of radiation induced cross-linking is that this process performance the generation of the hydrogel and its sterilization at the same time (Caló und Khutoryanskiy 2015).

From all the possible synthesis methods, the one that concerns to this project applies UV light as photoinitiator.

Selecting the appropriate source, a big variety of hydrogels can be produced, existing three kinds of polymers (Dumitriu 2002):

- **Neutral**. With neutral monomers and polymers, hydrophilic or hydrophobic groups may be available. Even the most of them are hydrophilic, some hydrophobic portions are added to improve the mechanical strength
- **Acidic or anionic**. This type will minimize calcification when the hydrogels are implanted

- **Basic or cationic**. The positive charge of cationic hydrogels will make anions more permeable.

Ideal networks (with covalent-bond tetra-functional cross-links) are very difficult to obtain and different defects can appear. In real networks, multifunctional junctions or physical molecular entanglements might show up (Ratner 2013) as it shown in Figure 2
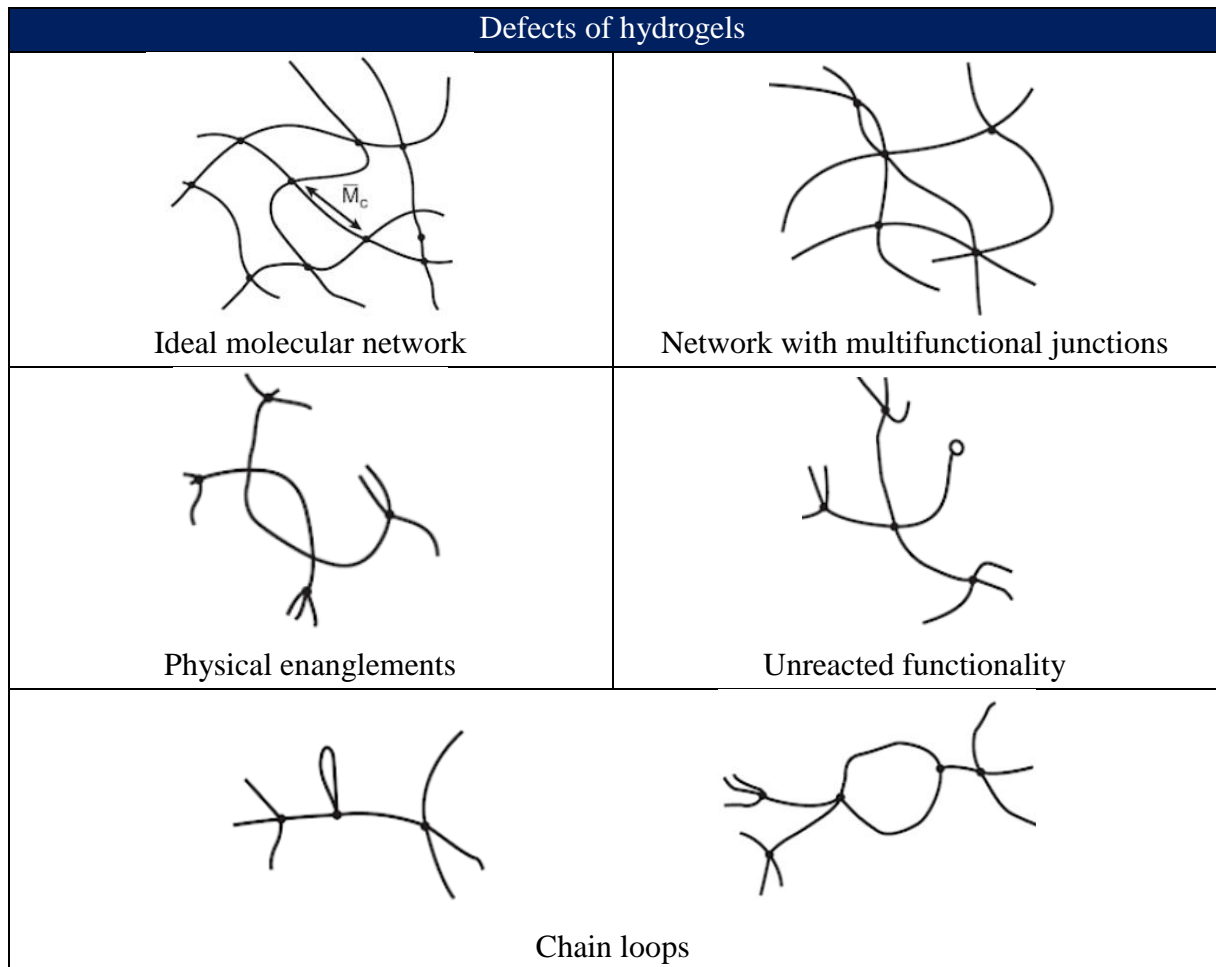
Figure 2. Defects of hydrogels (Ratner 2013)

2.3.4 Properties

<u>Swelling property</u>

The most basic theory for the performance of hydrogels is given by the Flory rubber elasticity theory, which says that, for one hand, the polymer chains of hydrogels try to expand to the complete solvated state by interacting with the solvent molecule. At the same time, the cross-linked structure reacts with a retroactive force to pull back the polymer chain inside. It is this counterbalance of the expanding and reacting force what remains the equilibrium (Dumitriu 2002).

Introduction

The swelling behaviour is described by the swelling ratio or the water content in the most of the cases, which are expressed like (Dumitriu 2002):

$$swelling\ ratio = \frac{weight\ of\ swollen\ gel}{wieght\ of\ dry\ gel}$$

$$water\ content = \frac{weight\ of\ water}{weight\ of\ water + wieght\ of\ dry\ gel} \times 100$$

When the water content is higher than 90%, the material is called superadsorbent hydrogel (Dumitriu 2002).

Hydrogels are called "reversible" or "physical" gels if molecular entanglement and/or secondary forces such as ionic, H-bonding or hydrophobic forces play the main role in forming the network. Physical gels are often reversible and it is possible to dissolve them by changing environmental conditions, such as pH, and the ionic strength of solution or temperature. In "permanent" or "chemical" gels, the network of covalent bonds joining different macromolecular chains can be achieved by cross-linking polymers in the dry state or in solution. There gels may be charged or non-charged depending on the nature of functional groups present in their structure. Charged hydrogels usually exhibit changes in swelling upon variations in pH, and it is known that they can undergo changes in shape when exposed to an electrical field (Caló und Khutoryanskiy 2015).

The desired swelling properties can be achieved through the combination of a basic hydrophilic monomer and others more or less hydrophilic monomers. Knowledge of the swelling characteristics of a polymer is of utmost importance in biomedical and pharmaceutical applications since equilibrium degree of swelling influences:

- The solute diffusion coefficient through these hydrogels
- The surface properties and surface molecule mobility
- The optical properties
- The mechanical properties

Permeability

The permeability of target molecules in hydrogels allows them to be used in a lot of medical applications: releasing of drugs and proteins for drug delivery systems, oxygen permeation for contact tens, etc (Dumitriu 2002).

Technical features

The ideal hydrogel would present the next list of functional features (Ahmed 2015):

- The highest absorption capacity (maximum equilibrium swelling) in saline.
- Desired rate of absorption (preferred particle size and porosity) depending on the application requirement.
- The highest absorbency under load (AUL).

- The lowest soluble content and residual monomer.
- The lowest price.
- The highest durability and stability in the swelling environment and during the storage.
- The highest biodegradability without formation of toxic species following the degradation.
- pH-neutrality after swelling in water.
- Colourlessness, odourlessness, and absolute non-toxic.
- Photo stability.
- Re-wetting capability (if required) the hydrogel has to be able to give back the imbibed solution or to maintain it; depending on the application requirement (e.g., in agricultural or hygienic applications).

Another properties

As a small rheological review, when the entanglement between polymers in low or intermediate concentration on water solutions is weak, the compound has a Newtonian behaviour. However, as higher is the cross-linked network concentration, as high is the elasticity, arriving to viscoelastic or even purely elastic behaviour (Akhtar et al. 2016).

The most important disadvantage of this material is its poor mechanical stability at swollen state (Kamoun et al. 2017).

such as reversible swelling/deswelling behaviour, sorption capacity, novel mechanical property, high ionic conductivity, high environmental sensitivity or permeability)4

2.3.5   Smart hydrogels

Even every hydrogel exhibits a different swelling behaviour depending on the external responses, some of them present a significant change in this property compering with others. This sensitive gels are called stimuli-responsive hydrogels (Ratner 2013).

Depending of the type of hydrogel, the stimuli for generating a swelling response will be different. Physical gels will response to temperature, electric field, magnetic field, light, pressure and sound. In the other hand, chemical hydrogels will perform in front of pH variations, ionic strength, solvent composition and molecular species (Ahmed 2015), as it is shown in Figure 3
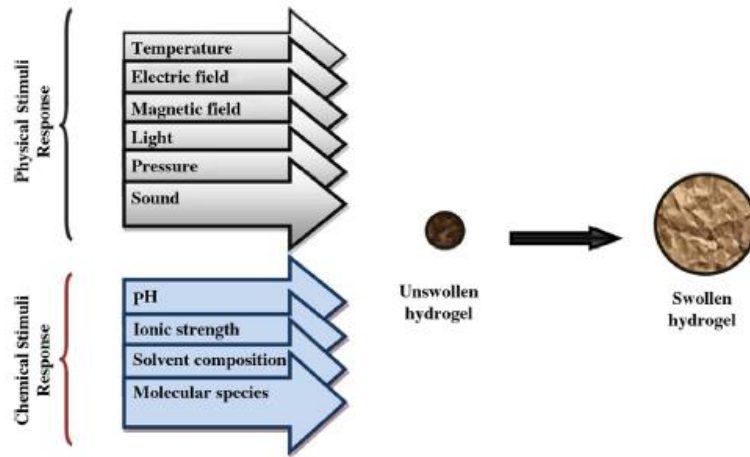
Figure 3. Stimuli response swelling hydrogel (Ahmed 2015)

2.3.6 Applications

Since water is the greatest component of the human body, a hydrogel, which can absorb large quantities of water, is considered to have great potential when applied for biomedical purposes.

The structure and properties of a specific hydrogel are extremely important in selecting which material is suitable for the specific application. In fact, due to their wonderful properties (such as reversible swelling/deswelling behaviour, sorption capacity, novel mechanical property, high ionic conductivity, high environmental sensitivity or permeability), hydrogels can be used for microfluidic control, biomimetic biosensor/bioactuator, separation process and artificial muscle (Barbucci 2010; Li 2010).

Another exceptional promise of the hydrogels are their biocompatibility and biostability potentials, by which the hydrogels become excellent substitution for the human body tissues. There are also extensive explorations of the hydrogels in the medical and pharmaceutical applications, such as drug delivery systems, articular cartilage, biomaterial scaffold, dentistry, corneal replacement and tissue engineering (Li 2010).

For example, in recent years, injectable hydrogels have emerged as a promising biomaterial for therapeutic delivery of cells and bioactive molecules for tissue regeneration in dentistry and medicine because of their tunable tissue-like properties, controllability of degradation and release behaviour, adaptability in a clinical setting for minimally-invasive surgical procedures, and ability to conform to the three-dimensional (3-D) defect upon gelling (Seong Toh 2014).

Lubricant

Hydrogels are applied in dry surfaces of biomaterials for lubricating. Some of these applications are the dry surface of gloves or the drainage tubes used to evacuate collections of

fluid within body cavities. If the surface is not slippery enough, the mechanical friction between the tube and the body tissue may cause injuries (Dumitriu 2002).

Contact lens

A contact lens is a medical device to correct the optical function of the eye with intimate contact to the eye. There are two types of contact lenses according to their elasticity: soft (flexible) and hard contact lenses and hydrogels are used for producing the first type, where the cross-linking level is very low. Even hard lenses have longer service life, they used to be wide less accepted by the wearers and require a lengthier adaptation period. That is why soft contact lenses gained popularity fast (Caló und Khutoryanskiy 2015): flexible lenses are more comfortable and easier to fit. In the other hand, they are more easily damaged and require more hygienic care (Dumitriu 2002).

Hydrogel contact lenses may obtained with different methods such a mold-casting, spin-casting and lathe-cutting, being the last one the only one made by a free-radical initiators polymerization (Caló und Khutoryanskiy 2015).

The main characteristics that a polymeric hydrogel should show to be used at this application are (Caló und Khutoryanskiy 2015):
- Luminous transmittance
- Refractive index
- Sufficient oxygen-permeability
- Wettability and permeability to water
- Stability
- Excellent mechanical properties
- Biocompatibility

The first hydrogel for contact lenses were made of poly(2-hydroxyethyl methacrylate) (PHEMA) and it is still the most used material for contact lenses even when other hydrogels are been developed for the same application, such as N-vinyl-2-pyrrolidone (NVP) or silicon hydrogel (SiHy) (Caló und Khutoryanskiy 2015; Dumitriu 2002).

In addition to the applications of soft contact lenses in correction of vision, they can potentially be used for drug delivery to the eye. However, conventional hydrogel-based contact lenses exhibit relatively low drug loading capacity and often show a burst release upon ocular administration.

Developing safe and cost-effective contact lenses is the focus of the eye care industry. Contact lens materials with optimal characteristics such as oxygen permeability, comfort, compliance, hygiene and disinfection have still not been achieved, which opens exciting opportunities for further developments in this area.

Wound dressing

Introduction

A wound is a defect or a break in the skin which can result from trauma or medical/physiological conditions. Wounds can be classified, depending on the number of skin layers and on the area of the skin affected as (Caló und Khutoryanskiy 2015):

- **Superficial**. If only the epidermis is involved
- **Partial-thickness**. If the epidermis and deeper dermal layers are affected
- **Full-thickness wounds**. When subcutaneous fat and deeper tissue has been damaged
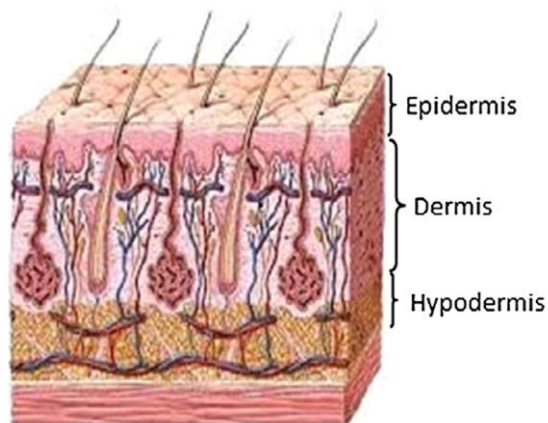
Figure 4. Schematic representation of normal skin structure (Ahmed 2015)

Design of effective dressings relies on an understanding of the healing process, as well as the specific conditions of a patient and the effect that each material used could have on the wound. Wound healing can be hindered by various factors such as desiccation, infection or abnormal bacterial presence, maceration, necrosis, pressure and trauma (Caló und Khutoryanskiy 2015).

The main characteristics of membranes as wound dressings to endorse the skin healing and to defend the skin defect zone from infection, have progressively investigated and applied in the clinical sector since early eighties.

The mechanism of hydrogels as wound dressings can be ascribed as follows (and it is shown in Figure 5). Hydrogels can absorb and retain the wound exudates, which promote fibroblast proliferation and keratinocyte migration. The last two processes are very necessary for complete epithelialization and healing of the wound. In addition the tight mesh size of hydrogels structure protects the wound from infection and prevents microorganism and bacteria to reach the wound area. However, hydrogels structure allows transporting bioactive molecules e.g. antibiotics, and pharmaceuticals to wound centre. Such molecules can be entrapped into hydrogel networks during gelling process, while these molecules can be exchanged with absorbing the wound exudates during the sustainable release process after contacting hydrogels with the wound surface. The significant tissue-like water content of hydrogels provides the needed flexibility and elasticity to adapt wounds located in different body sites (Caló und Khutoryanskiy 2015).
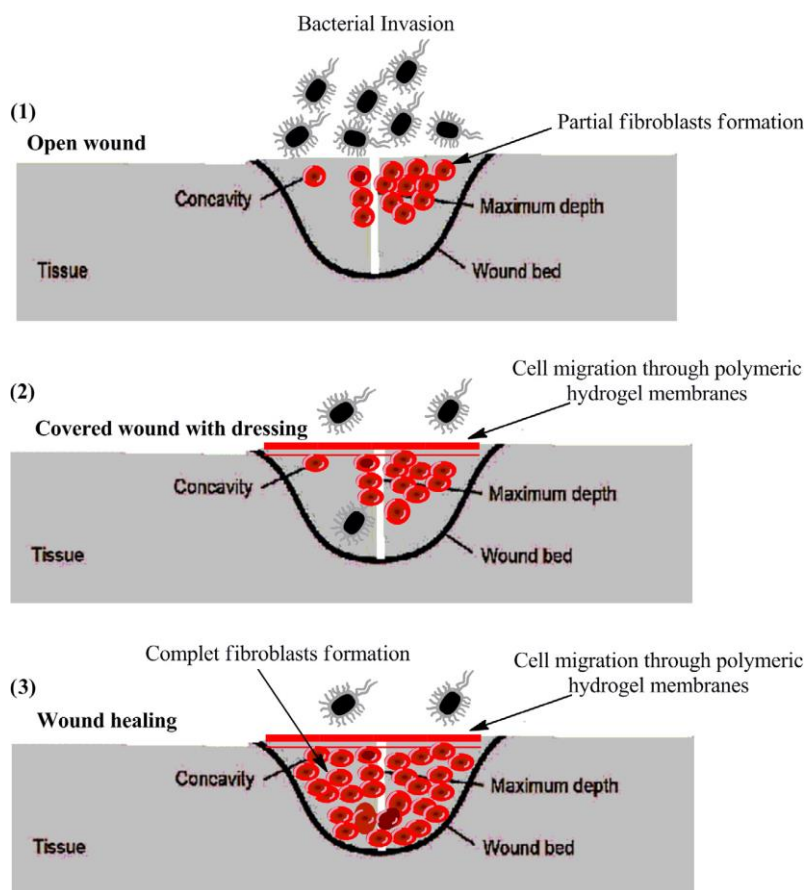
Figure 5. Schematic representation of the role of hydrogel membrane materials for enhancing and accelerating the wound healing phases (Kamoun et al. 2017)

The 'ideal' wound management product should absorb excess exudate and toxins, keep a good moisture between the wound and the dressing, preserve the wound from external sources of infection, prevent excess heat at the wound, have good permeability to gases, be supplied completely sterile and be easy to remove without further trauma to the wound (Caló und Khutoryanskiy 2015).

Recently, the wound dressing industry highlighted the importance of providing comfort and conformability of dressings, the need for infrequent changes, cost effectiveness and a long shelf life. The choice of the right dressing to suit a particular wound is therefore fundamental for optimum healing and the quality of life of the patient. The majority of the currently available products can be classified as low adherent dressings, semipermeable films, hydrocolloids, hydrogels, alginates, foam dressings or antimicrobial dressings. Although plain gauze is still one of the most commonly employed products in hospitals, new wound dressing research and development have produced advanced materials with better physical and chemical properties (Caló und Khutoryanskiy 2015).

Future developments in wound care products will depend on continued demands from public and healthcare professionals [79]. The important challenge for the future is to establish the appropriate wound care strategy for every single patient, and this can be achieved only by

offering the optimal products. Innovative dressings need to be developed while their production costs must be kept low (Caló und Khutoryanskiy 2015).

Drug delivery

Hydrogels are being considered as controlled release carriers of drugs and proteins because of their good tissue compatibility, easy manipulation under swelling condition and solute permeability. The high porosity that characterizes them can easily be adjusted by controlling the density of cross-links in their matrix and the affinity to water. Their porous structure also allows drugs to be loaded and then released. The advantages offered by hydrogels for drug delivery applications include the possibility for sustained release, which results in maintaining a high local concentration of an active pharmaceutical ingredient over a long period [9]. The drug can be loaded into a hydrogel and then its release may proceed through several mechanisms: diffusion controlled, swelling controlled, chemically controlled and environmentally-responsive release (Dumitriu 2002; Caló und Khutoryanskiy 2015).

The net effect of the swelling process is to prolong and linearize the release curve (Ratner 2013).

There are types of systems:
- **Rapidly swelling, diffusion-controlled devices**

  The diffusion controlled release systems can be represented by reservoir or matrix devices. Both allow the drug release by diffusion through the hydrogel mesh or the pores filled with water. A reservoir delivery system (Figure 6) includes a drug-containing core coated with a hydrogel membrane, commonly available as capsules, cylinders, spheres or slabs. The concentration of the drug is higher in the centre of the system to allow a constant release rate (Caló und Khutoryanskiy 2015).
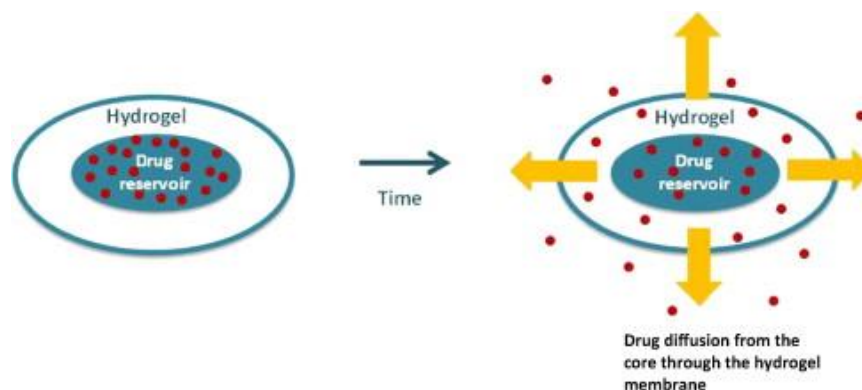
Figure 6. Scheme of drug release through a hydrogel membrane in a reservoir system (Caló und Khutoryanskiy 2015)

  In matrix systems the drug is dispersed or dissolved uniformly throughout the three-dimensional structure of the hydrogel (Figure 7). Drug release is achieved through the macromolecular mesh or the pores, and the initial release rate in this case

is proportional to the square root of time, rather than being constant and time independent as happens in reservoir systems (Caló und Khutoryanskiy 2015).
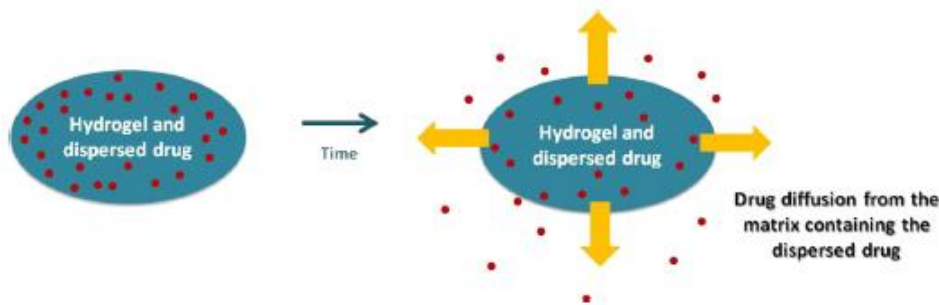
Figure 7. Drug release from matrix systems (Caló und Khutoryanskiy 2015)

- **Slowly swelling, swelling-controlled devices**

    In swelling controlled systems, the bioactive agent is dispersed into the polymer to form nonporous films, disks, or spheres. Upon contact with an aqueous dissolution medium, a distinct front (interface) is observed that corresponds to the water penetration front into the polymer and separates the glassy form the rubbery (gel-like) state of the material. Under this conditions, the macromolecular relaxation of the polymer influences the diffusion mechanism of the drug through the rubbery state. This water uptake can lead to considerable swelling of the polymer, with a thickness that depends on time (Ratner 2013).

In both cases, an initial burst effect is frequently observed in matrix devices, especially if the drying process brings a higher concentration of drug in the surface (Ratner 2013).

The hydrogel-based dosage forms can have different designs and shapes depending on the route of drug administration (Caló und Khutoryanskiy 2015):

- Peroral: spherical beads, discs, nanoparticles
- Rectal: suppositories
- Vaginal: vaginal tablets, torpedo-shaped pessaries
- Ocular: contact lenses, drops, suspensions, ointments, circular inserts
- Transdermal: dressings
- Implants: discs, cylinders

Novel biorecognizable sugar-containing copolymers have been investigated for use in targeted delivery of anti-cancer drugs (Ratner 2013).

Tissue engineering

Tissue engineering involves the improvement or replacement of specific tissues or organs using engineered materials and synthetic strategies. Combination of cells and a immunoisolative membrane forms biohybrid organs, which become a permanent part of the

host organ by acting as the functional analog of the original organ by continuously supporting the organ. (Caló und Khutoryanskiy 2015; Dumitriu 2002).

High water content gels that may be synthesized with degradable backbone polymers, with added advantage of being able to attach cell adhesion ligands to the network polymer chains, are very attractive for this field (Ratner 2013).

Tissue engineering is a more recent application of hydrogels, in which they can be applied as space filling agents, as delivery vehicles for bioactive substances or as three-dimensional structures that organize cells and present stimuli to ensure the development of a required tissue (Figure 8). Drugs can be delivered from hydrogel scaffolds in numerous applications including promotion of angiogenesis and encapsulation of secretory cells. Additionally, hydrogel scaffolds have also been applied to transplant cells and to engineer many tissues in the body, including cartilage, bone, and smooth muscle. Hydrogels scaffolds are used for cell-sheet and tissue production (Caló und Khutoryanskiy 2015).
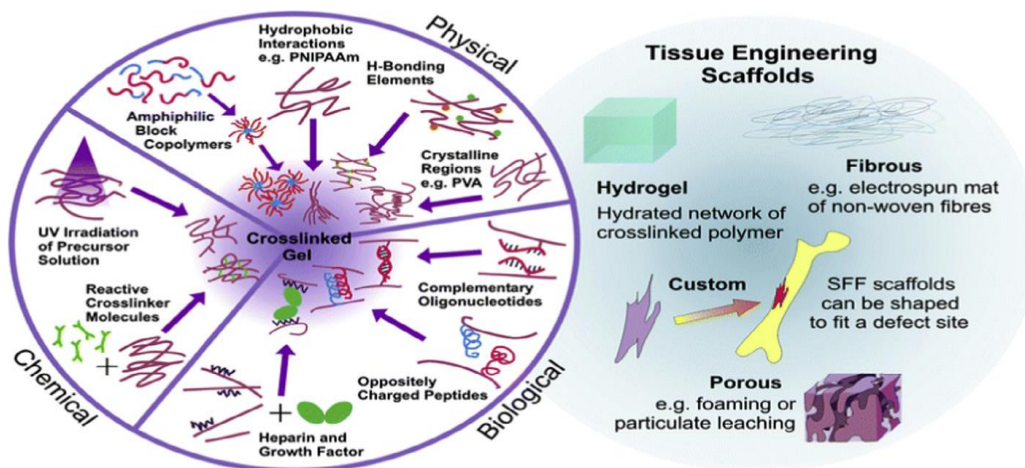
Figure 8. Hydrogels in tissue engineering (Caló und Khutoryanskiy 2015)

One very interesting observation with hydrogels that has recently been reported is that they may stimulate stem cell differentiation; that is, when stem cells are deposited on some hydrogel surfaces, depending on the composition and/or mechanical stiffness of the surface, differentiation of the stem cells into certain phenotypes may occur (Ratner 2013)

However, successful tissue regeneration by the use of growth factors has not always been achieved. One of the reasons for this is the very short half-life periods of growths factors in the body to sustain biological activities. Thus, it is highly necessary to contrive the dosage of growth factors for enhancing the in vivo efficacy (Dumitriu 2002).

One of the most important future challenges in tissue engineering is how polymers could be used to stimulate the blood vessel network formation in the desired tissue, essential to supply its needs. Hydrogels could represent a valid option to effectively control the vascularization process, by local delivery of both angiogenic factor and endothelial cells to the intended area [120]. Additionally, many types of tissue such as bone, muscle or blood vessels are located in areas requiring excellent mechanical properties that the majority of the currently available hydrogels do not show, so new approaches should be investigated in the future to achieve better results (Caló und Khutoryanskiy 2015).

3 Materials

For understanding what is happening during the investigation and giving good conclusions, it is important first to know the materials we are going to use. In general, there are seven: three of them for preparing the composite and another four (solutions) for studying its properties.

3.1 Materials for preparing the polymer

3.1.1 Polyvinylpirrolidone (PVP)

The polyvinylpirrolidone (PVP or polyvidone) is one of the most used polymers in the world. As it will be explained later, its special properties make this material perfect for a lot of applications where the non-toxicity and biocompatibility are need like the medical fields.

Polyvidone is soluble in a plenty organic solvents (including alcohols, some chlorinated compounds, nitroparaffins, and amines) and water as is shown in the (Teodorescu und Bercea 2015):

Table 2. Solubility of PVP in various solvents (Teodorescu und Bercea 2015)

Soluble in		Insoluble in	
Water	Di(ethylene glycol)	Ethyl acetate	Carbon tetrachloride
Methanol	Poly(ethylene glycol)	Acetone	Light petroleum
Ethanol	Propylene glycol	Dioxane	Toluene
Propanol	1,4-Butanediol	Pentane	Xylene
Butanol	Glycerol	Cyclohexane	Liquid paraffin
Cyclohexanol	N-Vinylpyrrolidone	Cyclohexanol	Mineral oil
Chloroform	Triethanolamine		
Dichloromethane	Formic acid		
1,2-Dichloroethane	Acetic acid		
N-Methylpyrrolidone			
Ethylenediamine			

Its solubitily is so good that the applicationin practical purposes is only restricted by the resulting viscosity of the solution and it is due to the fact that PVP has hydrophilic as well as hydrophobic functional groups (Bühler 2005; Schwarz 1990).

The viscosity of PVP aqueous solutions is only slightly changed over a wide pH range. However, viscosity increases in concetrated hydrochloric acid, and in concetrated nitric acid PVP dorms stable gel (Schwarz 1990; Teodorescu und Bercea 2015; ASHLAND 2014).

The enthalpy of solution is -4,81 kJ/mol and the pH in water solution is between 4 and 5 (slightly acid) and quite stable (Teodorescu und Bercea 2015; Schwarz 1990).

Because of its unique chemical nature, PVP would be expected to be biologically inert apart from exerting osmotic activity. A large number of animal and human studies support the metabolic inertness of this polymer and hence, the safety of PVP (ASHLAND 2014).

During World War II, PVP was used as a blood plasma extender making a saline solution with it for the army (Sandler 2012). In the early 50's it was successfully used for first time in hairsprays (Polymer Science Learning Center).

Some of the applications of this polymer are: foods and beverages, cosmetics and toiletries, photographic products, dyeing applications and inks, detergents and surfactants, suspensions and emulsions, production of plastics, adhesives, paper auxiliaries or membranes (Schwarz 1990; Acton 2013).

Pharmaceutical industry and medicine are the domains that benefit more of the polyvidone because of its excellent biocompatibility and capability to form stable association compounds and complexes with many active substances (Teodorescu und Bercea 2015): tabletting auxiliary, coatings for medical devices, contact lenses, orthopaedics, antiseptics, cardiology, biomedical diagnostics, oncology or gene therapy

Focusing on drugs delivery applications, chitosan blends with PVP were investigated for oral gingival delivery system, showing miscibility in all ratios studied, compared to the another polymers, its range of compositions for a good miscibility was smaller (Adikwu 2010).

At the same time, PVP has been tried in blends for wound healing (biological process that involves tissue repair and regeneration) improving the effects of the medicaments combining them (Adikwu 2010).

In dentistry, PVP bioadhesive films were developed for the buccal delivery of fentanyl. Researchers concluded that buccal PVP films of only 1–2 cm^2 surface area provide a therapeutic effect equivalent to a 10 cm^2 transdermal patch, with a much shorter lag-time making these systems more advantageous (Teodorescu und Bercea 2015).

3.1.2 Histidine

Histidine is an amino acid. Amino acids are made up of a weak acid molecule group – a chemical fragment containing carbon, oxygen and hydrogen- in conjunction with a strong basic animo molecule group – chemical fragment containing nitrogen (which make it able to repair and built tissue, for example). The mild basic or acidity of amino acids is too minimal to affect acid-basic balance in the body, which is preserved by multitudes of protective buffer systems (Braverman et al. 2003; WebMD 2005).

Histidine is an essential amino acid abundant in hemoglobin that is required for the growth and repair of tissue. It is also crucial for the survival of premature babies. Children and adults can make small amounts of histidine in their bodies, but most of the histidine processed in the body must come from the food. Inadequate levels of histidine are found in the blood of individuals with rheumatoid arthritis (Braverman et al. 2003).

One of the most important function of histidine in the body is its conversion to histamine, which can take place in many parts of the body. It is a potent vessel dilator that plays a central role in allergic reactions and other functions of the immune system. It acts as a major neurotransmitter in the brain, especially in the hippocampus, and throughout the autonomic nervous system, and also as a stimulator to the secretion of pepsin and hydrochloric acid, which are important for digestion (Barnes und Gray 2003).

Histidine has mild anti-inflammatory properties; it helps bind with trace minerals and copper, thus aiding their removal from the body.

This amino acid is a generally considered to be a polar amino acid, however it is quite unique with regard to properties, meaning that it does not particularly substitute well with any other amino acid (Braverman et al. 2003).

Histidine has a pKa near to that of physiological pH, meaning that it is relatively easy to move protons on and off of the side chain (i.e. changing the side chain from neutral to positive charge). This flexibility has two effects. The first is that it means histidine is rather ambiguous about whether it prefers to be buried in the protein core, or exposed to solvent. The second is that it is an ideal residue for protein functional centres (University of Arizona 2003).

3.1.3   Hydroxyapatite (HAp)

Hydroxyapatite is an inorganic compound, the most widely used member of the family of calcium orthophosphates (CaPs), biocompatible ceramics present in bones, teeth, deer antlers and the tendons of mammals, giving these organs hardness and stability. There are eleven known non-ion-substituted calcium orthophosphates with a Ca/P molar ratio between 0.5 and 2.0. This is known as stoichiometric hydroxyapatite and its atomic ratio Ca/P is 1.67. Its chemical formula is $Ca_{10}(PO_4)_6(OH)_2$, with 39% by weight of Ca, 18.5% P and 3.38% of OH. (Šupová 2015; Rivera-Muñoz 2011) (
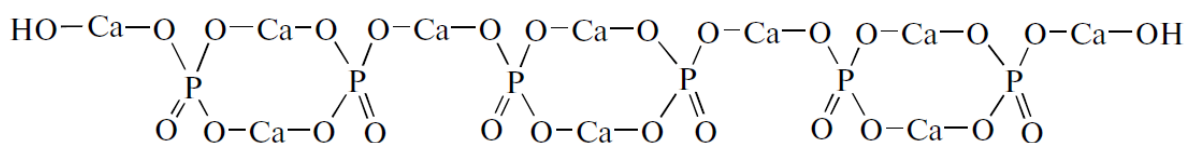
Figure 9. Hydrosyapatite (Šupová 2015)

An important characteristic of hydroxyapatite is its stability when compared to other calcium phosphates. Thermodynamically, hydroxyapatite is the most stable calcium phosphate compound under physiological conditions as temperature, pH and composition of the body fluids (Rivera-Muñoz 2011)

Another aspect we must consider is the degree of crystallinity. It has been observed that the crystallinity in the tissues for the tooth enamel is very high, while in the cases corresponding to dentin and bone, it is very poor. This means that the reactivity depends on the degree of crystallinity, since the reactivity in dentin and bone is higher than in tooth enamel (Rivera-Muñoz 2011).

Materials

Hydroxyapatite bioceramic (HAp) has the highest biocompatibility of all synthetic inorganic implant materials. Because of the similarity to chemical composition of natural apatite in bone, hydroxyapatite does not irritate the surrounding tissue, does not cause acute or chronic inflammation, instead it stimulates bone repair processes, which enable the creation of chemical bond at the implant-bone interface (Sobczak-Kupiec et al. 2018).

Hydroxyapatite bioceramic material can be assigned to nontoxic and biologically active materials because it is characterized by a unique bioactivity e the surface layer of hydroxyapatite reacts with a physiological medium and creates chemical bonds between the implanted material and the bone. Hydroxyapatite stimulates intense bone ingrowth into bone pores and provides the biological stability of the reconstructed tissues by osteoconductive action (Sobczak-Kupiec et al. 2018).

Hydroxyapatite is a topic for itself: the bibliography is very wide and the interest for the investigation with this additive is still growing. Some of the applications are (Mucalo 2015):
- Bone fillers
- Bone tissue engineering sacffolds
- Bioactive coatings
- Hard and soft tissue repairs
- Rapid fractionation of proteins, nucleic acids and antibodies
- HAp materials are also potential candidates for use in fluorescence labelling, imaging and diagnosis materials, etc.
- Cell targeting

Focus on drug delivery applications, HAp materials can serve as carriers for drug/protein delivery and gene therapy due to their excellent biocompatibility, easily tunable physical-chemical properties (size, morphology, porous structure or surface composition), low toxicity, low production cost, excellent storage stability, inertia to microbial degradation and pH-dependent dissolution (Mucalo 2015).

With the decrease of the pH value from alkaline to acidic conditions, the degradation rate of HAp increases apparently, which accelerates the release of the drug molecules from the HAp surfaces. The feature of the pH-dependent dissolution property is particulary interesting since pH of about 7,4 is observed in normal tissues, and the values about 5 are found in extracellular environments of solid tumours (Mucalo 2015).

As the acidic gastric environment can degrade HA, it is mainly used as a skeletal drug delivery system in bone disorders (such as osteoporosis, bone tumours) rather than oral therapeutic systems. Porous HA loaded with the appropriate drug allows its precise delivery to the affected area. Hydroxyapatite is mainly used to deliver directly to hard tissues antibiotics and also other drugs, such as anti-cancer substances, anti-inflammatory drugs, anti-osteoporotics substances and others, like vitamins, hormones, proteins, and growth factors. The local and sustained release of drugs allows shortening of the prolonged therapies and can accelerate the bone healing process, as well minimize the extent of surgical removal of the affected bone.(Szcześ et al. 2017)

In dentistry is being used, for example, for remineralization of early caries lesion combined with NaF, due to the similarities of HAp and the teeth composition, increasing the degree of remineralization comparing with tradition methods (Kim et al. 2007)

But not only for regeneration. HAp is used too for prevention and restorativation. In a research, after a few days, the nano-HA/fluorapatite added to cement showed greater hardness to compression, a higher hardness to diametrical tension and a higher hardness to biaxial flexibility, compared to the control group (Pepla et al. 2014).

But HAp is not perfect and presents some important weaknesses:
- Low mechanical properties
- Low degradation rate
- Lack of osteinductivity, which occurs in materials with high affinity with bone tissue, which promote the formation of new tissue, but are also capable of directing their growth, depending on the structure they have. It is known that these materials should have high porosity (the order of hundreds of microns) to allow the development of bone within and across them. (Rivera-Muñoz 2011; Mucalo 2015).

3.2   Solutions for chemical studies

3.2.1   Ringer's solution

Sydney Ringer, a British physician who studied mechanisms or cardiac-contraction, introduced a solution in 1880 and it consisted of calcium and potassium in a sodium chloride diluent and was intended to promote the contraction of isolated frog hearts. This solution slowly gained in popularity as an intravenous fluid (Marino und Sutin 2007).

This solution is a crystalloid solution, an intravenous fluid similar to plasma. It contains sodium as its osmotically active particle and distributes throughout the entire extracellular space in such a way that approximately 25% to 30% of the infused volume remains in the intravascular compartment (Marino und Sutin 2007).

Ringer's solution contains potassium and calcium in concentrations that approximate the free (ionized) concentration in plasma. The addition of their cations requires a eduction in sodium concentration for electrical neutrality, so the solution has a lower sodium concentration than either isotonic saline or plasma (Marino und Sutin 2007).

However, Ringer's solution is also characterized by some disadvantages. The calcium present in the mentioned solution can bind to certain drugs and reduce their effectiveness, diminishing bioavailability. Drugs that should not be infused with Ringer's solution for this reason include aminocaproic acid, amphotericin, ampicillin and thiopental (Marino und Sutin 2007; Corry et al. 1996).

In the other hand, the same calcium can also bind to citrated anticoagulant in blood products. This can inactivate the anticoagulant and promote the formation of clots in donor blood. Actually, it is contraindicated as a diluent of blood (Corry et al. 1996; Marino und Sutin 2007).

Materials

Ringer's liquid is mainly used to (Wilkins 2007):
- provide hydration
- provide calories
- provide electrolyte replacement
- protect protein from being used as a source of energy (when solutions containing dextrose are used)
- replace ECF losses (normal saline solution)

3.2.2 Artificial saliva

Artificial saliva is formed by an aqueous solution of mineral salts and some other component designed to emulate the organoleptic properties of physiological saliva (Consejo General de Colegios Oficiales de Farmacéuticos 2015).

The ideal artificial saliva should have a neutral pH (to prevent demineralisation of the teeth), and contain fluoride (to enhance remineralization of the teeth) (Davies und Finlay 2005).

It also should be characterized by a high viscosity because it is beneficial for the lubrication of tissues and slows its removal from the mouth by swallowing (Carpenter 2014).

Some artificial saliva solutions also contain bioactive subtances that are added to replicate some of the bacteriostatic or antibacterial effects of natural saliva. Since natural saliva is already naturally high in calcium, the addition of this ion would seem obvious (Carpenter 2014).

In addition to the viscosity mentioned earlier, natural saliva has many unique physical properties and artificial fluids cannot simulate (or even some) these properties or composition, but the best approach is to create a solution with some specific properties of saliva for specific studies. For example, the influence of saliva's buffering capacity on foods material properties can be studied using simple buffering solutions that maintains a neutral pH. The impact of the polymeric components can be studied using dilute solution of polyelectrolytes such a mucin solutions, etc. While such studies are time consuming, it is a way to isolate the different factor that may play a role in the changing properties of food during oral processing (Chen und Engelen 2012).

3.2.3 Hemoglobin

Human hemoglobin A (Hb) is the main protein component of red blood cells, making up to 97% of their dry content. Hb plays a crucial role in vertebrates, as it carries oxygen from the lungs to the tissues for their oxidative metabolism (Kim und Greenburg 2013).

Human hemoglobin from outdid blood is an obvious source of oxygen-carrying blood substitute, as it is available in a relative large quantity. The idea of substituting the whole blood with purified free human Hb emerged at the end of nineteenth century. Chemical modifications have been explored to overcome the toxic effects of free hemoglobin, including polymerization or the use of nanoparticles (Kim und Greenburg 2013). In this project, hemoglobin solution will be used as a simulation of real one.

4   State of the art

A very small amount of bibliography about hydrogels with hydroxyapatite have been found from years before 2006, as it is shown in Figure 10. Hydrogels with hydroxyapatite researches increased in the last 12 years, which means we are in front of a very new topic with a lot of no-found possibilities.
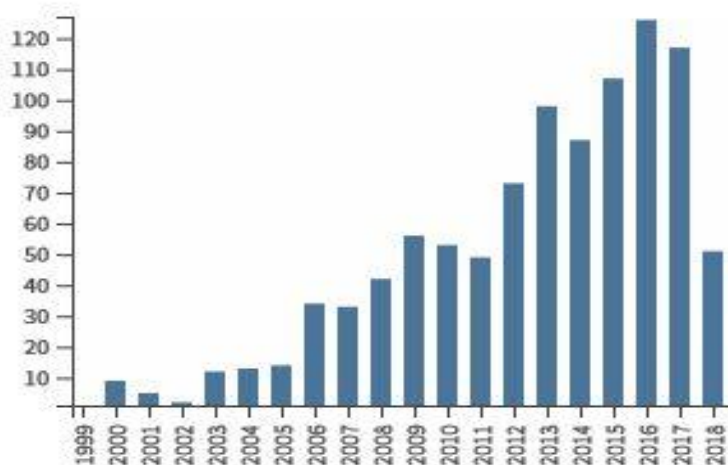

Figure 10. Number of publications per year (Web Of Science, 2018)

Dimitriu (2002) talks about the use of this type of hydrogel for applications where bone bonding is required because, as a biomedical material, calcium phosphate os considered bioactive and to have good osteoconductive properties (Tachaboonyakiat et al. 2001). And explains how a few researchers have attempted to create polymer-HAp composites for orthopaedic and tissue adhesive applications, being optimistic about implant applications if this hydrogels-HAp composites can be formed. The main problems he saw was the unclarified calcification mechanism.

There were many factors to study, including chemical formula, functional group, swelling ratio, and so on. Imai et al., who used PHEMA copolymer hydrogels, found that the amount of calcified deposits depends of HEMA content in the hydrogels and that their chemical structure was more important factor in calcification than hydrophilicity. Using various hydrogels that carried hydroxyl groups, the relationship between hydrogel characteristics and HAp formation was studied. It was revealed that there were two factors (the bound water content and the swelling ratio of a hydrogel) that affect HAp formation. The higher bound water content will provide a large number of nucleation sites for HAp on/in a hydrogel. On the other hand, the higher ratio becomes, the larger amount of ions is supplied into a hydrogel matrix form a solution in order to gro HAp nuclei on/in a hydrogel (Dumitriu 2002).

But HAp it is not only appropriated for bone tissue: One agent that has been recognized as an important signaling factor for the appropriate regeneration of nerves is calcium. Studies have shown that the calcium ions play a pivotal role in the formation of functional connections of the nervous system and development of individual nerve cells. Calcium-mediated signalling

pathways take part in the regulation of axonal outgrowth, the specification of neurotransmitter subtype and axonal migration and extension (Salehi et al. 2017).

4.1   Orthopedic applications

Orthopaedic biomaterials are generally limited to those materials that withstand cyclic loadbearing applications. While metals, polymers, and ceramics are used in orthopaedics, it remains metals, which have over the years uniquely provided appropriate material properties (Ratner 2013).

Polymers are most commonly used in orthopaedics as articulating bearing surfaces of joint replacements and as an interpositional cementing material between the implant surface and bone. Polymers used as articulating surfaces must have a low coefficient of friction, and low wear rates when articulating contact with the opposing surface, which is usually made of metal (Ratner 2013).

Dosset and Totten (2002) notice that the most common materials used in total replacement joints are metals, ceramics, and UHMWPE (Ultra-high-molecular-weight polyethylene), what leaves hydrogels off the board.

4.2   Dental implants

From all types of dental materials used in this field (composite restorative materials, dental cements, denture teeth, orthodontic wires…), hydrogels with HAp could be an option for pit and fissure sealants, where silicon carbide have been tested.

4.3   Tissue adhesives applications

Adhesive is a general term and in specific contexts may be replaced by designations such as cement, glue, paste and bonding agent. .Some adhesives may be designed to exhibit further functions, such as antibacterial action, delivery of drugs or beneficial ions, such as the antibacterial ion Ag^+ or fluoride (F^-) (Ratner 2013).

For Ratner (2013), hydrogels can be used as adhesives mostly for soft-tissue attachment such as wound closure, where copolymers of polyethylene glycol (PEG), poly(lactic acid) and acrylate esters are the most common.

Feng et all (2018) propose a PVA (polyvinyl alcohol) hydrogel with polyethylene glycol and nano-hydroxyapatite (n-HA) to enhance phase separation. However, for being used in wound dressing applications, a macroporous structure should be presented (Feng et al. 2018).

4.4   Tissue engineering scaffolds

However, the great promising application for this type of hydrogels is synthetization of scaffolds for tissue engineering.

In recent years, one of the significant difficulties in tissue engineering field is the manufacturing of acceptable biomaterials that could be utilized as substrate for cell bonding, multiplication, development and reproduction. A standout among the most basic issues is to develop appropriate three-dimensional (3D) biomechanical scaffolds as tissue replacement. To accomplish this task, the perfect scaffold should meet some particular criteria, including biocompatibility, convenient porous structure, adequate mechanical quality, and controllable biodegradation (Tohamy et al. 2018).

Polymeric scaffolds gained a magnificent attention in the last 20 years due to their biocompatibility, durability and simplicity of fabrication. A lot of strategies have been used for fabrication of biopolymer scaffolds. For example, natural polysaccharides- especially alginate- had been attractive due to their impressive properties. They are renewable, cheap, biodegradable, non-toxic upon in vivo administration and abundant available natural polymers (Tohamy et al. 2018).

Controlling the scaffolds degradation is one of the biggest challenges facing the tissue engineering scientists, thus it would certainly ensure simultaneous tissue replacement upon scaffold degradation (Tohamy et al. 2018).

Scaffolds offer the opportunity to create environments that help guide cells and create neotissue. Synthetic hydrogels are promising for their reproducible properties (e.g., mechanical and swelling) and tunable rates of degradation both of which can affect the cells and their ability to synthesize neotissue (Carles-Carner et al. 2018). Hydrogels are used for the regeneration of different tissues, including bone and cartilage, because they have physical properties simi-lar to extracellular matrix (ECM) in human tissue (Park et al. 2017).

One simple approach to create an osteoinductive environment is through the incorporation of hydroxyapatite particles into a hydrogel. Studies have shown that introducing hydroxyapatite particles into a hydrogel enhances osteogenesis of mesenchymal stem cells when cells are seeded on top of or encapsulated in hydrogels containing hydroxyapatite particles (Carles-Carner et al. 2018).

Carles-Carner et al. developed a MMPsensitive photoclickable PEG hidrogel with HA. Incorporation of hydroxyapatite nanoparticles did not affect the hydrogel properties as measured by compressive modulus and equilibrium swelling. Overall, osteogenesis was observed in the hydrogels with hydroxyapatite nanoparticles in growth medium but was enhanced in differentiation medium (Carles-Carner et al. 2018).

A novel starch-based hybrid hydrogel was formed by physical and mild steps by Xiao y Xu. SM/PVA hydrogel exhibits high stability, which is essential for the subsequent biomimetic

formation of hybrid hydrogel. To demonstrate the SM/ PVA hydrogels that contained various content of SM, they have been immersed in the buffer solutions with different pH values for some time (Xiao und Xu 2013).

4.4.1   Types of scaffolds

Tissue-specific cells are isolated from a small biopsy from the patient, expanded in vitro and seeded into a well-designed scaffold. Then, the cell-loaded scaffolds are subsequently transplanted into the patient either through direct injection with the aid of a needle or other minimally invasive delivery technique, or through implantation of the fabricated tissue at the desired site in the patient's body using surgery as it is shown in Figure 11 (El-Sherbiny und Yacoub 2013)
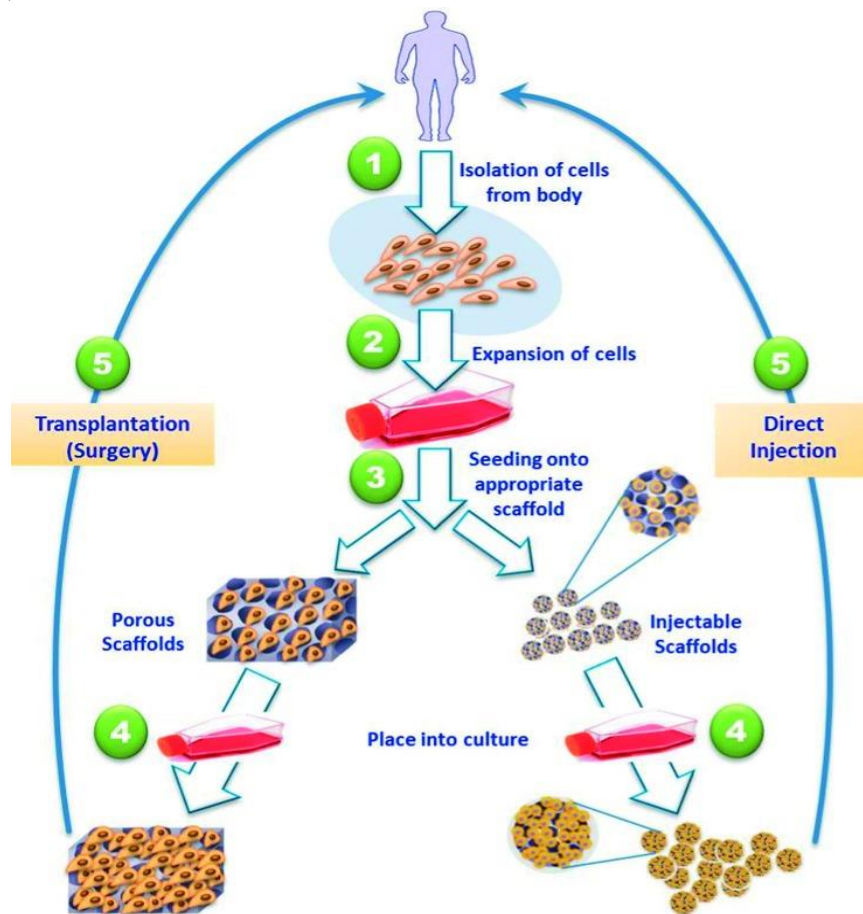

Figure 11. Schematic with the most common tissue engineering approaches (El-Sherbiny und Yacoub 2013)

Especially, studies on the injectable hydrogel usingthermo-sensitive materials have received a great deal of attention. By using injectable hydrogel the injected CaP could allow localinjury to heal without additional surgery, CaP brittleness to redeem,and its low bio-absorbability to enhance. (Park et al. 2017)

However, Carles-Carner et al opted for hydrogel disks of 4.5mmdiameter and 2mmheight were madein cylindrical molds by polymerizing the hydrogel precursor solution with a photoinitiator (Carles-Carner et al. 2018).

4.4.2 Surface characteristics

The surface of hydrogel scaffolds is the initial and primary site of interaction with surrounding cells and tissues. Therefore, both physicochemical and topographical surface characteristics of scaffolds are vital parameters in controlling and affecting cellular adhesion and proliferation. As most cells used in the engineered tissues are anchorage-dependent, the hydrogel scaffolds should be designed in such a way to facilitate their attachment. For this reason, hydrogel scaffolds with relatively large and accessible surface area are advantageous in order to accommodate the number of cells required to replace or reinstate tissue or organ functions. Surface characteristics of hydrogel scaffolds can be selectively improved by various approaches including thin film deposition and immobilizations of adhesive biomoieties such as RGD peptides, growth factors (like bFGF, EGF), insulin, fibronectin and collagen (Figure 12). This modification can enhance the biocompatibility of the hydrogel scaffold and consequently, cells can specifically recognize the scaffold. The adhesive biomoieties can either be covalently linked, electrostatically absorbed, or self-assembled on the surface of hydrogel scaffolds (El-Sherbiny und Yacoub 2013).
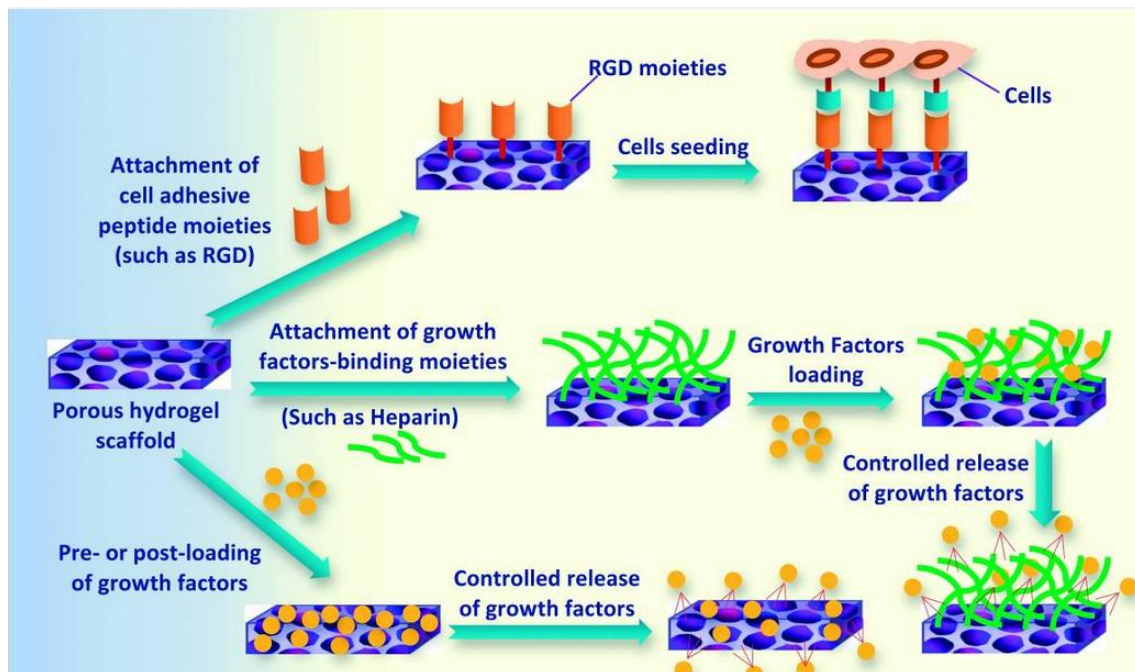

Figure 12. Some approaches for selective enhancement of surface characteristics of hydrogel scaffolds (El-Sherbiny und Yacoub 2013)

5 Experimental part

The experimental part can be divided in three big blocks: preparing of the solutions for chemical later tests, preparing the first set of samples and testing them mechanically and preparing the second one after choosing the best option from the first and performing swelling, incubation and traction tests on them.

5.1 Solution for chemical studies

The polymers will be tested in three different solutions simulating human body fluids, and in distilled water (as a reference point). For the different tests, one litter of every solution is need, and the preparation of each is described in this chapter.

5.1.1 Distilled water

As water doesn't need any special treatment, it will be taken directly from the source when it is need.

5.1.2 Ringer's solution

This isotonic to plasma solution doesn't need temperature for achieve a proper blend but a good mixing power is recommended.

The needed compounds and their amounts are shown in Table 3

Table 3. Ringer's solution composition

Compound	Theoretical (g/L)	Experimental (g/L)	Error (%)
NaCl	8,60	8,650	0,580
KCl	0,30	0,309	2,91
CaCl$_2$	0,48	0,494	2,83
Water	1000	1032	3,10

5.1.3 Artificial saliva

The same details applied for artificial saliva, i.e. room temperature and mixing.

The needed compounds and their amounts used for the preparation of artificial saliva solution are shown in Table 4

Table 4. Artificial saliva composition

Compound	Theoretical (g/L)	Experimental (g/L)	Error (%)
NaCl	0,400	0,404	0,99
KCl	0,400	0,408	1,96
Na$_2$S·9H$_2$O	0,005	0,007	28,57
N$_2$H$_2$PO$_4$·9H$_2$O	0,780	0,789	1,14
Urea	1,000	1,041	3,94
CaCl$_2$	0,684	0,684	0,00
Water	1000	1015	1,48

5.1.4 Hemoglobin

As the amount of hemoglobin need is quite big and it comes as a not-well soluble powder, it is recommended to mix this substance with water little by little and with a high blending power. In

Figure **13** it is possible to see how the powder remains in the surface and only the column of water over the paddle is moving enough for grabbing some hemoglobin.
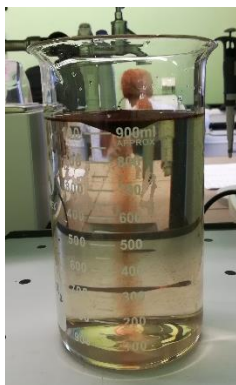

Figure 13. Blending of hemoglobin and water

The needed compounds and their amounts for preparation of haemoglobin solution are shown in Table 5

Table 5. Hemoglobin composition

Compound	Theoretical (g/L)	Experimental (g/L)	Error (%)
Hemoglobin	20	20,053	0,26
Water	1000	1014	1,38

A positive aspect of the solutions prepared is that they last in perfect conditions for a relative long time, allowing to work with the same product for a few weeks at least.

5.2 Preparation of composites based on PVP and histidine

5.2.1 Synthesis

As both compounds are given as powder, the first step is to prepare the main solutions: PVP 10% and histidine 1%. Other component used for the synthesis of composites are, diacrylate poly(ethylene glycol) is used as cross-linker and 2-hydroxy-2-methylpropiophenone used as photoinitiator, (it is important to keep in a dark place as long as possible).

Once all solutions are ready, we combine them as Table 6 shows:

Table 6. Composition samples

Number	PVP 10% (ml)	Histidine 1% (ml)	Cross-linker (ml)	Photoinitiatior (ml)
1	15	15	4,8	0,25
2	10	20	4,8	0,25
3	20	10	4,8	0,25
4	25	5	4,8	0,25
5	30	0	4,8	0,25

Experimental part

The order of combination is important: first PVP with histidine, then cross-linker and, only in the moment before the polymerization, we add the photoinitiator.

Next step is photopolymerization, where mixtures are poured on dishes and located near the radiation source, using a special lamp for this process as it is presented in Figure 14

Figure 14. Photopolymerization

During polymerization, it is important to rotate carefully the dish for keeping a homogeneous polymerization grade.

Samples were obtained and cut one by one, making sure that polymerization time is enough but not too much with a simple visual testing.

Once polymerization is over, polymer is cut with a paddle-shape samples, for the mechanical test and the rest is kept for another analyses, trying to make circular samples as it is shown in Figure 15.

Figure 15. Samples of composites based on PVP and histidine

It is important to let them dry for a few days at least. Samples for mechanical tests are kept under pressure for making sure the flatness of them.

First observations are about the aspect of the polymers.

On one hand, it is possible to observe how the amount of PVP affects the colour of the prepared materials, turning the polymer more yellow, as it is shown in Figure 16, where sample 2 contains 10 ml of PVP and sample 3 contains 20 ml of this compound.
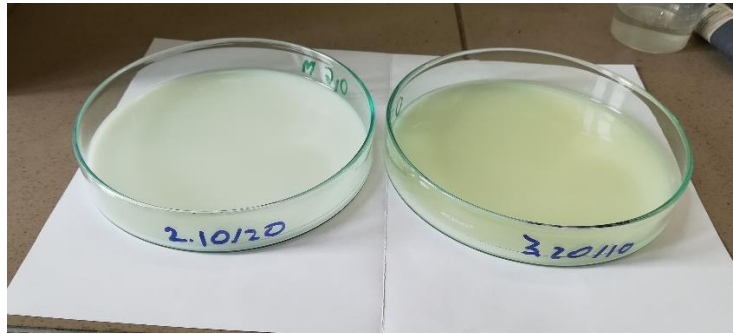

Figure 16. Comparison of colour of obtained polymers

On the other hand, all samples have soft surface and are flexible, sometimes even too flexible for being mechanically tested as it is explained below.

5.2.2 Mechanical studies of composites

The mechanical tests performed are tensile strength tests.

For these tests a universal testing machine was used, exactly The Brookfield CT-3 Texture Analyzer, as it is shown below in Figure 17. In this Figure it is also possible to see determining the dimensions of the sample during the test.

Figure 17. Measurements conducted during mechanical test

Experimental part

It is possible to conclude that aging of materials takes place in dry conditions. As it is shown in Figure 18 and Figure 19, the polymer is aging quite fast in dry conditions, losing at least 25% of its size.

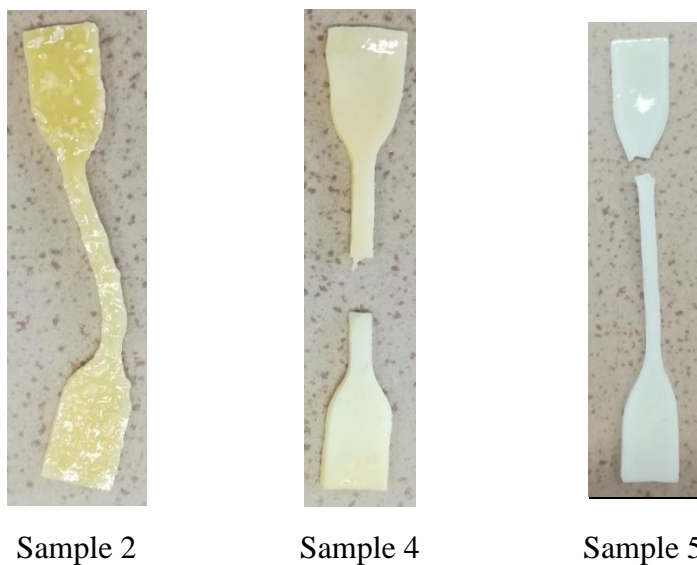**Figure 18. Circular samples aged**

Sample 2   Sample 4   Sample 5

Figure 19. Paddle-shape samples aged

Samples 2 is shown before mechanical test and 4 and 5 after it.

This give us an idea of the different aging rate of every substance, being faster as much histidine contain the polymer. Such variation should be considered for dry applications.

Knowing PVP is already being used for a lot of medical applications, the interesting part of this investigation is the effect of histidine on it. So histidine is considered just as an additive.

Experimental part

Mechanical studies of PVP-histidine materials were conducted to choose the best composition for further modification.

The results obtained are shown in Figures 8, 9, 11 and 12 and Table 7.

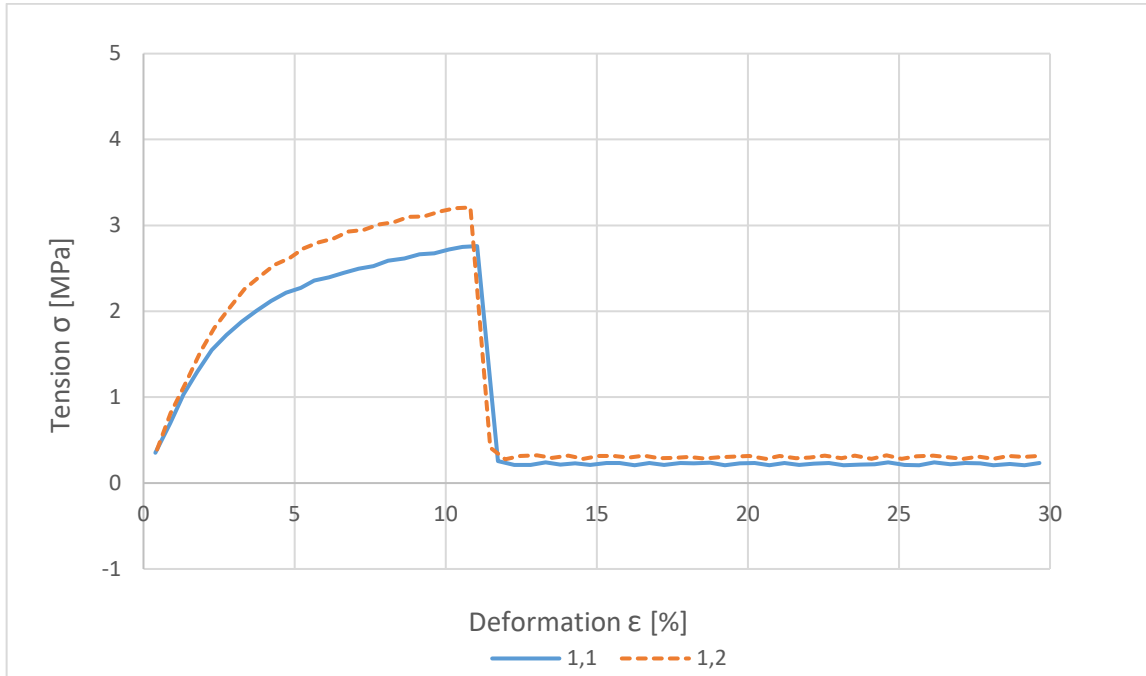Test of sample based on 15 ml PVP and 15 ml histidine

Figure 20. 15 PVP - 15 Histidine - mechanical test

Both experiments with 15/15 composition show a very similar performances: a short elastic region is followed for a longer plastic behaviour and a breaking point almost at the same elongation but with a little different tensions.

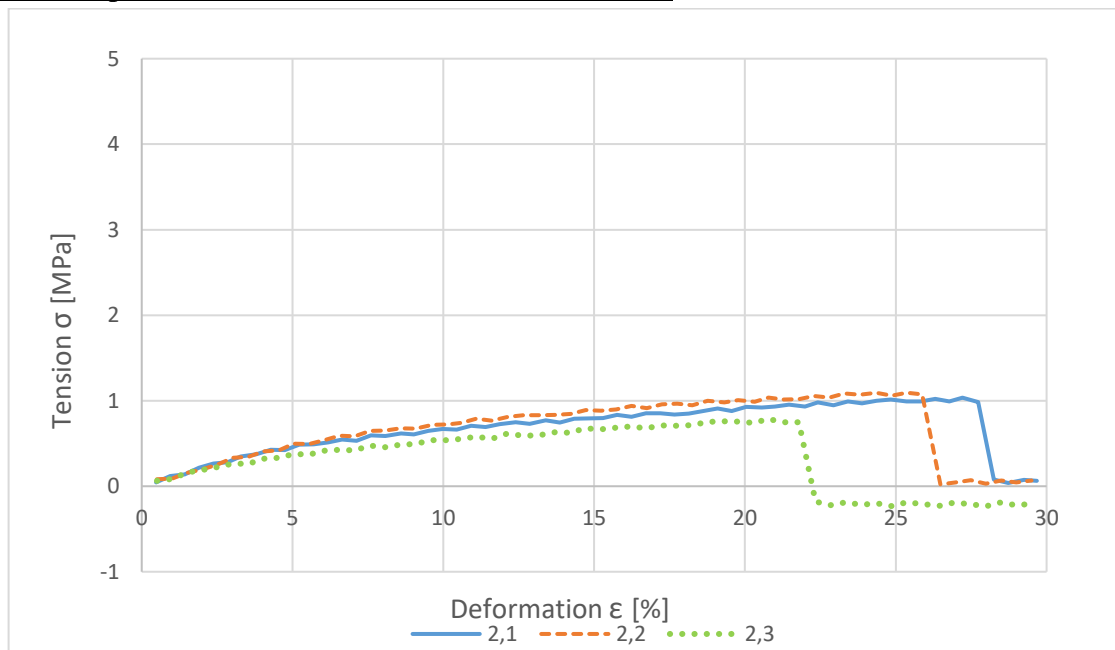Test of sample based on 10 ml PVP and 20 ml histidine

Figure 21. 10 PVP - 20 Histidine - mechanical test

39

When quantity of histidine increases, behaviour changes completely: polymer becomes weaker, without any elasticity but a plasticity range, maybe due to the own nature of histidine or at a worst interaction with PVP.

On the other hand, the behaviour of the experiment 2.3 is completely different from the others, showing a really lower result. This can due to the sample was not absolutely straight as it's possible to see in Figure 22.

This inclination should make necessary more tension for breaking the sample, because one part of the tension is being applied in the transversal axe. But, surprisingly, the maximum achieve is lower than the rest. As the used compounds were powder and the polymer was poured in the vessel for polymerization as homogeneous as possible, the directionality of the polymer is discarded.
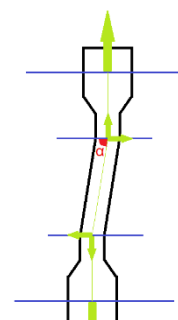
Figure 22. Sketch of forces transmition

So, the only possibility is given for the paddle-shape of the sample, prepared only for tension efforts, not for shear forces.

Test of sample based on 20 ml PVP and 10 ml histidine

The viscosity of the composite varies in function of the composition, the most viscous materials are samples with 20/10 and 25/5, where amount of PVP is higher but not only in such conditions. Actually, it was impossible to test any samples of polymer with 20/10 rate because they were breaking during the extraction.

At the end, these reasons of this behaviour can be a result of a bad interaction between both polymers or because to the fact that PVP is characterized by higher viscosity than histidine, what makes it less appropriate for the mechanical requirements needed.

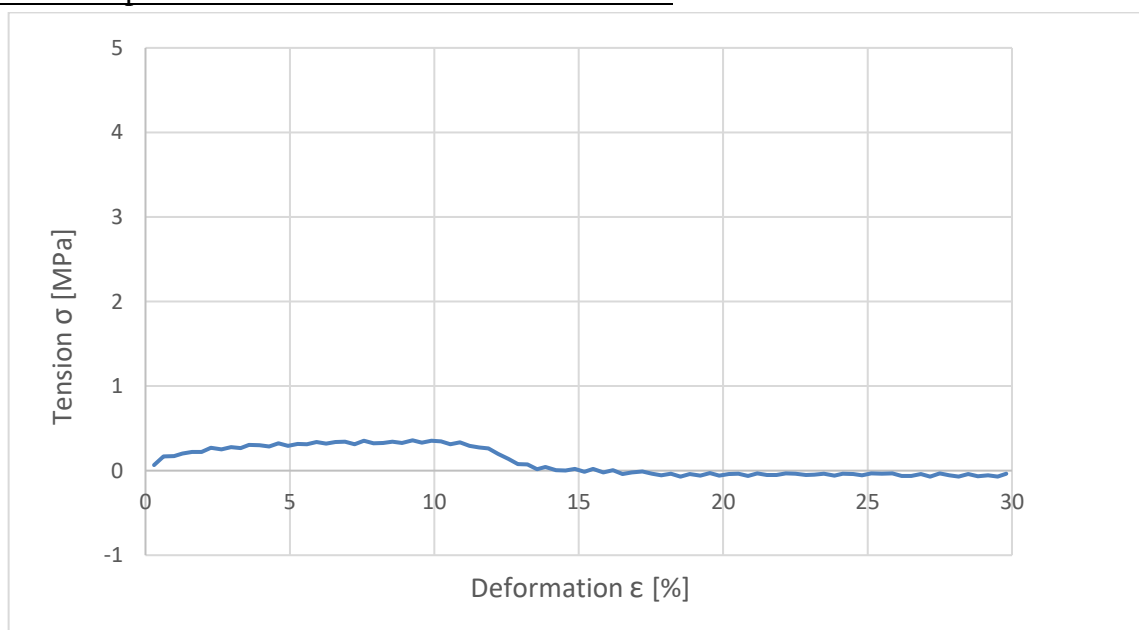Test of sample based on 25 ml PVP and 5 ml histidine

Figure 23. 25 PVP – 5 Histidine - mechanical test

Sample with rate 25/5 follows the behaviour of the sample 20/10, showing a barely existent break tension, but enough for doing the mechanical test.

The test of 25/5 (and others like one of the 30/0), shows negative loads after breaking point, due to the unexpected high viscosity misscalibration of the equipment happens. Anyway, as this data are not useful for the study, this loss of calibration during test is not important.
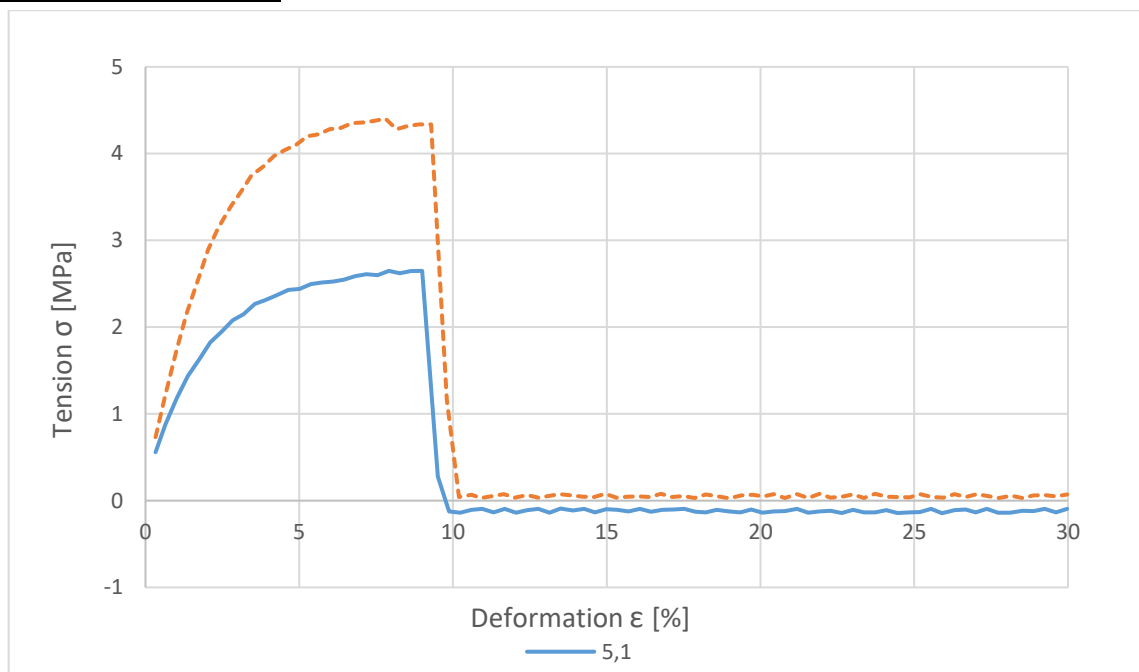
5. 30 PVP – 0 Histidine

Figure 24. 30 PVP - 0 Histidine - mechanical test

The performance is similar to sample 1 (15/15) but ranges of tensions achieved are quite different.

This situation can be related to the area of the polymer where the sample is cut and due to the fact polymerization was not completely homogenous: depending of the light and the inclination of the vessel, properties can be different. Maybe one part of the polymer receives more light, or, even when the amount of light is the same, maybe not the thickness in all the plate, making some areas not polymerized to the same extent than others.

Selection

In this preliminary study, we are looking for the best mechanical behaviour, and for that, we use Table 7, where we compare resultant tension of a given elongation. In this case we will study the tension at 5% elongation of every test made for ever sample. For some polymers, like 10/20, was possible to perform until three tests but for anothers (like 25/5), only one test could be performed.

The best result is shown by sample 5.2 (which rate is 30/0), but, the other sample with the same composition has such a different value (5.1), this data is not reliable

Table 7. Tension at 5% elongation

	Elongation (%)	Tension (MPa)
1.1 (15 PVP – 15 HIS)	5,204	2,273
1.2 (15 PVP – 15 HIS)	5,287	2,732
2.1 (10 PVP – 20 HIS)	5,223	0,485
2.2 (10 PVP – 20 HIS)	5,043	0,496
2.3 (10 PVP – 20 HIS)	5,043	0,378
4.1 (25 PVP – 5 HIS)	4,934	0,295
5.1 (30 PVP – 0 HIS)	5,007	2,441
5.2 (30 PVP – 0 HIS)	4,898	4,102

So, the best mechanical properties are shown by the composition 1, 15 PVP – 15 HIS.

5.3 Synthesis of composites containing hydroxyapatite

The preparation of the composites containing hydroxyapatite is similar to procedure used for the synthesis of unmodified materials was introduced into the polymer in a form of solid and compositions of new polymers are shown in Table 8.

Table 8. Polymers containing hydroxyapatite composition

Number	PVP 10% (ml)	Histidine 1% (ml)	Hydroxyapatite (g)	Cross-linker (ml)	Photoinitiatior (ml)
1	15	15	0,25	4,8	0,25
2	15	15	0,50	4,8	0,25
3	15	15	0,75	4,8	0,25
4	15	15	1,00	4,8	0,25
5	15	15	1,25	4,8	0,25

Before addition of crosslinking agent and photoinitiator to the reaction mixture, solution of PVP, histidine and Hap should be mixed thoroughly

In this case, the applied polymerization time was between 2-3 minutes (decreasing the time in every sample). Only sample 1 needed a longer time (about 4 minutes) of photopolymerization and the result was a polymer which was slightly too hard and easily-breakable during the cutting with the shape at it is shown in Figure 25.
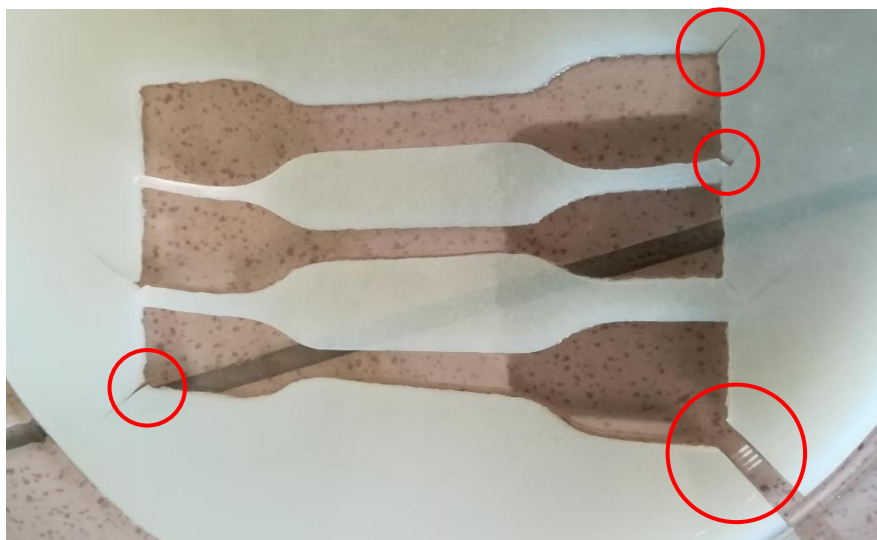
Figure 25. Sample 1 cut

The expected behaviour was that HAp would stay in suspension, even would remain in the surface. But, instead of that it falls down to the bottom of the vessel and remains in a quite homogeneous layer.

This layer makes the polymer heterogeneous with two clearly differentiated phases: the top surface, where the composition is mainly PVP and histidine with some Hap dissolved, and the bottom part, where the most part of Hap remains. The contrast is easy visible and shown in Figure 26

Figure 26. Both surfaces of polymer containing hydroxyapatite

The top surface is wet, glossy, soft touch, transparent and yellow (probably it is caused by the presence of PVP) and bottom surface is white, opaque, rough and dry, due to the undissolved HAp that gathered at the bottom of reaction vessel. This property can be used for make easier the contact between two incompatible materials.

As the same amount of PVP-Histidine blend can absorb the same amount of HAp and, from the smallest amount of HAp, the solution cannot take all the powder in, the layer of HAp is growing with the amount of powder added. In Figure 27 it is possible to anotice how whiter (layer of HAp) area is turning more visible.

Figure 27. Samples containing hydroxyapatite

5.4 Studies of modified composites containing hydroxyapatite

In this case, characterization is more complete, including two chemical studies (swelling and incubation) and another tensile strength study.

5.4.1 Mechanical studies

Mechanical test for modified composites containing hydroxyapatite are performed exactly the same as modified composites based on PVP and histidine, with the same equipment and same conditions

But, in this occasion, the grater stiffness of the polymer was achieveed –probably because of the HAp layer-, Results of mechanical studies are shown in Figure
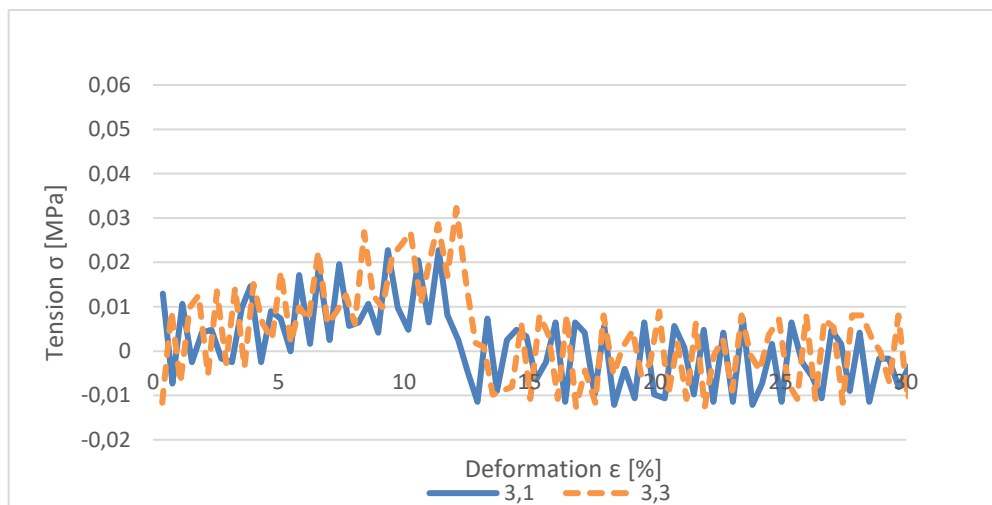

Figure 28. Results of mechanical studies of sample 3 (0,75 g HAp)

A smoothing formula has being applied in all the results with a factor of 70% of smoothness, which are possible to see in Figure 29, 18, 19, 20 and 21. This makes the results useless for quantitative conclusions, but good enough for obtaining a quite-accurate overview of the material.
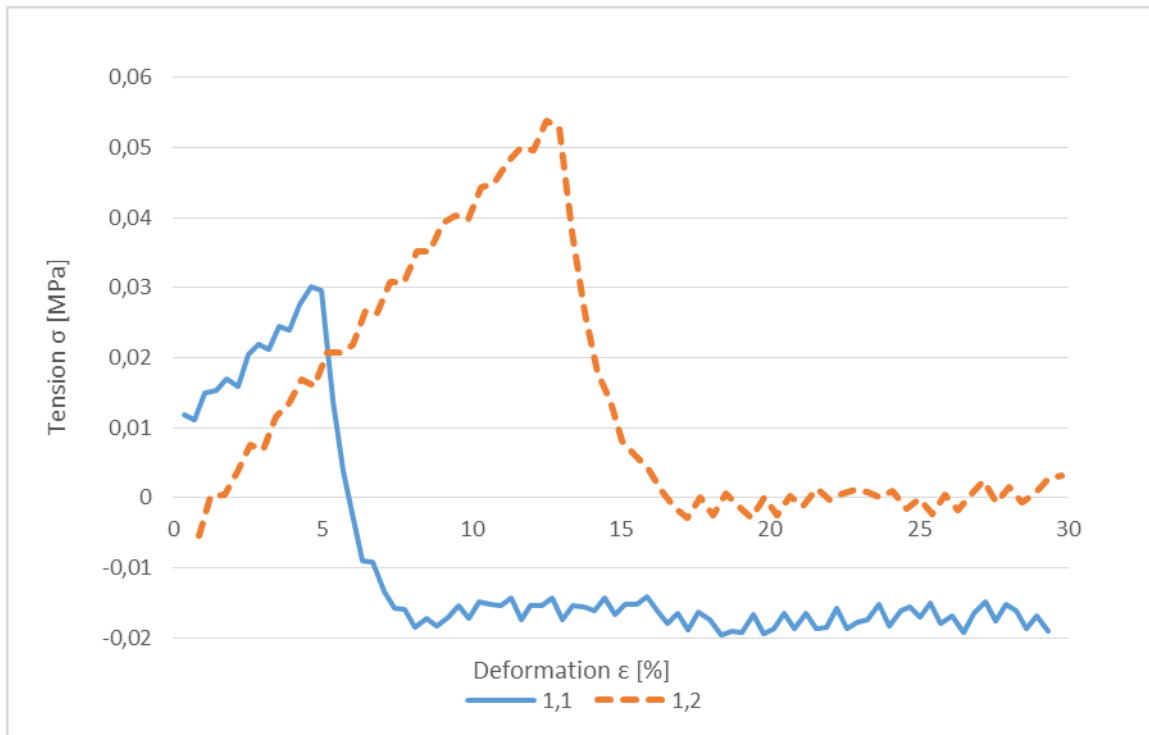
Figure 29. Sample containing 0,25 g hydroxyapatite - mechanical test

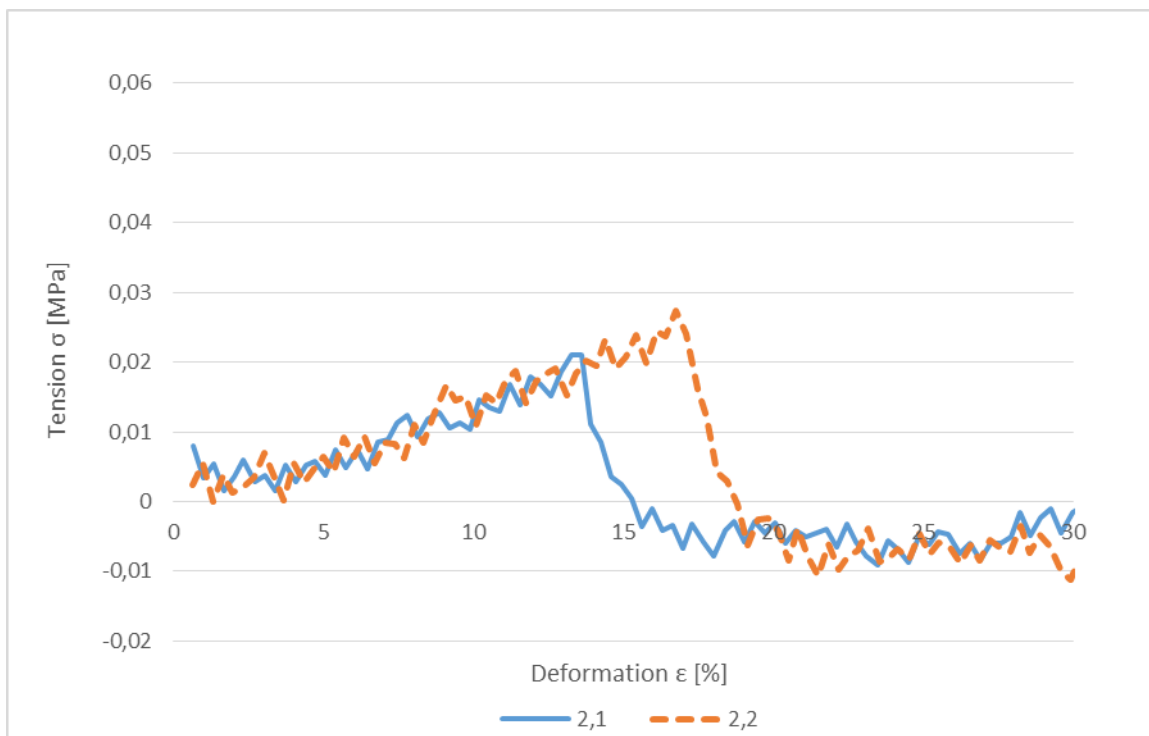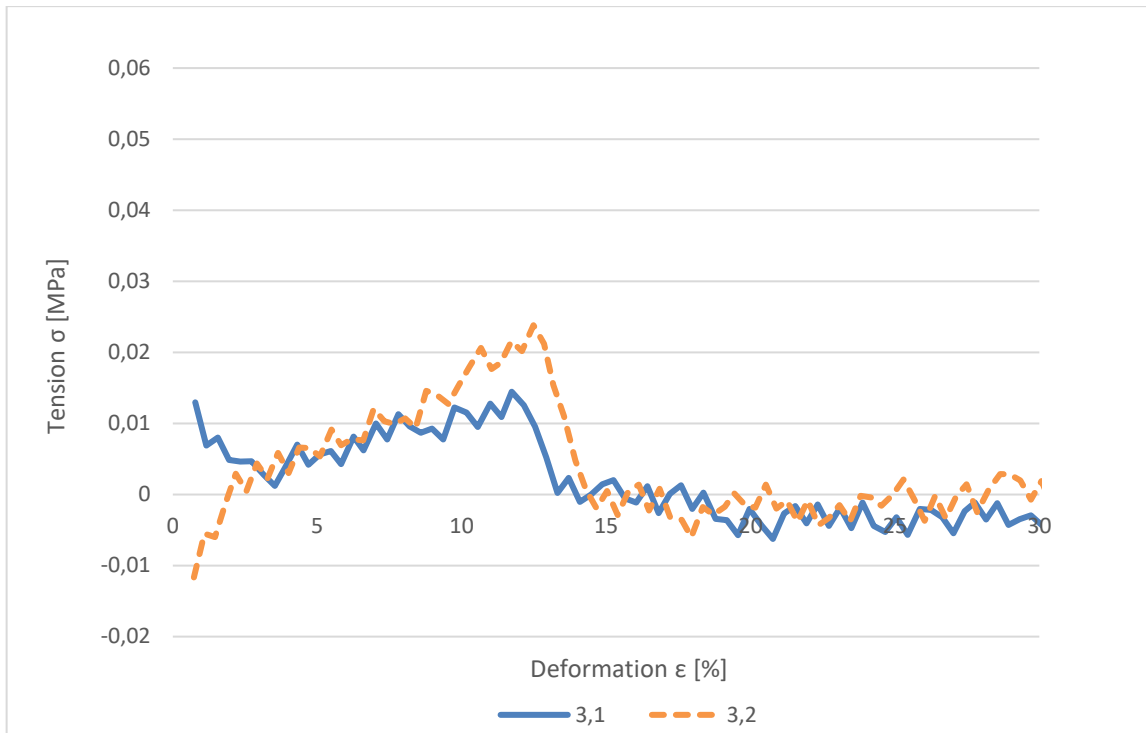**Figure 30. Sample containing 0,5 g hydroxyapatite - mechanical test**

Figure 31. Sample containing 0,75 g hydroxyapatite - mechanical test
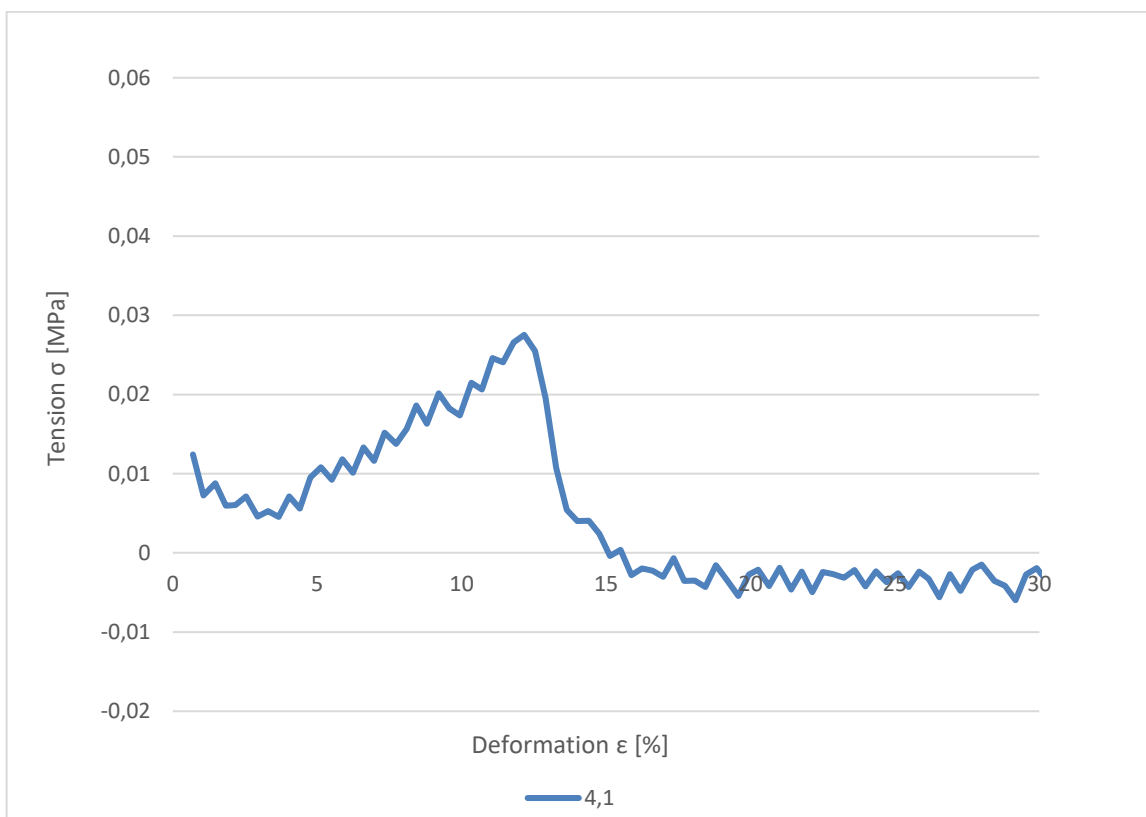

Figure 32. Sample containing 1,0 g hydroxyapatite - mechanical test
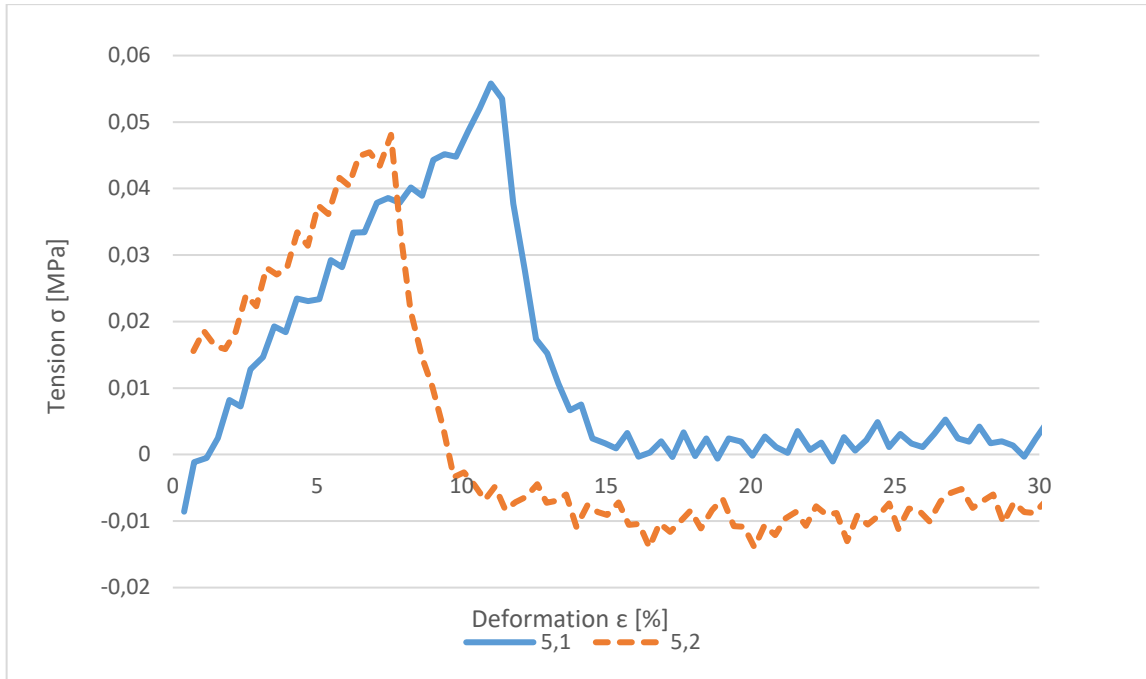
Experimental part

Figure 33. Sample containing 1,25 g hydroxyapatite - mechanical test

First of all, checking Table 9 and comparing with Table 7 (it means, comparing tension at 5% of elongation) maximum tension have fallen down in an extreme way.

Table 9. Maximums mechanical test composites containing hydroxyapatite

	Elongation (%)	Tension (MPa)
1.1 (0,25 g HAp)	4,939	0,028
1.2 (0,25 g HAp)	5,133	0,021
2.1 (0,5 g HAp)	5,068	0,016
2.2 (0,5 g HAp)	4,945	0,005
3.1 (0,75 g HAp)	5,087	0,007
3.2 (0,75 g HAp)	4,866	0,011
4.1 (1,0 g HAp)	5,132	0,005
5.1 (1,25 g HAp)	5,076	0,024
5.2 (1,25 g HAp)	5,025	0,033

This just means elasticity decreases now and polymer breaks with almost any effort, what can be good or acceptable for applications where mechanical requirements are low like drug delivery. The mentioned behaviour can be due to the bad interaction between HAp and one of the other components or both.

Comparing all the maximums (without smoothing formula), the maximum tension is given by the sample 5, where HAp layer is the thickest. This means HAp is more resistant for itself than with the composite PVP-Histidine.

For another hand, in every sample, the experiments have being given big differences between them, what is actually only true in part: as the material breaks so easily, the breaking point arrives quickly and the range is very small, making differences very big.

5.4.2 Swelling test

For swelling and incubation tests, one portion of every sample was immersed in vessels with one every solution (Ringer's liquid, artificial saliva and haemoglobin) and distilled water.

Figure 34. Vessels with immersed samples for swelling and incubation tests

In swelling test, the experiments are kept at room temperature for 48 hours and measurements are performed after 1, 24 and 48 hours for comparison of masses of dry and swollen materials to determine their sorption capacity in different liquids of the body.

Swelling ability of the tested materials is determined by calculating the swelling rate, which shows the degree of swelling (g/g) for each sample. The formula for this parameter is

$$Q = \frac{w_t - w_0}{w_0}$$

where w_t and w_0 are the sample's weight in swollen state after certain period of time (1, 24, and 48 h), and in the dry state, respectively.

A very interesting case is the hemoglobin test, where, due to the dark red pigment of the compound, it is possible to observe the unequal absorption of the surfaces.

First of all, in Figure 35, three different samples are shown and, in all of them, the behaviour is the same: in the areas where HAp is less concentrated, more hemoglobin is absorbed. In the samples, the most part of hemoglobine is in the edges or in pores in the surface. But, in the first and the third, the HAp layer is not homogeneous and the thinner part is redder.

Figure 35. Photographies of samples after 1 h of swelling in hemoglobin

This behaviour keeps until all the polymer satures, as shows in Figure 36. This sample comes from incubation studies and shows the state of the sample after 7 days in haemoglobin.
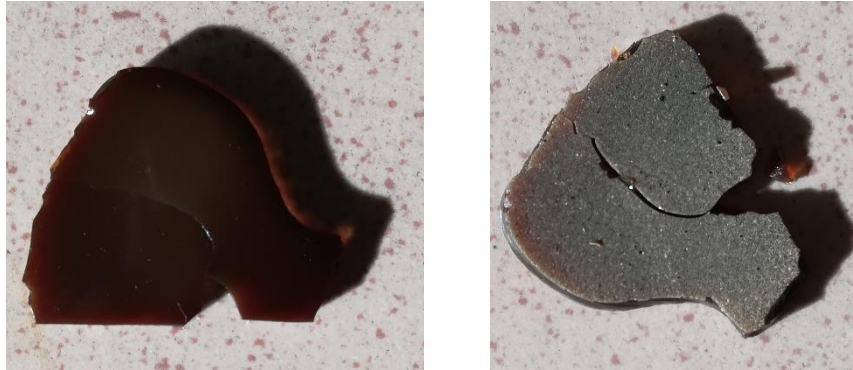

Figure 36. . Photographies of samples after 7 days of swelling in hemoglobin

Here, all the polymer phase is completely dyed with the colour of hemoglobin and the HAp layer remains gray, showing its less capacity of absorption. This could be because HAp is less absorbent than the polymer or because of the fact that HAp is located in the pores of the material on this side and therefore swelling is limited and colour is less intensive.

In Figures 25, 26, 27 and 28 results of swelling studies are shown.
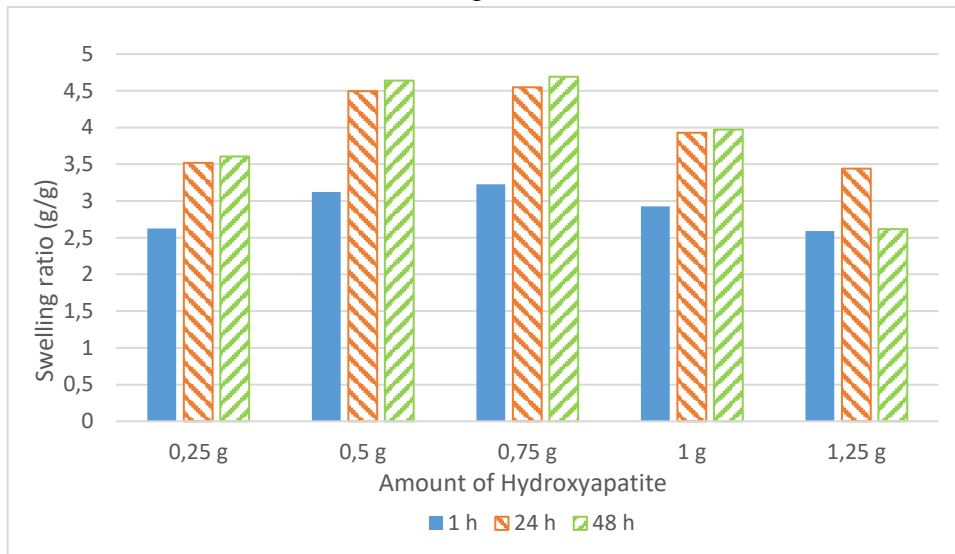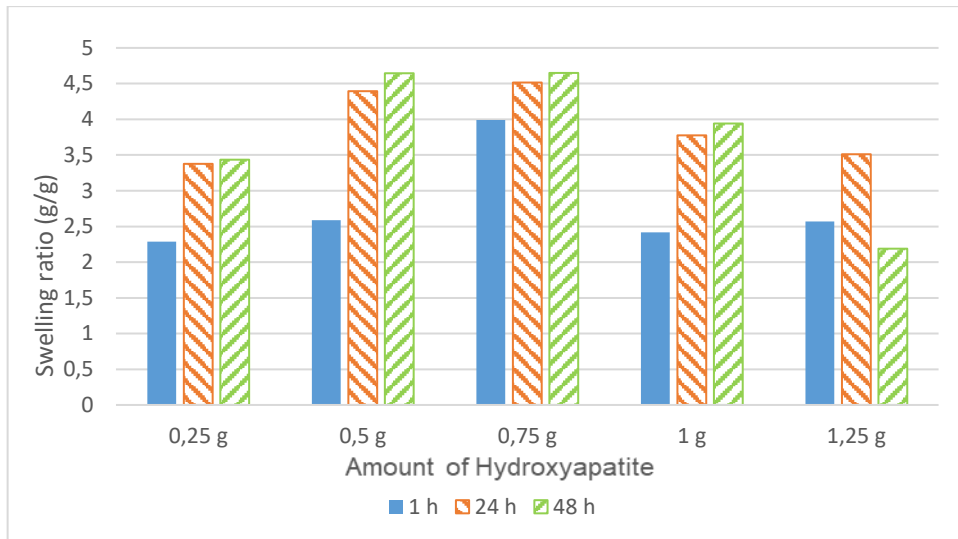

Figure 37. Swelling test - Water
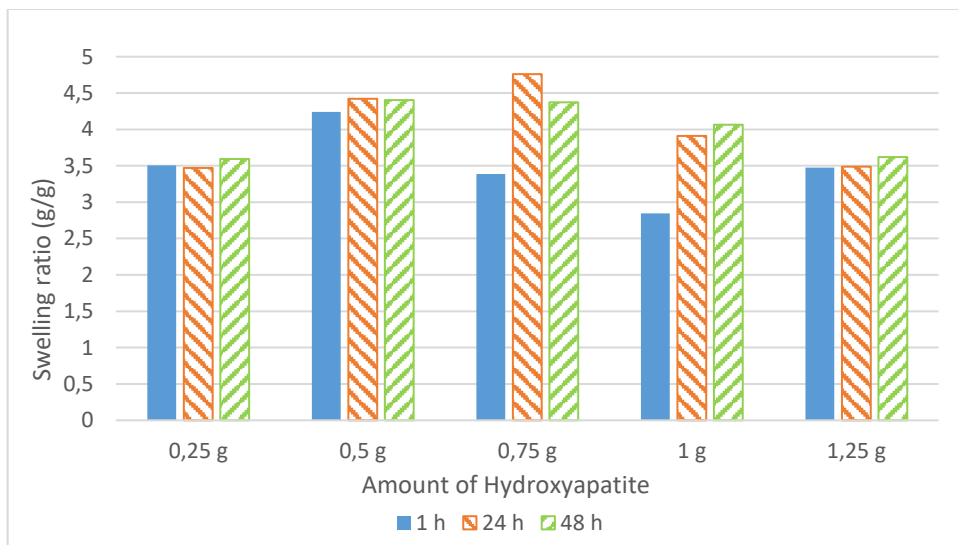
Figure 38. Swelling test - Ringer's solution

Figure 39. Swelling test - Artificial saliva

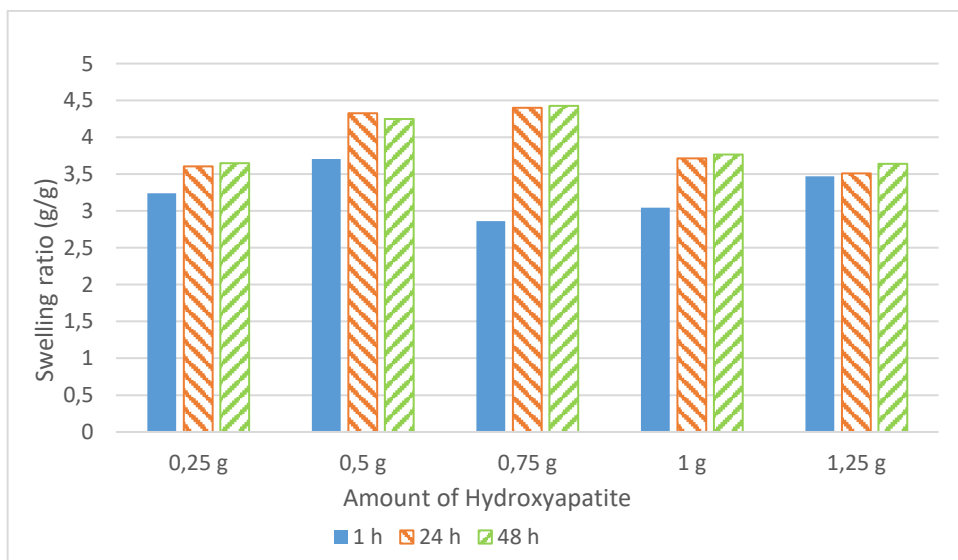**Figure 40. Swellinf test - Hemglobin**

The first thing that can be observed in all cases is how, after only 1 hour, the sample recovers its size and keeps growing a little bit more after 24 hours. After that, in some cases the mass of the samples remains almost stable (performing a slightly increase) or decrease. This not-unanimous behaviour probably means that HAp doesn't have a general effect over all solutions on the swelling properties and it will be good to check the behaviour dividing the solutions in function of their main difference relative to pH.

In all cases, anyway, the highest ratio is always for the sample containing 0,75 g HAp sample, followed nearly for the sample containing 0,5 g HAp. The explanation of this phenomenon is probably not given for HAp, but for the polymerization grade: the time of exposition was being changing, as is explained in the experimental process. So, it is possible in case of samples containing 0,5 and 0,75 g HAp, we achieve the optimum polymerization time for swelling properties (if we are looking for to maximize this ratio).

On the other hand, the different tendencies for the sample 5 (1,25 g HAp) in every solution make us to think about the nature of them: water and Ringer's solutions are inorganics and saliva and hemoglobin are organics (at least, in part).

In inorganic solutions (water and Ringer's liquid), HAp is eluted after 48 hours when the layer is the thickest. This is because its buffering properties derive in elution in inorganic media, being the compound itself an inorganic mineral. For organic solutions, elution is not happening and the material keeps swelling a little bit.

This behaviour becomes the composite in a selective material: it will be appropriate for organic solutions for long periods of time and for inorganic solutions for shorts periods of time.

5.4.3 Incubation test

As we already mentioned, samples for incubation studies are prepared in the same way that for swelling (immersing of samples in the solutions), but in contrast to swelling studies, incubation studies are carried out in 37°C (human body's temperature) and at least 24 hours before the first measurements of pH.

Incubation test regards the effect of the composite over the solutions. Behaviour is different for every solution, having always a common starting: from the first to the second point, and increase of pH happens. During that time, the solution is finding a new balance, with the ions given by the polymer.

From this point, every solution is a little different and will be analyzed separately.

In Figure 41 results of incubation studies in distilled water are presented
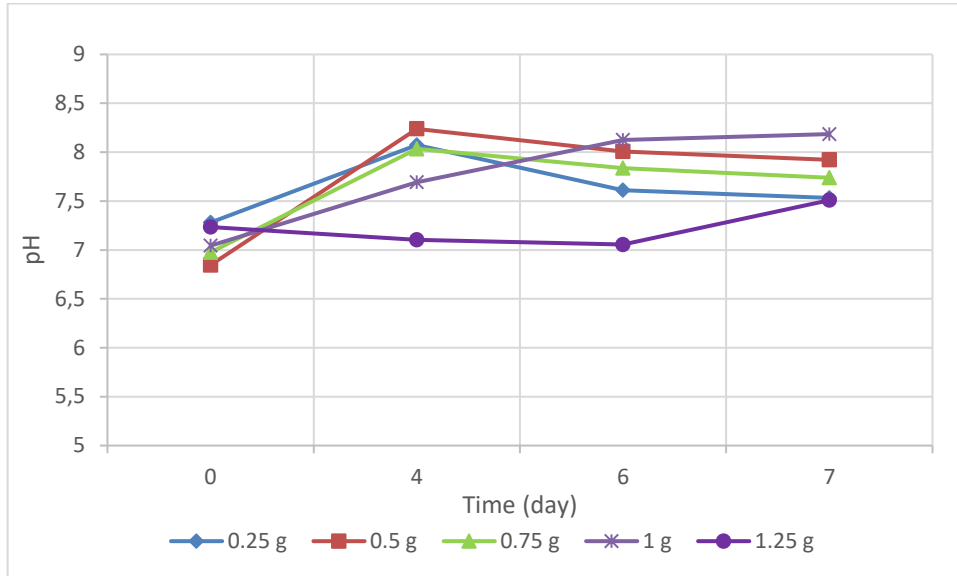

Figure 41. Incubation test - Water

In water, until composite containing 1 g of HAp, the pH follows the already mentioned behaviour: starts with an increase and tends to stabilization due to hydroxyapatite, which performs as a buffering compound and keeps the pH balanced. This additive is delivered little by little from the porous of the polymer and from the bottom layer.

For composites containing 1,25 g of HAp, there is enough additive from the beginning of the study for starting the buffering effect without waiting for it delivery of the HAp from the pores.

The second liquid is Ringer's solution. Results of incubation in this liquid are shown in Figure 42, where the general tendency after the general firs increase is to decrease. This is due to the additive is buffering, not only ions coming from the polymer, but the owns from the solution (which is a isotonic liquid for plasma) when the polymer has been enough dissolved for delivering the additive. This changes the general pH. The mentioned behaviour is more prominent for the maximum amount of HAp.

In Figure 42 results of incubation studies in Ringer's solution are presented.
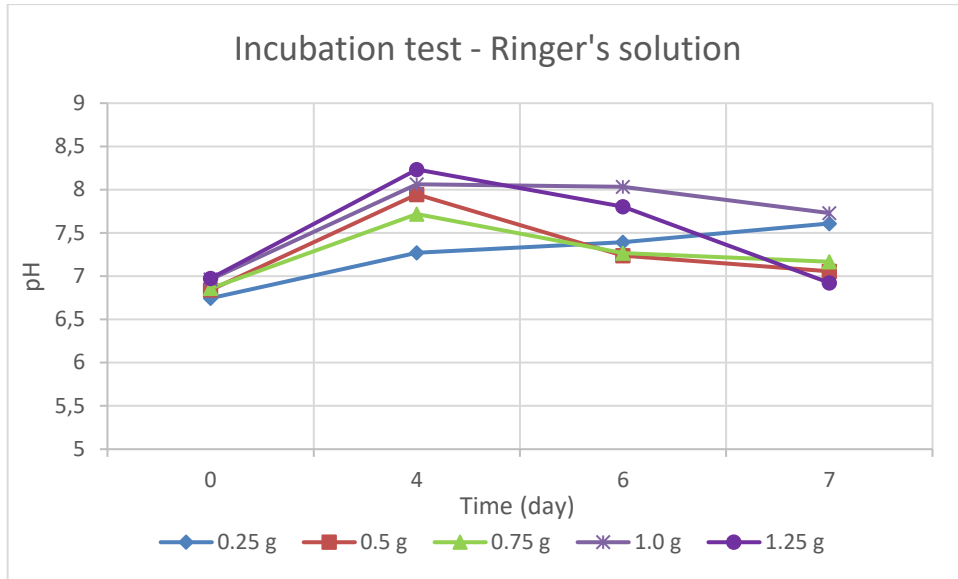
Figure 42. Incubation test - Ringer's solution

On the other hand, when composite contains only 0,25 g of HAp, the tendency is completely different and remains growing. The reason of that is the polymer has not enough additive for buffering even the ions from itself. So, we found the limit of buffering for HAp in Ringer's solution: an amount between 0,25 g and 0,5 g.

In case of samples containing bigger amount of HAp then 0.25 g, similar pH values are observed during the whole period of incubation.

In Figure 43 Figure 42results of incubation studies in artificial saliva are presented.
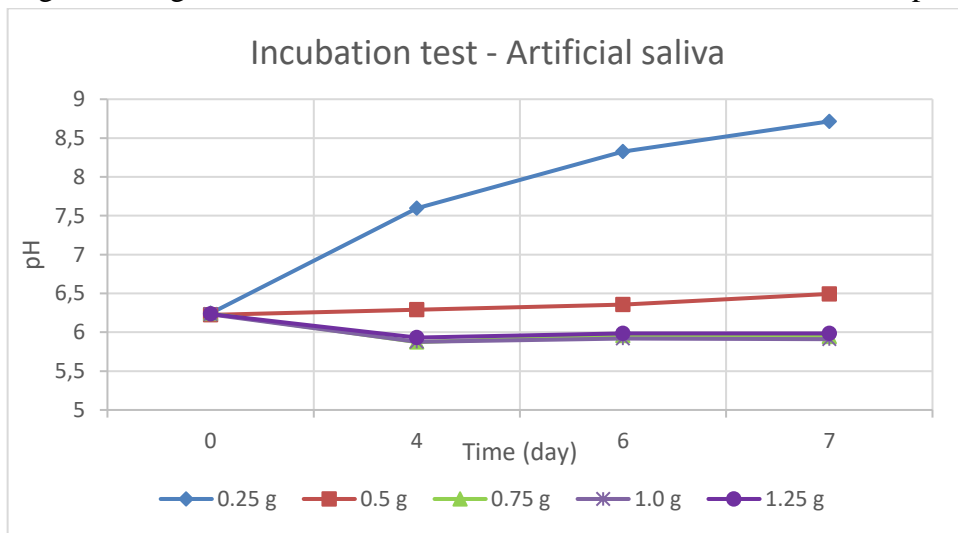

Figure 43. Incubation test - Artificial saliva

As is possible to see, the behaviour is the same as in Ringer's solution but in a more radical way: for the polymer with the smallest amount of additive (0,25 g) pH increase quickly – until more than 2 units- and, on the other hand, the rest of experiments keep stable from the

beginning. This shows how the solution is not able to take charge of the polymer for itself, which means a big dependency of the HAp.

In case of samples containing bigger amount of HAp then 0.25 g, similar pH values are observed during the whole period of incubation.

This radicalization of the situation comes from the nature the solution: both are very ionic liquids but Ringer's solution is inorganic and elution is happening while artificial saliva is organic, and elution doesn't take place.

In Figure 44Figure 42 results of incubation studies in hemoglobin are presented.
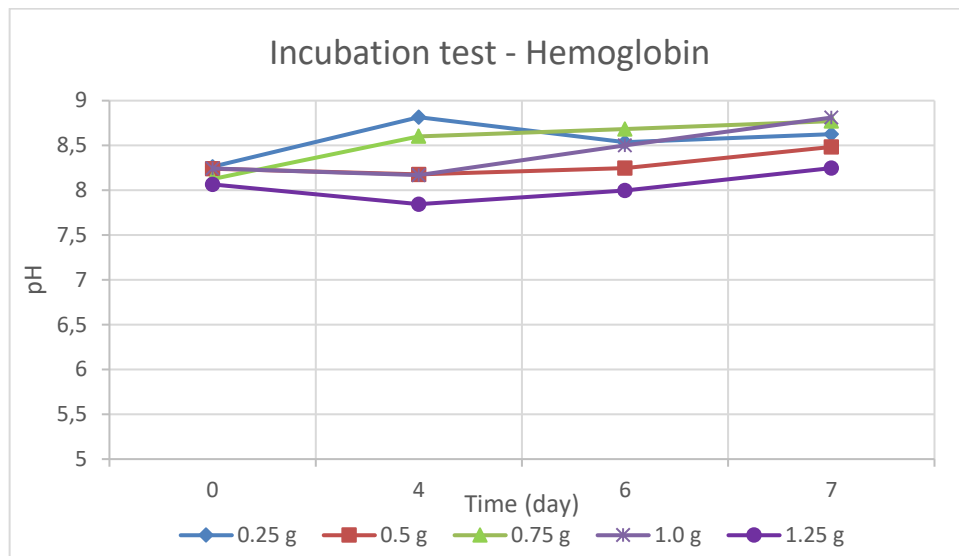
Figure 44. Incubation test – Hemoglobin

Based on the results a stable tendency from the beginning for all amounts of HAp can be observed, which is mostly due to the fact that this solution is not too ionic (the same as water) and, at the same time, is organic, so elution is not happening like happens in water. So, from the beginning, there are not too many ions to break the balance.

In general, pH is stable enough for a short period of time in all liquids. But such results include a range of use too small for being accepted for the most of medical applications. So, we should take this experiments like a previous step to a long term experiment with periods more in accordance with the expected time in real applications (weeks, months…).

6 Possible applications and future works

From the begging, all possible applications have been summarized in: orthopaedics, dental implants, tissue adhesives and tissue engineering scaffolds.

As the mechanical resistance of the composite is so low, applications with high mechanical requirements as orthopaedics and mental implants are discarded.

On the other hand, the polymer have not shown any remarkable adhesion capacity, being not possible to be used as tissue adhesive.

However, this hydrogel is promising for bone tissue engineering due to its raw and properties: all materials (PVP, histidine and HAp) are suitable and enhance the tissue regeneration, being the HAp a great option for bone healing. At the same time, the polymer has good porosity and stability of the polymer in front different body fluids.

The two-differentiated surfaces achieved polymer proposes a methodology of use, being advisable to place the more concentrated HAp face in contact with the damaged tissue.

Nevertheless, all these assumptions are only theoretical and a wide range of studies is needed to be able to determine with precision the applicability of the hydrogel, like biocompatibility studies (in vivo and/or in vitro), microscopy (SEM, Raman, X-Rays) or thermal analysis (TGA, TMA, DSC).

7 Conclusions

From the first part of the study, characterization of composites based on PVP and histidine, conclusions are:

- ➢ All samples have soft touch and are flexible, sometimes even too weak for being tested.
- ➢ The polymer is aging quite fast in dry conditions, losing at least 25% of its size.
- ➢ When quantity of histidine increases, behaviour changes completely: polymer becomes weaker, without any elasticity but a plasticity range, maybe due to the own nature of histidine or a worst interaction with PVP.
- ➢ The viscosity of the composite varies in function of the composition, being the most viscous materials when the content of PVP is higher than content of histidine.
- ➢ Homogeneity of polymerization affects the properties.
- ➢ The best mechanical properties are shown by the following composition 15 PVP – 15 HIS.

From the second part of the project, characterization of composites containing hydroxyapatite, it is possible to say that:

- ➢ Long-time polymerization turns composite slightly too hard and makes it easily-breakable during the cutting with the shape.
- ➢ Polymer has two clearly differentiated phases: the top surface, where the composition is mainly PVP and histidine with some Hap dissolved, and the bottom part, where the most part of Hap remains.

- ➢ The top surface is wet, glossy, soft touch, transparent and yellow (for the effect of PVP) and bottom surface is white, opaque, rough and dry, due to the undissolved HAp.
- ➢ Introduction of HAp resulted in obtainment of composites characterized by bigger elasticity.
- ➢ The composite has a great swelling behaviour: after only 1 hour, the sample recovers its size and keeps growing a little bit more after 24 hours.
- ➢ HAp doesn't have a general effect on the swelling properties over all solutions.
- ➢ When the polymerization time is optimal, swelling ability is the biggest.
- ➢ In inorganic solutions (water and Ringer's liquid), HAp is suffering elution after 48 hours when the layer is the thickest.
- ➢ For organic solutions, elution is not happening and the material keeps swelling a little bit.
- ➢ At the biginning of incubation studies pH of the tested solutions increases. During that time, the solution is finding a new balance, with the ions given by the polymer.
- ➢ When HAp content is enough for acting as a buffering agent, pH keeps stable. This amount depends on the type of tested solutions.
- ➢ Elution derives in the radicalization of the dependence of the pH with the HAp.
- ➢ Long term experiment with periods more in accordance with the expected time in real applications (weeks, months…) should be made after this study.

Finally, the main possible application for the polymer would be the tissue engineering scaffolds, being necessary, however, perform more studies for prove its aptitude.

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