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NOVEL POLYMER BASED HYDROGEL FOR BIOMEDICAL APPLICATIONS. SYNTHESIS, CLASSIFICATION AND RHEOLOGICAL INVESTIGATION OF THE HYDROGELS THIXOTROPIC BEHAVIOR.

IDROGELI POLIMERICI PER LE APPLICAZIONI BIOMEDICALI. SINTESI, CLASSIFICAZIONI E INVESTIGAZIONI REOLOGICHE DELLE PROPRIETÀ TIXOTROPICHE DEGLI IDROGELI.

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Abstract

This study was carried out in the period between 2013 and 2014, at the faculty of Chemistry and Pharmacy, under the supervision of Prof. Dr. Robert Luxenhofer, in Julius-Maximilians Universität, Würzburg, Germany and Prof. Michele Modesti, Industrial Eng. Dept., University of Padova, Italy.

The project is based on the synthesis and characterization of a novel polymer based hydrogel, for biomedical applications. The synthesis of the hydrogel was carried out starting from the mixture of a silicate nanoparticle (Laponite) with both synthetic and natural polymer: poly (ethylene oxide) and collagen I, respectively, in different mediums.

Then, the hydrogels were subjected to both rheological and swelling degree test, in order to be able to understand the mechanical or viscoelastic properties of these gels when deformed under a certain stress or strain, and also, to have a proper idea of the importance of the hydrogels swelling capacity in the area of solute or drug transportation in biomedical applications.

Factors, such as hydrogel inhomogeneity, ageing effect, temperatures, pH and ionic concentration of the medium used in the synthesis of the gels, that could affect the hydrogels rheology, swelling capacity and data reproducibility were also considered. All these factors are to be taken into account when the hydrogel system is needed for a certain application, for instance in drug delivery sector, wound dressing, tissue engineering field, injectable polymeric system and as well for technical products.

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Introduction

Hydrogels synthesis starting from the interactions in aqueous solutions between silicate nanoparticles and polymers, have received considerable attention within the recent years due to the ability of these materials to play a fundamental role both in biomedical and in the tissue engineering field. The mixtures of silicate particles with some polymers lead to the formation of the so called hydrogels systems, which exhibit interesting physical and chemical properties, and at a certain concentrations, some reversible network structures are formed in the hydrogels. The ability of these mixtures to form a reversible network structures (based on the type of chemical bonds among the systems) has led to the formation of a hydrogel structure with great mechanical properties; a very important aspect in the field of biomedical and tissue engineering.

So, understanding and controlling the mechanical properties of the hydrogels systems, for instance its elasticity or degree of softness, is very relevant for appropriate physiological function in numerous contexts. However, biocompatible synthetic materials have already found many applications, but a further combination of chemical compatibility with appropriate mechanical properties will increase the potential application of the hydrogels in tissue engineering field.

In this study, we have focused our attention on the process for obtaining the optimal mechanical properties and the classification of the phase behavior of these hydrogels systems, based on the variation of the concentration of its components. The viscoelastic properties of these hydrogels were also classified, by studying the rheology of these materials, through the use of the dynamic and static method approach.

A correlation between the hydrogels mechanical properties and their degree of swelling was also studied, and we were able to outline the factors affecting these two properties.

The first chapter introduce the definition of novel hydrogels, their characteristics and classifications, based on the type of bonding existing in the gel structure. And the field of applications of the hydrogels: biomedical and tissue engineering field and others.

In the second chapter, there is the description and properties of the materials that were used for the synthesis of the gels, the structures, the mechanical properties, samples preparation and experimental conditions were also discussed in this chapter.

The third chapter consist mainly in the techniques for the characterization of the hydrogels, where the hydrogels rheology, their swelling behavior (water contents, pore and permeation) and finally the hydrogel biocompatibility properties were discussed.

And finally, in chapter four, the importance of hydrogels mechanical and viscoelastic properties was discussed, followed by the description of the methods that were used for the measurement of the hydrogel mechanical properties with the rheometer; both dynamic and static method of measurement. A ternary representation of some hydrogels samples was also given in this chapter, and the result of the gels swelling behaviour in different mediums with different compositions was also explained. And finally, the hydrogel reproducibility, ageing effect, together with the temperature effect on the gel rheological properties were also discussed.

Chapter 1

Novel hydrogel: characteristic and applications

1.1 Novel hydrogel

The terms hydrogels and gels are used interchangeably by food and biomaterials scientists to describe a three dimensional cross-linked polymer network, which has the capacity to hold water within its porous structure. The water holding capacity of the hydrogels arise mainly due to the presence of the hydrophilic groups; carboxyl and hydroxyl groups in the polymer chains. The amount of water present in a hydrogel may vary from 10% to thousands of the weight of the xerogel (Hoffman, 1997). A xerogel may be defined as a polymeric network devoid of water. The water holding capacity of a xerogel is dependent on the number of the hydrophilic groups and crosslinking density. Higher the number of the hydrophilic groups, higher is the water holding capacity, and as the crosslinking density increases, there is a subsequent increase in the hydrophobicity and a corresponding decrease in the stretchability of the polymer network.

Gels are defined as a substantially dilute cross-linked system, and they are categorized principally as weak or strong gel, depending on their flow behavior in steady state (Ferry, 1980). The formation of weak or strong gels, depends strongly on the concentrations and the interactions among the main components of the hydrogel system: Laponite-polymers.

Hydrogels are basically obtained from synthetic and or natural polymers, which can absorb and retain significant amount of biological fluids and swell. When swelled, they are soft and rubbery and resemble the living tissue, exhibiting excellent biocompatibility. They are able to respond to the fluctuations of the environmental stimuli (temperature, ionic strength, pH, electric field, presence of enzyme and so on.). These biomaterials are widely used in different field of pharmaceutical and biomedical engineering, today, drug delivery experience several challenges where hydrogel could be one potential answer to those.

Thanks to the unique properties of hydrogel for which they are widely exposed to different biomedical fields. The use of hydrogel for biomedical applications dates back to 1960 when the pioneers; Wichterle and Lim developed crosslinked poly (hydroxyethyl methacrylate, pHEMA) and poly (methyl mathacrylate). Apart from these synthetic polymers, the use of natural polymers often termed as biopolymers, for the development of hydrogels have gained a substantial importance over the years. Alginate and chitosan are the two biopolymers which have been extensively studied in the recent past. The use of hydrogels is not only limited to pharmaceutical and nutraceutical delivery, but recently, it has also been extended to regenerative medicine.

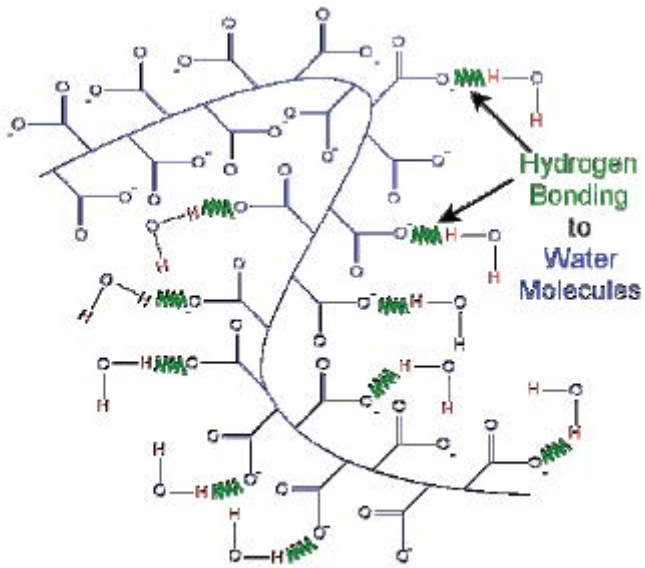

Figure 1. *Structure of a cross-linked polymer network hydrogel*

The mechanism of network formation or gelation process, might be refer to as the linking of macromolecular chains together which initially leads to progressively larger branched of soluble polymers, depending on the structure and conformation of the starting material. A later mixture of the poly-disperse soluble branched polymers is called Sol. The continuation of the linking process with time, results in increasing the size of the branched polymer with decreasing solubility, to form the so called “infinite polymer” also known as “gel or network”, which is permeated with finite branched polymers. The transition from a system with finite branched polymer to infinite molecules is called “sol-gel transition” or

gelation. And the critical point where the gel first appears is called the “gel point” (Rubinstein & Ralph, 2003).

1.2 Classification of hydrogels

Hydrogels are mainly classified through the mechanism with which they are formed, either by chemical crosslinking (chemical gelation) or by physical crosslinking (physical gelation). The crosslinking may take place in two types of environments: in vitro, during the preparation of a hydrogel in a specific container (glass tube) or in vivo (in situ), the gelation occur directly after application or injection in a precise location, for instance in human body.

The in vitro chemical crosslinking takes place in a vial once the hydrogel components are brought together and subsequently mixed together by a vortex system or simply by agitating the vial with hand.

While the usual technique for crosslinking a hydrogel in vivo (in situ) is photo-polymerization, which aim is to inject directly the polymer mixture, and the liquid to solid transformation takes place when the cross-linker appears (usually laser exposure, 514nm). This method is utilized for instance in dentistry to form sealants and dental restorations in situ. There are several advantages of photo-polymerization over traditional technique: spatial and temporal control of polymerization, fast curing rates at physiological temperature and minimal heat production.

Chemical crosslinking is also known as a process through which permanent gels are formed, due to the presence of a covalently crosslink networks, by replacing hydrogen bond with a stronger and stable covalent bond. These types of gels attain an equilibrium swelling state, which depends on the polymer – water interaction parameter and the crosslinking density (Rosiak & Yoshii, 1999).

Physical crosslinking or reversible gel, are gels that are formed when the networks of the system are held together by molecular entanglements, and or secondary forces including ionic, hydrogen bonding or hydrophobic interactions. In physically cross-linked gels, dissolution is prevented by physical interactions, which exist between different polymer chains (Hennink & Van Nostrum, 2002). Hydrogels that are formed through physical interactions are mostly reversible, and can be disrupted by changes in physical conditions or application of stress (Rosiak & Yoshii, 1999). With respect to the chemical gels, which are mostly irreversible, due to the formation of covalent bonds in its structure, physical gels can

be sub categorized into strong and weak physical gels. Strong physical gels are mainly made up of strong physical bonds between polymer chains and they are effectively permanent at a given set of experimental conditions, so for this reason they are basically similar to the chemical gels. Some examples of strong physical gels might be those of lamellar microcrystals, glassy nodules or double and triple helices. While weak physical gels are mainly composed of reversible links that are formed from some temporary associations between chains, and they are categorized with a finite lifetimes, breaking and reforming process continuously, depending on the conditions at which they are subjected. Some examples of weak physical bonds are hydrogen bond, block copolymer micelles and ionic associations.

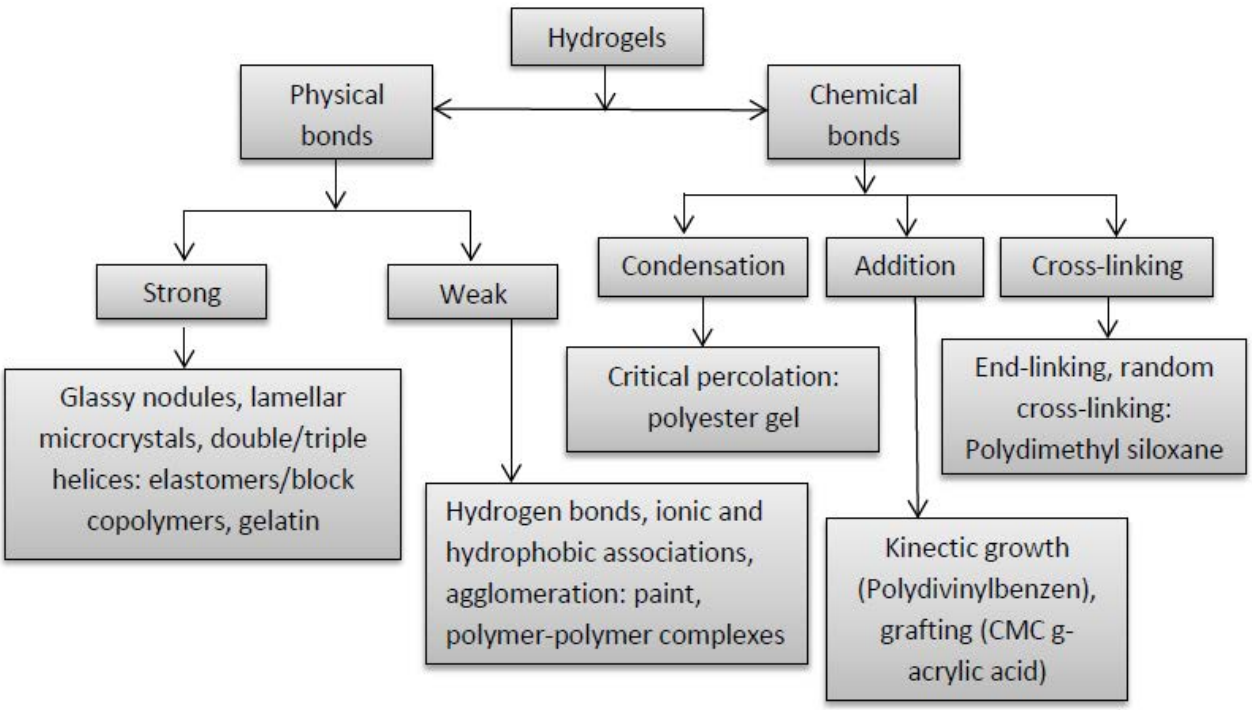

Figure 2. *Classification of hydrogel gelation mechanism, and some relevant examples* (Syed & Saphwan, 2013).

In this study, most of the hydrogels prepared are physically cross-linked, because under deformation, the polymer chains may attach or detach from the nanoparticles. The hydrogel usually shear thin, a property that makes some of them injectable via syringe. And after cessation of shear, the macrostructure and rigidity of the hydrogel recovers completely within seconds, suggesting a self-healing property. Most of these gels are not homogeneous,

due to the fact that some clusters of molecular entanglements, hydrophobic or ionic associated domains, might create in-homogeneities.

Other techniques for the classification of the hydrogels are those stated below:

- Classification based on source

  Hydrogels are classifieds in this case into two groups, based on the fact they have been prepared through the use of natural or synthetic polymers.

- Classification based on configuration

  The classification of hydrogels that depends on their physical structure and chemical composition can be as follows:

  - Amorphous (non-crystalline)
  - Semi-crystalline: a complex mixture of amorphous and crystalline phases.
  - Crystalline.

- Classification according to polymeric composition

  Hydrogels are mostly differentiated through the methods with which they are prepared, and these leads to the formation of some important classes of hydrogels with different properties. These can be classified as follow:

  - Homo-polymeric hydrogels are referred to polymer network derived from a single species of monomer, which is a basic structural unit comprising of any polymer network. Homo-polymeric hydrogels may have cross-linked skeletal structure depending on the nature of the monomer and polymerization technique.
  - Co-polymeric hydrogels are comprised of two or more different monomer species with at least one hydrophilic component, arranged in a random, block or alternating configuration along the chain of the polymer network.
  - Multi-polymer Interpenetrating polymeric hydrogel (IPN), these are important class of hydrogels that are made up of two independent cross-linked synthetic and or natural polymer component, contained in a network form. Normally, in a semi-IPN hydrogel, one component is a cross-linked polymer and the other is a non-cross-linked polymer.

- Classification based on the network electrical charge

  Hydrogels may be categorized into four groups on the basis of the presence or absence of an electrical charge that is located on the cross-linked chains.

- Ionic (including anionic or cationic)
- Non-ionic (neutral)
- Amphoteric electrolyte (ampholytic) containing both acidic and basic groups.

• Classification according to the physical appearance

Hydrogels appearance as matrix, film or microsphere depends on the technique of polymerization involved in the preparation process: by linking polymer chains through chemical reaction, physical interaction, or by using ionizing radiation to generate main-chain free radicals which can recombine as cross-link junctions.

1.3 Applications of hydrogel: biomedical and tissue engineering field and others

Hydrogels have been of great interest to biomedical scientists for many years, due to their hydrophilic character and potential to be biocompatible; compatibility with the immune system of human being, water holding capacity and permeability are the most important characteristic features of the hydrogels that makes them applicable in biomedical and tissue engineering field.

In 1980, with the important and influential work of Lim, one of the pioneer in the synthesis of hydrogels it was possible to demonstrate the successful application of calcium alginate microcapsules for cell encapsulation. Later in the 1980s, arises the first incorporation of natural polymers, such as collagen and shark cartilage into hydrogels for use as artificial burn dressings. Nowadays, hydrogels based on both natural and synthetic polymers, have been developed for cells encapsulation, and most recently such hydrogels have become attractive to the new field of tissue engineering as matrices for repairing and regenerating a wide variety of tissues and organs.

The evolution of tissue engineering, started with the treatment, reparation and replacement of parts or the whole of a fail or mal-functional tissues or organs, with a synthetic or natural substitute or by regeneration. And this is normally limited to those situations where surgical methods and implants have achieved success. Total replacement of the diseased or mal-functioning organ or tissue with a natural substitute requires transplantation of an acceptable and healthy substitute, and there is a limited supply of such organs and tissues. Thus tissue engineering holds out great promise for regeneration of organs.

Hydrogels designed for use as a tissue engineering scaffolds may contain pores, large enough to accommodate living cells, or they may be designed to dissolve or degrade away, releasing growth factors and creating pores into which living cells might penetrate and proliferate. Due to the significant amount of water in hydrogels, hydrogels possess a degree of flexibility similar to that of a natural tissue. And it is possible to change their chemistry simply by controlling their polarity, surface properties, mechanical properties and swelling behavior. One of the most significant advantages with the use of hydrogels as tissue engineering matrices is the ease with which one may covalently incorporate cell membrane receptor peptide ligands, in order to stimulate adhesion, spreading and growth of cells within the hydrogel matrix. Another advantage with the use of hydrogel is that of the biodegradability effect, hydrogels can be easily degradable simply in the presence of enzymes, hydrolytic solution and through the effect of the ambient condition: pH, temperature and electric field.

However, it is necessary to take into account some of the negative aspects, such as low mechanical strength, causing significant difficulties in handling. Another issue might be that of the sterilization. So, it is necessary to overcome most of these disadvantages before the hydrogels will become practical and used in the field.

Table 1. *Advantages and disadvantages of hydrogels as tissue engineering matrices* (Hoffman, 2002)

Advantages
Aqueous environment can protect cells and fragile drugs (peptides, proteins, oligonucleotides, DNA)
Good transport of nutrient to cells and products from cells
Might be easily modified with cell adhesion ligands
Can be injected in vivo as a liquid that gels at body temperature
Usually biocompatible
Disadvantages
Can be hard to handle
Usually weak mechanically
May be difficult to load drugs and cells and then crosslink in vitro as a prefabricated matrix
May be difficult to sterilize
In-homogeneities of the gels might lead to an increase in the rate of drug delivery in a system with respect to another.

The combination of synthetic and natural polymers with some certain types of alginate (clay) for the production of hydrogels have mainly found their application in the biomedical and tissue engineering field, but also, thanks to their high water absorption capacity and biocompatibility, they have also been used in other fields, such as in the pharmaceutical field, agriculture, sanitary pads, dental materials and others. A list of hydrogels with their corresponding applications and polymers used is shown in the table below.

Table 1.2. *Applications of hydrogels, types of polymers and references*

Application	Polymers	References
Drug delivery, pharmaceutical	Poly(vinylpyrrolidone), starch, poly(acrylic acid), chitosan, αβ-glycerophosphate, k-carrageenan	(Benamer, 2006) (Campo, 2009)
Wound care	Polyurethane, poly(ethylene glycol), poly(propylene glycol), carboxymethyl cellulose, alginate, hydrocolloids	(Rosiak & Yoshii, 1999) (Kim & Park, 2005)
Tissue engineering, implants	Poly(vinylalcohol), poly(acrylic acid), collagen	(Rosiak & Yoshii, 1999) (Drury & Mooney, 2003)
Dental materials	Hydrocolloids (Ghatti, Karaya, Kerensis gum)	(Al-Assaf & Dickson, 2009)
Injectable polymeric system	Polyesters, polyphosphazenes, polypeptides, chitosan	(Yan & Altunbas, 2010)
Technical products (cosmetic, pharmaceutical)	Starch, gum Arabic, carrageenan, chitin, chitosan	(Trksak & Ford, 2008) (Phillips & Plessis, 2003)
Agriculture waste treatment, separation, etc.)	Starch, polyvinyl alcohol, poly(vinyl methyl ether)	(Jeremie & Markov, 1999)

Chapter 2

Materials and experimental methods

2.1 Description and properties of the materials used, structures and chemical properties

The main scope of this study is to delineate the structures and properties of hydrogels made predominantly from synthetic materials, and to figure out a way for enhancing the mechanical properties of the hydrogels, which is one the issue with the application of hydrogels in biomedical and tissue engineering field. One of the strategy followed consist in utilizing nanoclays as cross-linkers and thus forming nanocomposite hydrogels with enhanced mechanical properties such as elongation, toughness and tensile strength. Nanoclays are inorganic silicate particles that are used as rheological additives to control viscosity and flow properties of an aqueous solution.

We have synthesized hydrogels starting from a binary mixture (nanoclays and polymer) or from a ternary mixture (nanoclays, polymer and collagen), in order to determine whether the incorporation of a highly inorganic clay particles (Laponite XLS) with the polymer (poly ethylene oxide, PEO) could improve the overall mechanical properties of a chemically or physically cross-linkable hydrogel system. The choice of the polymer (PEO) is based on their extensive application in the biomedical field and their ability to exhibit physical interactions with Laponite nanoparticles, to form a physical aggregates, that is characterized by high viscoelasticity and forming a reversible shear force-sensitive gels (shake gels) in aqueous solution. The formation of physical gels under shear force was initially attributed to the enhanced interactions between Laponite nanoparticles through their increased surface area under shear force, and this clearly indicate that the Laponite nanoparticles act as multifunctional cross-linkers through secondary interactions with polyethylene oxide chains.

2.1.1 Synthetic hectorite clay: Laponite

Laponite is a synthetic disc-shaped crystalline silicate particles, with 25 nm in diameter and 1,0 nm of thickness, 2,53 g/cm^3 of density and with an empirical formula of $Na^{+0.7}[(Si_8Mg_{5.5}Li_{0.3})O_{20}(OH)_4]^{0.7-}$, which is used basically to modified the rheological properties of liquids or colloidal solutions and as a film former. As a rheological modifier, Laponite may be added to the formulation of many waterborne products such as surface coatings, household cleaners and personal care products. It often impart thixotropic shear, sensitive viscosity and improve stability and syneresis control. While as a film former, Laponite is also used to produce electrically conductive, antistatic and barrier coatings systems. Laponite (hydrous sodium lithium magnesium silicate) is a synthetic crystalline layered silicate colloid, with crystal structure and composition closely resembling the natural smectite clay hectorite. It is manufactured by Rockwood Additive Ltd, Cheshire UK, and Southern Clay Products, Inc., Gonzales, Texas.

Laponite is chemically composed of SiO_2, 62.82%; MgO, 30.15%; Na_2O, 3.2%; LiO_2, 0.83%. And the unit cell of the crystal is comprised of six octahedral magnesium ions sandwiched between two layers of tetrahedral silicon atoms, with these groups balanced by twenty oxygen atoms and four hydroxyl groups. When Laponite is dispersed in water, the Na+ ions are released into solution, creating a double layer that causes the particles to repel each other, initially stabilizing the particles, the particles or the suspensions are stable up to concentrations of 2.5-3% by weight, at these concentrations or greater in water, gels are formed rapidly.

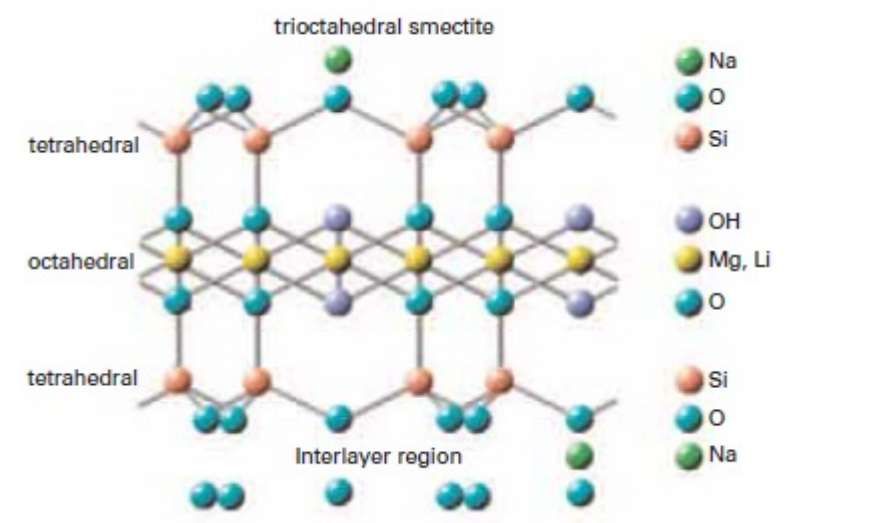

Figure 2 *Laponite idealized structural formula (Rockwood Additives Limited).*

Gelation process occurred when Laponite is dispersed in water because the electrostatic attractions draw the sodium ions which go into solution towards the crystal surface and osmotic pressure from the bulk of water pulls them away, this lead to the formation of an electrical double layers, because an equilibrium state is established where the sodium ions are held in a diffuse region on both side of the dispersed Laponite crystals. So, when two particles approach, their mutual positive charges repel each other and the dispersion exhibits low viscosity and Newtonian type rheology. Addition of polar compounds in solution, for instance, simple salts, surfactants or coalescing solvents, to the dispersion of Laponite will reduce the osmotic pressure holding the sodium ions away from the particle surface. This makes the electrical double layer to contract and allows the weaker positive charge on the edge of the crystals to interact with the negative surfaces of adjacent crystal, leading to an increase in the formation of gel structure that is held together by some weak electrostatic forces, and the gel is characterized by a strongly thixotropic behavior.

Laponite shows a greater degree of shear thinning than other commonly used thickeners or clay, because the gel structured that is formed when dispersed in water is held together by an ionic bonds, and the gel structured can be easily broken down on application of shear stress, followed by a rapid decreased in the viscosity of the structure under shear. After the shear stress is removed, the gel reforms and the rate of restructuring depend on the composition, electrolyte level, age of the dispersion and the temperature of the system.

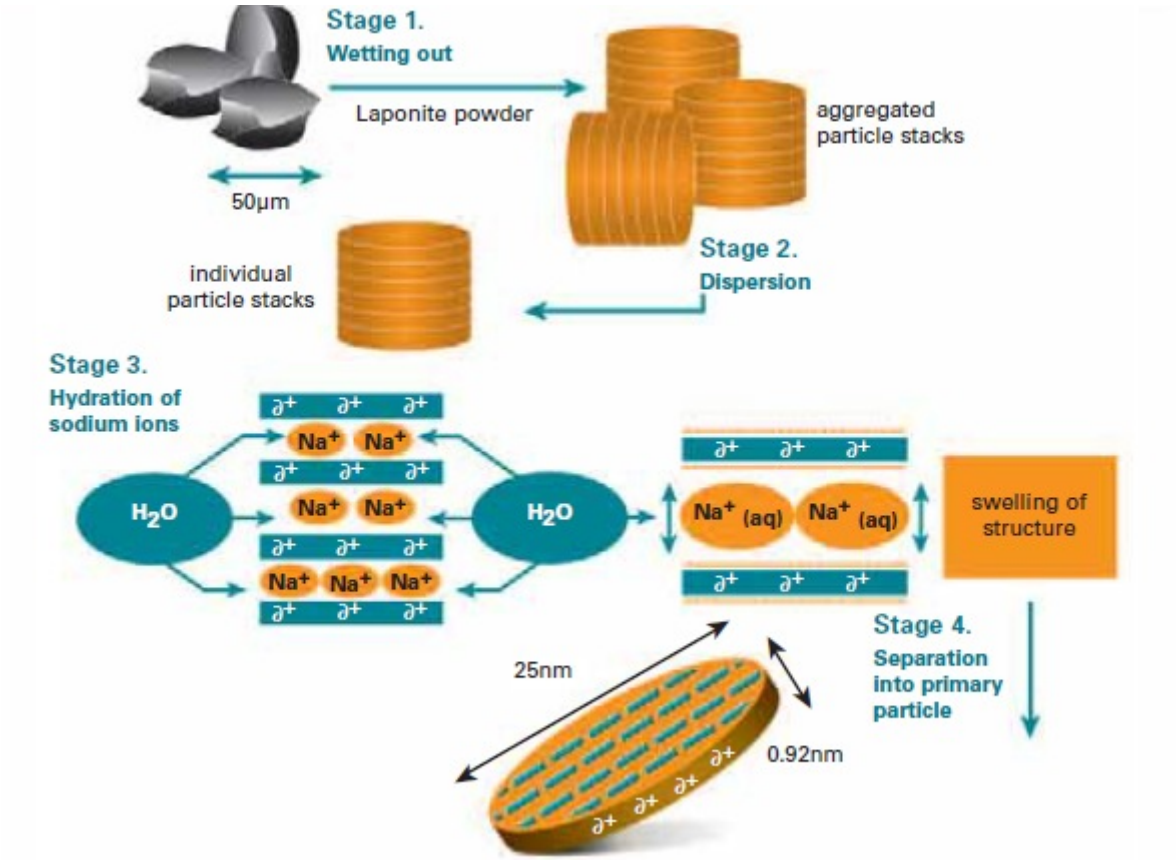

Figure 2.1 *Schematic representation of the electrostatic interaction that occurred when Laponite is dispersed in water (Rockwood Additives Limited).*

Table 2. *Properties and benefits of the dispersion of Laponite in water (Rockwood Additives Limited).*

Properties	Benefits
Synthetic layered silicate	High purity Colorless dispersion Excellent consistency Free from abrasives
Inorganic material	Cannot support microbial growth Not affected by high temperature Non toxic Non flammable
Colloidal sized primary crystal	Produces clear gels or sols in water Disperses rapidly in water without the need for high shear. Elevated shear thinning behavior

2.1.2 Polymer (polyethylene oxide)

The premier material used today for both drug delivery, cell encapsulation and as adhesion promoters is Poly (ethylene glycol) hydrogels, (PEG). PEG, otherwise known as poly (oxyethylene) or poly (ethylene oxide), is one of the most widely used hydrogels in medicine and biomedicine. Hydrogels based on its derivatives: polyethylene glycol methacrylate (PEGMA), polyethylene glycol dimethacrylate (PEGDMA) and polyethylene glycol diacrylate (PEGDA) are also used in the stated fields.

Materials with molecular weight lesser than 100,000 g/mol are usually called PEGs, while those with higher molecular weight are called PEOs. And these materials are known as hydrophilic monomers that provide a distinct advantage in both fabrication and application of hydrogels. They are characterized by their high biocompatibility, flexibility, lack of toxic influences on surrounding tissue and solubility in water, which makes them good candidates for drug delivery system applications.

In this study, we have evaluated and analyzed the interactions between Laponite nanoparticles, collagen and polyethylene oxide polymer chains, by quantifying the amount of physical gels that are formed under some certain experimental conditions, by varying the Laponite nanoparticles concentrations with different molecular weight of PEOs chains: one with an average molecular weight (Mw) of 100,000 g/mol and the other with 600,000 g/mol. We have found out that the PEOS chains with the Mw of 100,000 g/mol can indeed interact with Laponite nanoparticles in water to form physical gels, but the amount of physical gel formed was dependent both on the concentration of Laponite and on the Mw of the PEOs chains.

Meanwhile, the physical gels formed with the low Mw PEOs polymer were not mechanically robust and stable, as they could not withstand rigorous shear for extend period of time. While the amount of physical gel that were formed for the same concentration of Laponite with the high Mw PEOs chains was higher as compare to the low Mw counterparts. And this is due to the enhanced interactions between the PEOs chains with high molecular weight and Laponite nanoparticles.

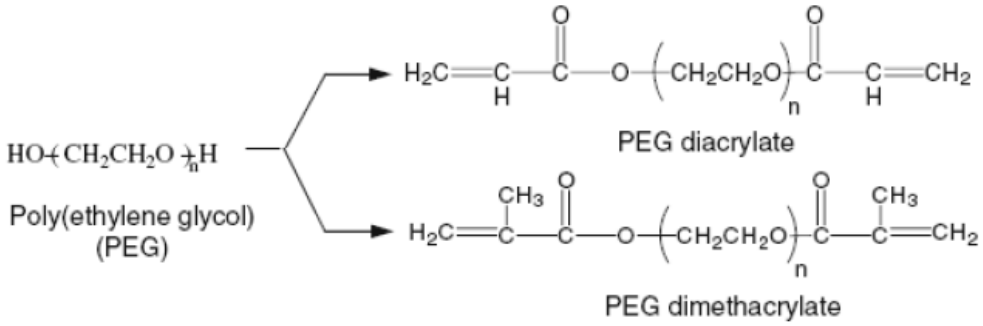

Figure 2.2 *Poly (ethylene glycol) and its derivatives (Iwona & Helena, 2010).*

Poly (ethylene oxide) hydrogels drug release systems are stimuli sensitive and react in the presence of a physical or chemical (biological) agent. Due to their unique properties, hydrogels used for these kinds of drug release systems are often called “intelligent gels” or “smart gels”. Some examples of the physical stimuli can be that of the temperature, solvent, light, radiation, a magnetic, acoustic or electrical field, while the chemical and biological stimuli include pH, specific ions and molecular recognition events (Miyata & Uragani, 2002). PEOs polymer hydrogels that are formed through a chemically cross-linked process are often used in the manufacture of synthetic, biologically active hydrogel scaffolds for protein recombination and functional tissue production. But the main field of PEO hydrogel applications is in the drug delivery system, as drug carriers in the efficient and controlled release systems of drugs, proteins, biomolecules and growth factor distribution (Park & Kim, 2002).

2.1.3 Collagen

Recently, the formation of hydrogels with the used of some naturally derived polymers: collagen, alginate, agarose, fibrin, gelatin and others, have found a great interest in tissue engineering applications, due to the fact that they are either components of or have macro-molecular properties similar to that of the natural extracellular matrix (ECM). Collagen is the main component of mammalian connective tissue, and it is the most abundant protein in mammals, comprising of about 25% to 35% of the whole body protein content. Collagen in the form of elongated fibrils, is mostly found in fibrous tissues such as tendons, ligaments and skin, and also abundant in corneas, cartilage, bones, the gut, intervertebral discs and blood vessels. Along with soft keratin, collagen is responsible for the skin strength and elasticity,

and its degradation leads to wrinkles that accompany ageing. It strengthens blood vessels and play important role in tissue development.

Collagen is an attractive material for biomedical applications as it is the most abundant protein in mammalian tissues and is the main component of natural extracellular matrix. There are about almost 28 types of collagen, but about 80-90% of the collagen in the body are consists of types I, II, and III. These collagen molecules are packed together to form long thin fibrils of similar structure. The basic structure of all collagen is composed of three polypeptide chains, which wrap around one another to form a three stranded rope structure. The strands are held together by both hydrogen and covalent bonds. Collagen fibers and scaffolds can be created and their mechanical properties can be enhanced by introducing various chemical cross-linkers (formaldehyde, carbodiimide) or through crosslinking with physical treatments (UV irradiation, freeze-drying, heating) and also by blending them with other polymers: PEO and chitosan (Park & Kim, 2002).

We made used of collagen type I in this study for the synthesis of the hydrogels, and collagen type I is the major structural component of extracellular matrix consisting of 300kDa molecules, which is found in connective tissues and internal organs, but it is most prevalent in the dermis, tendons and bone.

For these reasons, collagen type I is exploited to promote cell attachment, proliferation, differentiation, migration and tissue morphogenesis during development. The collagen I (6 mg/ml) was extracted from a rat tail, courtesy of the department of Tissue Engineering, University of Würzburg. Some basic characteristic of collagen is that it is highly biocompatible and can be easily injected in vivo, it has the correct properties for tissue regeneration such as pore structure, permeability, hydrophilic and it is stable in vivo. Collagen scaffolds are also ideal for deposition of cells, such as osteoblasts and fibroblasts, and once inserted, growth is able to continue as normal in the tissue. Recently, it has also been used as a natural wound dressing. Because it has properties that artificial wound dressing materials do not have. It resist against bacteria, which is of vital importance in wound dressing, so its helps to keep the wound sterile, due to its natural ability to fight infection.

2.1.4 Phosphate buffered saline solution

Phosphate buffered saline (PBS) is a buffer solution which is commonly used in biological application, it is a water-based salt solution, comprised mainly of sodium phosphate, sodium chloride, potassium chloride and potassium phosphate. PBS has many applications because it is isotonic and non-toxic to most cells, while the osmolarity and the ion concentrations of the solutions match those of the human body (isotonic).

Table 2.1. *Composition of the phosphate buffer saline solution (1x)*

Salt	Concentration (g/l)	Concentration (mmol/l)
NaCl	8,0	137
KCl	0,2	2,7
Na_2HPO_4	1,44	10
KH_2PO_4	0,24	1,8

All the salts components are dissolve in 800ml of de-ionized water, and the pH of the system is taken to 7.4 by adding an acid (HCl) or a base (KOH), and later brought to a final volume of 1L, by adding 200ml of de-ionized water.

2.2 Samples preparation and experimental conditions

The formulation and combination of synthetic and natural polymers with nanoparticles and biomolecules synergistically allows combining advantageous chemical, physical and biological properties to produce novel hydrogels that support the reparation and regeneration of human tissues and body functions. Incorporation of silicate nanoparticles such as Laponite with a solution of natural and synthetic polymers: collagen and poly(ethylene oxide), adds mechanical strength to hydrogel materials. However, charged nanoparticles, such as silicates, may exfoliate easily in water due to the colloidal interactions that stabilize the resulting gel. Meanwhile, nanoparticles that are neither charged nor stabilized by salt or polymer usually formed aggregates, and these aggregates strongly affect the morphological structure of a novel hydrogel and its mechanical properties. So, in order to prepare a stable hydrogel, the nanoparticles need to be well dispersed and the resulting large scale needs to be well controlled.

The typical Laponite concentration used are in between 2.5-5% by weight, above which they formed an irreversible gel, for instance, a Laponite stock solution of 5% was

prepared by adding 5 grams of Laponite powder to 100 ml of de-ionized water or to a phosphate buffer saline solution (PBS) and later mixed together with a vortex system for few seconds. The solution is initially turbid, but becomes clear in about 30 minutes, indicating that the particles are fully hydrated.

While the poly(ethylene oxide) stock solution we used is 2% by weight, in which 2 grams of the polymer was added to 100 ml of de-ionized water or the phosphate buffer saline solution. This solution was put to rest and allowed to dissolve for a few days. The Laponite and polymer stock solutions are mixed together by adding the polymer stock solution to the Laponite stock with a pipette and then gently tilting the mixture back and forth to ensure complete mixing. Different samples are prepared in various ratio, but with a total concentration or volumetric fraction of 2.5% (for a sample of 1,9/0,5/0,1, means it contains 1,9% of Laponite, 0,5% of poly(ethylene oxide) 600 and 0,1% of collagen I), in order to be able to explore the wide range of the hydrogels behavior.

Once the samples are shaken vigorously several times for a period of 5 seconds with a vortex mixing system, the resulting hydrogels formed, show an increase in the viscosity, turbidity and mostly, we observed the appearance of bubbles suspended within the sample, showing the difficulties in obtaining a completely homogenous hydrogel sample. At rest, all suspensions are clear and show low viscosity Newtonian-fluid-like behavior initially, where the viscosity is not a function of the applied shear rate. But a subsequent vigorous shaking of the samples with a vortex, makes the samples to exhibit an intriguing behavior; the non-linear shear created by agitating the samples, causes some the mixtures to form macroscopic, heterogeneous gels. And these gels are so elastic that even upon inverting the vials, the samples remain stationary and sometimes they might also flow down through the vial. If these samples are left undisturbed, the gels can relax back to the fluid state, in time scales that range from a few seconds to many hours and mostly to several days or months. But we have to take into account that all samples that formed shake-gels are reversible.

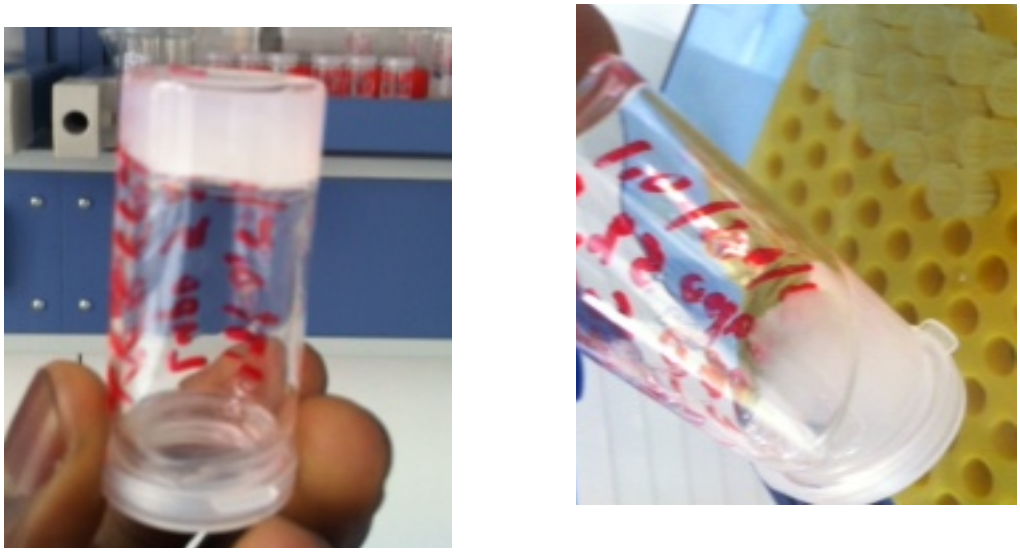

Figure 2.3. *Samples of solid and sol gel that were formed upon inverting the vials.*

In most cases, shear may induce the development of new structures in the hydrogels, but it is also possible that already existing structures might disappear in some cases. For example, the interactions between the PEO-Laponite aggregates in hydrogels can be easily broken up and disappear with the application of a critical shear. So, it is of great importance to find the optimal shear that must be applied to the system, in order to avoid the destruction of the interactions between the PEO-Laponite aggregates.

The hydrogel structures and viscoelastic properties can be tuned by changing the parameters such as composition, pH, temperature and ionic strength. For instance, varying the Laponite and the polymers compositions, it is easy to generate flowing gels, shake gels or permanent hydrogels. So, the shear induced gelation of shake gels is reversible and strongly depending on the concentration of the PEO, collagen, time and temperature.

At very low concentration of PEO, most of the chains are adsorbed and bridging can only occur over a small fraction of the silicate particles population. Because most of the particles are individual primary particles and the samples behave like pure clay dispersion of similar concentrations, and this is defined as the stable sol phase, which is characterized by a low viscosity.

At higher PEO concentrations but below the surface saturation (with Laponite concentration higher than that of the polymer), there is enough polymer to cause the macroscopic flocculation of the particles, and forming some bridges that are stable because the surfaces of the silicate particles are not saturated with polymer. This is evidenced

macroscopically as a turbid gel that shows no relaxation is formed, also known as permanent gel and with high viscosity. A further increase in the concentration of the polymer (with Laponite concentration lower than that of the polymer), lead to the formation of a sol gel, a low viscosity liquid state, due to the fact that there is a lack of silicate particles or surfaces on which the polymer can be adsorbed.

So, the interaction between silicate or Laponite nanoparticles and the polymer are strongly influenced by shear and composition of the hydrogel components, which contributes to the complex behavior of the hydrogels. The soft, rubbery consistency and the flexibility in varying the mechanical properties make these hydrogels potential candidates for many technical applications, especially in the biomedical field.

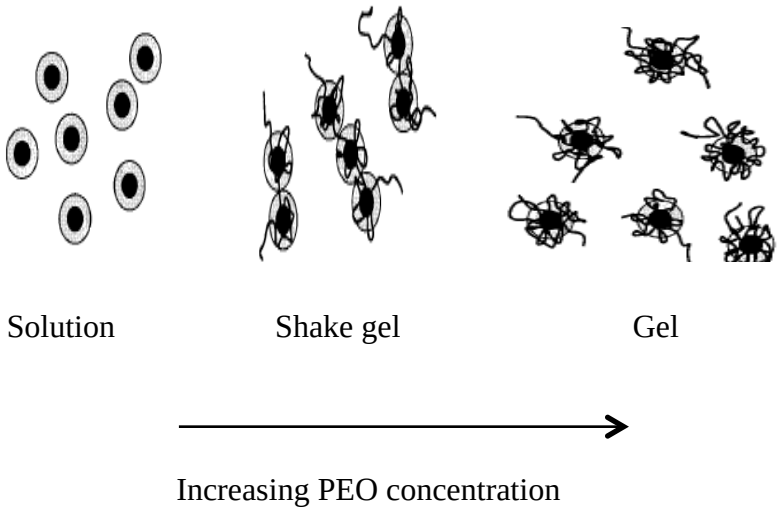

Figure 2.4. *Gelation process during the mixture of the silicate nanoparticles with the polymer.*

Another method we have used for the preparation of the hydrogels is with the used of the so called Sulzer Mixpac AG syringe system, a system that was constructed mainly for the mixing of two components, in our case, Laponite and the polymer (PEO) in different ratios. The main purpose we have used this system is to figure out the possibility of obtaining a homogeneous hydrogel structure, without the formation of any bubble in the system. Meanwhile, this was not always possible, and the degree of gelation was lower than that of the hydrogels prepared in the vials and mixed together with the use of a vortex system.

However, the situation was slightly improved when the length of the tip of the mixing section was reduced.

*Figure 2.*5. *Sulzer Mixpac syringe system for the preparation of the gels.*

Chapter 3

Techniques for the characterization of the hydrogels

3.1 Mechanical properties

Mechanical properties of hydrogels are very important from the biomedical and pharmaceutical point of view, and the evaluation of the mechanical properties is essential in various biomedical applications, such as in ligament and tendon repair, wound dressing material, cartilage replacement, matrix for drug delivery and as well in the field of tissue engineering. Understanding and controlling the mechanical properties, specifically softness, is very important for appropriate physiological function in numerous contexts. And mostly, hydrogels mechanical properties should be such that it can maintain its physical texture during the delivery of therapeutic moieties for the predetermined period of time.

The desired mechanical properties of a hydrogel might be obtained by incorporating crosslinking agents, co-monomers and by changing the degree of crosslinking, increasing the degree of crosslinking a stronger gel could be achieved, although, the higher the degree of crosslinking, the lower is the degree or the percentage of the hydrogel elongation, and the resulting hydrogel formed in this case, will be characterized by lower elasticity and formation of brittleness structure. Elasticity of the gel is very important to give higher flexibility to the cross-linked chains and to facilitate the movement of the incorporated bioactive agent. Thus a compromise between mechanical strength and flexibility is necessary for appropriate use of the hydrogels, and in order to do this, it is necessary to find the optimal degree of crosslinking for the gels.

The mechanical characterization is mainly done through the relaxation experiments (normal stress relaxation at constant frequency) to determine the hydrogel linear viscoelastic range and to define the relaxation spectra and Young modulus by using the generalized

Maxwell model. And the basis of Young modulus and Flory's theory, it was possible to determine the hydrogels crosslinking density.

Most research in the field of tissue engineering has focused their attentions on the biochemical agents that determine tissue function, with resulting mechanical properties considered a byproduct of the necessary biological functions. Concentrations, concentration gradients and spatial orientation of an immense number of growth factors, extracellular matrix molecules, steroids, hormones and adhesion molecules are critical mediators of the interactions between cells and their environments. Numerous dysfunctions and disease states can be viewed in part as a failure of the mechanical components of tissues. For instance, emphysema, a chronic alveolar lung disease is characterized by a loss of mechanical elasticity, induced by both biochemical changes to the extracellular matrix of the lung and forces produce during respiration.

Healthy lung tissue has been shown to have an elastic modulus in the range of 5-30 kPa when deformed at physiological condition, whereas tissue treated with proteases to mimic progression of alveolar disease showed a loss in mechanical rigidity between 33% and 47 %. Similarly, lung fibrosis is characterized as stiffening of the lung parenchyma, and is concomitant with an increase of almost 50% in the mechanical resistivity of lung tissue (Ilya & Penelope, 2007).

Despite the inherent complexity of hydrogels stiffness, many different hydrogels have been tested, by using a variety of experimental modalities, and comparisons of stiffness can be made from measurements at similar time-scales and strain magnitudes. Due to the large differences in stiffness between some tissues, most mammalian organs have an elastic modulus between the values of 100 Pascal for the softest organs such as the brain, to tens of thousands of Pascal in muscle tissue, and sometime on the order of Megapascal for the cartilages (Ilya & Penelope, 2007).

Table 3. *Experimental elastic moduli of a variety of tissues, including the animal of origin of the tissue and the testing modality used to determine the elastic modulus (Ilya & Penelope, 2007).*

Tissue types	Animal	Elastic modulus	Testing method
Spinal cord	Human	89 kPa	Tension
Thyroid cancer	Human	45 kPa	Compression
Thyroid	Human	9 kPa	Compression
Breast tumor	Human	4 kPa	Compression
Premalignant breast	Human	2,2 kPa	Compression
Fibrotic liver	Human	1,6 kPa	Compression
Liver	Human	640 kPa	Compression
Lymph containing metastases	Human	330 Pa	Vibrational resonance
Lymph node	Human	120 Pa	Vibrational resonance
Mammary gland	Human	160 Pa	Compression
Fat	Human	17 Pa	Indentation
Achilles tendon	Rat	310 MPa	Tension
Articular cartilage	Bovine	950 MPa	Compression
Skeletal muscle	Rat	100 kPa	Tension
Spinal cord	Rat	27 kPa	Tension
Cardiac muscle	Mouse	20-150 kPa	Tension

3.1.1 Gel Rheology

The mechanical properties of the hydrogels have been evaluated through the rheological analysis. Since biological tissues are structurally complex and often anisotropic, rheological parameters are usually functions of time, the degree of deformation and the geometry of the applied deforming forces.

In this study, an instrument named Anton Paar Physica 301 Rheometer, was used to determine the viscoelastic behavior of the hydrogels, in particularly, two different methods are available for the description of these behavior: static and dynamic method. Static test involved the imposition of a step change in stress or strain and the observation of the subsequent development in time of the strain or stress. While dynamic method involves the application of a harmonically varying strain.

The scaling of time in rheology is achieved by means of the "Deborah number" and normally one with knowledge of the QWERTY keyboard will notice that the letter "R" and "T" are next to each other. One consequence is that any book on rheology has at least one

incorrect reference to theology. Meanwhile in the fifth chapter of the book of Judges in the Old Testament, Deborah is reported to have declared "The mountain flowed before the lord.." , in the field of rheology, the Deborah statement can be understand in the sense that " everything flows if you wait long enough, even the mountains! (Barnes & Hutton, 1993).

On the basis of this reference, in rheology, the dimensionless Deborah number was defined.

$$De = \tau / T \tag{3.1}$$

Where T is a characteristic time of the deformation process being observed and τ is a characteristic time of the material. The time τ is infinite for a Hookean elastic solid and zero for a Newtonian viscous liquid. High Deborah number corresponds to a solid-like behavior and low Deborah number to a liquid-like behavior. Meanwhile, Deborah dimensionless number was also used to describe the fundamental aspect of the swelling process, in this case, Deborah number is described as the ratio between the characteristic time of the polymer-solvent relaxation process, and the characteristic time of the diffusion of the solvent molecules in the system. In this case, when:

$De >> 1$, then there is no substantial changes in the polymer structure with time, during the hydration process.

$De << 1$, means that the polymer is subjected to a rapid structural changes.

For the above two situations, with $De >> 1$ and $De << 1$, the Fickian diffusion mechanism is predominant, while for process with Deborah number almost equal to one, the swelling process will no longer be controlled by the Fickian diffusion mechanism (non Fickian or anomalous transport), due to the fact the relaxation process is influencing the diffusion rate.

Rheology can be defined as the science that study the deformation and flow of matter, and this has been used for characterizing the viscoelastic properties of the hydrogels, in other words, hydrogels properties such as rigidity or storage modulus and viscosity can change with an applied stress. The change can occur either instantaneously or over a long period of time, and it can appear as either an increase or decrease of the material parameter. Most hydrogels exhibit a shear thinning behavior, a typical example of non-linearity, due to the fact that they undergo a decrease in the viscosity with increasing shear rate in steady flow. So, they are

classified as a “thixotropic” material; a time dependent shear thinning followed by a recovery state.

In the dynamic method experiment, rotational and oscillatory shear process were used to determine some viscoelastic properties of the hydrogels, by imposing a constant angular frequency of 1 rad/s and a variable strain (deformation) in the oscillatory test, while in the rotational test a variable shear rate was imposed. And the materials responses in terms of elastic modulus (G´), viscous modulus (G”), viscosity and shear stress were also measured.

The storage or elastic modulus (G´) and the loss modulus (G”) of the hydrogels have been used to differentiate an uncross-linked hydrogel from a cross-linked hydrogel. When G” is much higher than the G´, the hydrogel is said to be uncross-linked, and this is mainly due to the fact that the viscous property is higher than the elastic property over the entire period of deformation. With G´ higher than the G” for the partially cross-linked hydrogel, and at the same time, showing an increase in the slope of the elastic modulus curve, which indicates an elastic behavior of the gel.

For hydrogels, which are highly cross-linked polymer networks, both G´ and G” are very high and are nearly parallel to each other. The G´ and G” values of a hydrogel is measured in the linear viscosity range, and in the case of uncross-linked gel, the point where the G´ and G” intersects each other is known as the yield point or cross-over point, and it also denote the gel-sol transition point or deformation.

We have also observed that with the rheological studies, it was possible to study the physical interactions within the hydrogels; the addition of the natural and synthetic polymer to the silicate particles, leads to the formation of physical hydrogels, due to the formation of intermolecular hydrogen bonding among the molecules, when the concentration of the polymers were varied, with a subsequent development of a flow behavior typical to that of the non-Newtonian materials.

The determination of the mechanical properties of the hydrogels can be done through the use of four possible configurations or geometries; the parallel plate-plate system, the cone-and-plate geometry, coaxial cylinder and the coaxial double gap configuration. But, due to the difficulties in handling the hydrogels samples and for the total amount available, we have decided to measure the mechanical properties only with the use of the parallel plate-plate geometry, which configuration is shown in the following figure:

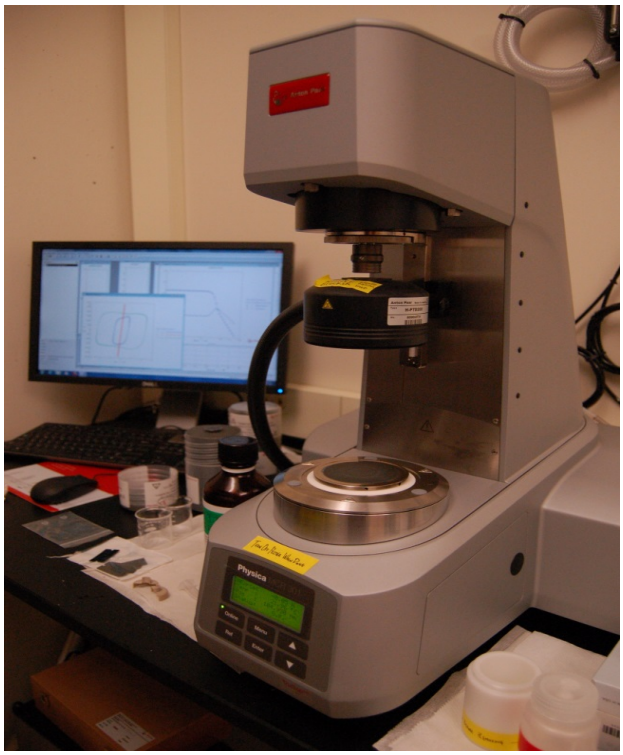

Figure 3.1. *Anton Paar Physica MCR 301 Rheometer, used for the measurement of the hydrogels mechanical properties.*

The figure above is referring to the Anton Paar Physica MCR 301 Rheometer that was used for the determination and the studies of the rheological properties of the hydrogels, both in dynamic and static method measurement. The major specifications of this instrument are listed below:

- Frequency range: 10^{-5} – 10^{2} Hz
- Temperature range: -40 – 200°C ± 0,01°C
- Shear rate range: 10^{-5} – 10^{4} s^{-1}
- Geometries: various geometries are available:
  - Cone – plate (CP50): R = 25mm; α = 0,991°, sample volume~ 0.6 ml
  - Plate-plate (PP25): R = 12.5mm, sample volume for 1 mm gap ~ 0.5 ml
  - Coaxial cylinder (CC27): gap width: 1.128 mm
  - Coaxial double gap (DG26.7): gap width: 0.42 mm and 0.46 mm; sample volume: 3.8 ml for low viscosity samples (0.4 mPas < η < 200 mPas)

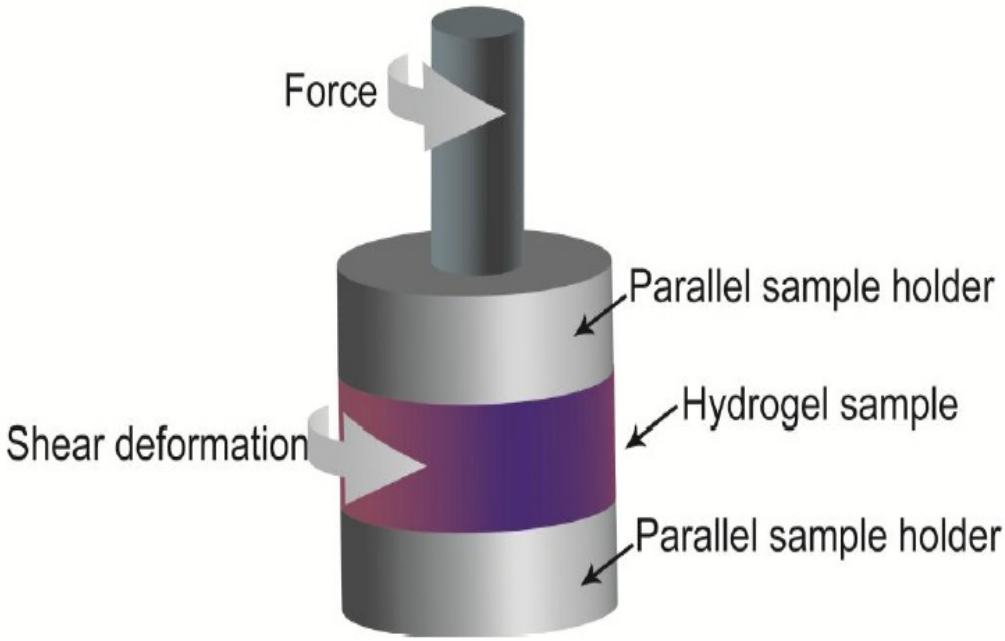

Figure 3.2. *Parallel plate-plate configuration for the rheological measurement (www.tainstruments.com)*

The above configuration in figure 3.2 shows the typical geometry of the parallel plate-plate configuration of the rheometer that was used for the measurement of the viscoelastic properties of the hydrogels. The upper and mobile plate that was used has a diameter of 25 mm, and the distance between the two plates was maintain constant at a value of 0,25 mm for all hydrogels samples. The reason for using this minimum distance value is due to the fact that increasing the distance between the two plates, a higher quantity of gel will be required in filling the space for the measurement.

3.2. Hydrogel swelling behavior, water contents, pore and permeation in hydrogels

One of the most important properties of hydrogels, which makes it favorable for various use in medical, biomedical and pharmaceutical field arise mostly from its cross-linked structure. The cross-linked structure of the hydrogels is determined mainly by the nature of the monomers, the nature of the crosslinking agent and finally, the method of preparation.

The study of the hydrogels swelling behavior is the most common approach which is used for understanding the cross-linked structures of the hydrogel. The swelling of the hydrogel has been studied and some certain parameters of swelling have been calculated. Meanwhile, the knowledge of the swelling characteristics of the gel is the first step in understanding the network structure of the gel and its capacity to function as a drug delivery carrier. Once the hydrogel is exposed to solvent, the gel swells, and the thermodynamically driven swelling force is counterbalanced by the retraction force of the cross-linked structure, leading to an equilibrium state. This swollen state allows widening of the gap between the crosslinks and mesh size, thus facilitating the transfer of different solutes through the gel. The transfer of solute is controlled by the swelling of the gel, and once this information is known, the gel can be manipulated by varying the mesh size and the property of the drug to enable diffusion of required drug in specific manner.

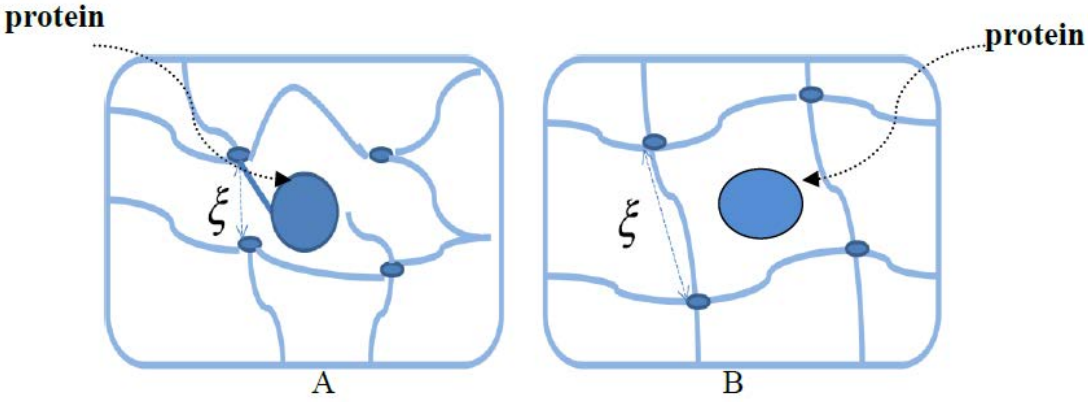

Figure 3.3. *Mesh sizes in hydrogels at (A) de-swollen state (B) swollen state. Where ε is the mesh size.*

As said earlier, the mesh size ε is one of the most important parameter in drug release process, and it can be calculated theoretically or measured through different techniques: microscopically, by light scattering, elasticity and swelling process measurement.

The determination of the amount of water imbibed within the hydrogel is an important criterion for characterizing the hydrogel for biomedical applications and is often represented by the degree of swelling (%S). The degree of swelling of the hydrogel is directly proportional to the amount of water imbibed within the hydrogel, and the amount of water imbibed within the hydrogel influences the diffusional properties of a solute through the hydrogel. Normally, the higher the degree of swelling, the higher is the amount of water imbibed and the higher is the diffusion rate of the solute. But other factors, such as the micro-

architecture of the polymer chain, may also play an important role in the diffusion rate of the solute through the gel.

Figure 3.4. *Swelling and de-swelling process of a hydrogel system*

Experimentally, the degree of swelling can be determined by weight difference method, and it is expressed by the following equation.

$$\%S = (W_s - W_d)/W_d * 100 \quad (3.2)$$

where, W_s = initial weight of the swollen gel, and W_d = final weight of the dry gel.

For the swelling degree analysis, we have considered two different methods for the preparation of the hydrogels, some hydrogels samples were prepared in a phosphate buffer saline solution (PBS), and others were prepared in de-ionized water. The reason this was done was to study the different swelling behavior of the gel in different medium.

3.2.1 Water in hydrogels

The water content in hydrogels is necessary for controlling the permeation of nutrients into the cells and of cellular products out of the hydrogels. Another interesting point is that it provide a moist environment, which for instance is of great important in wound healing. Dried hydrogels can swell in water or in a saline solution to about 1000 times their own weight.

The amount of water absorbed in the hydrogels is usually expressed as the equilibrium water content (EWC), which mathematical formulation is the following:

$$EWC = (W_w/W_t)*100\% \quad (3.3)$$

where W_w is equal to the weight of water in the gel, and W_t is the total weight of the hydrated gel.

The equilibrium water content is another significant property of hydrogels, since the amount of water in hydrogels structures, guarantee a unique property and make them a potential medium for different applications in biomedical fields. The swelling of hydrogels is quite a complicated process and consists of a number of steps. In the first step, water molecules entering the hydrogel matrix hydrate the most polar, hydrophilic groups, and these lead to the formation of a primary bound water. In the second step, hydrophobic groups are exposed, and they interact with water molecules, leading to the appearance of the so-called hydrophobically bound water or secondary bound water. Primary and secondary system are bound together to form a total water bound system. While in the third step, an additional amount of water is absorbed, due to the fact the osmotic driving force of the network towards infinite dilution is resisted by the covalent or physical cross-links.

So, the amount of water accommodated by a hydrogel structure can be classified into four types: the water in the outermost layer is called free and be easily removed from the hydrogel under mild conditions. Secondly, the interstitial water is the type of water which is not attached to the hydrogels network, but physically trapped in between the hydrated polymer chains. Thirdly, the bound water is directly attached to the polymer chain through hydration of functional groups or ions. The bound water remains as an integral part of the hydrogels structure and can only be separated at very high temperatures. Finally, the semi-bound water is a type of water with intermediate properties of a bound water and free water. Although other layers of water can be accommodated into the hydrogels structure, these have much weaker interactions with functional groups and ions as they are farther away from the functional regions. Meanwhile, the free and the interstitial water can potentially be removed from the hydrogels simply by centrifugation and mechanical compression (Kelvin, 2000).

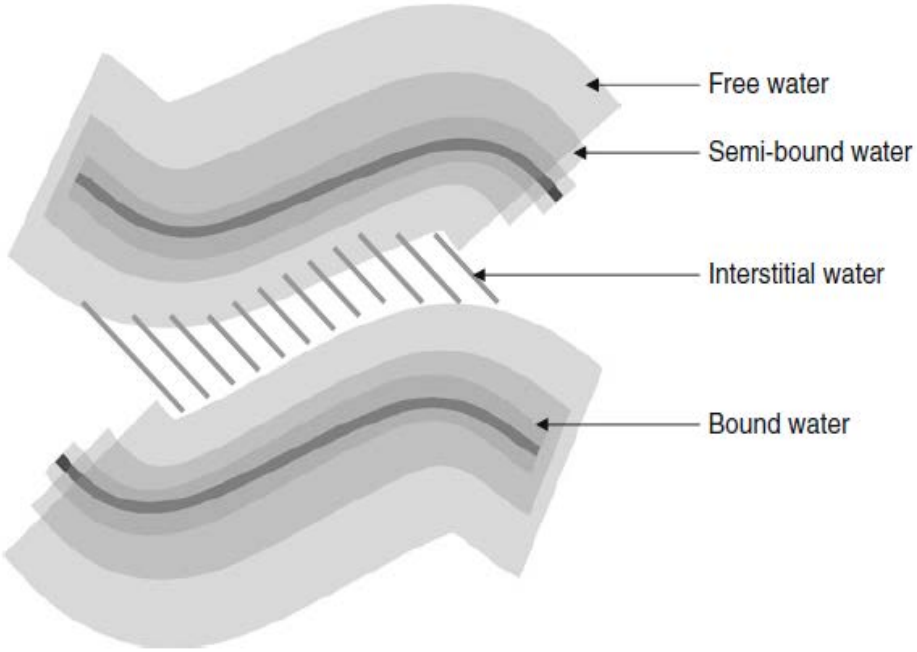

Figure 3.5. *Different phases of water distribution in hydrogels*

The water absorbed up to the equilibrium swelling level is called bulk or free water; it fills the space between the network chains and the centers of larger pores, macro-pores or voids. Considering the fact that water acts as a plasticizer in a hydrophilic polymer networks system, the swelling process of the hydrogels can be considered under rubbery state and can be described by the free energy of mixing ΔG_{mix} from the polymer and solvent interaction (the result of the spontaneous mixing of fluids molecules in the polymer chains, and is a measure of how compatible the polymer is with the molecules of the surrounding fluid) and the elastic $\Delta G_{elastic}$ from the cross-linked network (a contribution deriving from the elastic forces developed inside the gel).

The three forces; polymer-water interactions, electrostatic and osmosis, expand the hydrogels network. Hydrogels swelling, by definition, is the restricted solubility. In other words, infinite solubility of a hydrogel is prevented by elastic forces, which originate from the network crosslinking. The balance of these two different forces determines the equilibrium hydrogels swelling.

$$\Delta G_{system} = \Delta G_{mix} + \Delta G_{elastic} \tag{3.4}$$

$$\Delta G_{mix} = kT [n_1 ln v_1 + n_2 ln v_2 + X n_1 v_2] \quad (3.5)$$

where,

n_1 = moles of the swelling agent (water)

n_2 = moles of polymer

v_1 = volume fraction of the swelling agent

v_2 = volume fraction of polymer

K = Boltzmann constant

X = Flory polymer – solvent interaction parameter, dependent on the chemical structure of the system.

While the deformation process occurred without a reasonable change in the internal energy, thus, the internal energy can be neglected, and therefore, the elastic free energy is defined as followed:

$$\Delta G_{elastic} = - T \Delta S_{elastic} \quad (3.6)$$

where $\Delta S_{elastic}$ is the entropy variation in the deformation process. For isotropic swelling, the elastic free energy is defined as followed:

$$\Delta G_{elastic} = (\frac{kTv_e}{2})(3\alpha_s^2 - 3 - ln\alpha_s^3) \quad (3.7)$$

where v_e is the effective number of chains in the network, and α_s is the expansion factor, expressing the linear deformation of a network structure due to isotropic swelling.

The chemical potential of a solvent in a swollen gel is defined as followed:

$$\mu - \mu_0 = (\Delta\mu)_{elas} + (\Delta\mu)_{mix} + (\Delta\mu)_I + (\Delta\mu)_{elect} \quad (3.8)$$

$(\Delta\mu)_{elas}$ = Variation of the solvent chemical potential, due to the different configuration of the polymeric chain with respect to that of the polymeric network equilibrium.

$(\Delta\mu)_{mix}$ = Variation of the solvent chemical potential after the process of mixing with the polymer

$(\Delta\mu)_I$ = Variation of the chemical potential due to the variation in the mobile ion concentration within and outside the swollen gel (positive with respect to the swollen gel)

$(\Delta\mu)_{elect}$ = Variation of the chemical potential due to the electrostatic repulsive force between the fixed polymeric charges (positive with respect to the swollen gel).

When the swelling process starts, the $\Delta G_{mix} << 0$, $\Delta G_{elastic} > 0$, and $(\Delta G_{mix} + \Delta G_{elastic}) < 0$, so the swelling is favored and the solvent diffuses into the network. During the process of swelling, the ΔG_{mix} and the $\Delta G_{elastic}$ both increases until $|\Delta G_{mix}| = |\Delta G_{elastic}|$ and $\Delta G_{system} = \Delta G_{mix} + \Delta G_{elastic} = 0$, then the driving force for the swelling is exhaust, and the equilibrium swelling is reached, resulting in the cessation of the swelling process. So, an increase in the hydrogel network structure or in the intermolecular crosslinks density increases the impact of the elastic force on the liquid in the hydrogel, and this lead to an increase of the chemical potential of the solvent in the polymer, resulting in a decrease of the amount of water absorbed.

In order to be able to describe the swelling degree and the equilibrium water content of the hydrogels, we need to consider the following mathematical formulations:

$$m_{gel} = m_{poly} + m_{lapo} + m_{H2O} + m_{salt} \quad (3.9)$$

$$m_{(dry\ gel\ +\ salt)} = m_{poly} + m_{lapo} + m_{salt} \quad (3.10)$$

$$m_{dry\ gel} = m_{(dry\ gel\ +salt)} - m_{salt} \quad (3.11)$$

$$m_{H2O} = m_{gel} - m_{dry\ gel} \quad (3.12)$$

With m_{gel} equal to the mass or the weight of the initial swollen gel, m_{poly} is the weight of polymer in the sample, m_{lapo} is the weight of the silicate particles in the sample, m_{H2O} is the weight of the water in the sample, m_{salt} is equal to the weight of the salt content in the sample and finally, $m_{dry\ gel}$ is the weight of final dry gel after the sample has been exposed to the process of exsiccation.

Table 3.1. *Swelling degree for hydrogels samples prepared in de-ionized water, containing Laponite 2,5%, PEO 600 1,5%, and taking all samples to a final volume of 3ml with PBS.*

Volumetric fraction	Initial weight of swollen gel (g)	Weight of dry gel + salt (g)	Salt weight (g)	Final weight dry gel (g)	Swelling degree
1,9/0,25	2,57	0,058	0,0036	0,0544	4620
1,6/0,25	2,79	0,058	0,00031	0,0577	4740
1,3/0,25	1,38	0,039	0,01	0,0290	4660

Table 3.2. *Equilibrium water content for hydrogels samples prepared in de-ionized water, containing Laponite 2,5%, PEO 600 1,5%, and taking all samples to a final volume of 3ml with PBS.*

Volumetric fraction	Initial weight of swollen gel (g), W_t	Weight of dry gel + salt (g)	Weight of water in gel (g), W_w	Equilibrium water content (%)
1,9/0,25	2,57	0,058	2,512	97,7
1,6/0,25	2,79	0,058	2,732	97,9
1,3/0,25	1,38	0,039	1,341	97,2

Almost all the hydrogels samples that were prepared, has a density range that varies between the value of 0,009 – 0,9 g/ml, for the dried and swollen gel respectively.

The determination of the hydrogels swelling degree and its equilibrium water content described in table 3.1 and table 3.2 respectively, was made experimentally, through the use of an instrument named: IR35 Denver Instrument. All samples were subjected to a constant drying temperature of 80°C, and the final dry weight of the gels were obtained after a certain period of time, which varies and depending on the initial weight of the gel.

The Denver instrument moisture analyzer is basically used for fast and reliable determination of the moisture content of materials of liquids, rubbery and solid substances, by using a thermo-gravimetric method. It consists of a heating unit, a weighing system and a display and control unit. The functional aspect of this instrument is based on two procedures: the fully automatic mode and the timer mode. The fully automatic mode is referring to a process when loss of weight on drying follows a clearly delineated steps, which goes from the initial weight of the substance to the moisture phase (evaporation) and then to the final dry

weight, followed by automatic shutoff of the instrument. While the timer mode analysis ends as soon as the specified time has elapsed.

The following units can be selected for displaying analysis results when using the Denver IR instrument: Moisture (%M), Dry weight (%S), Ratio (%MS) and Residual weight (g).

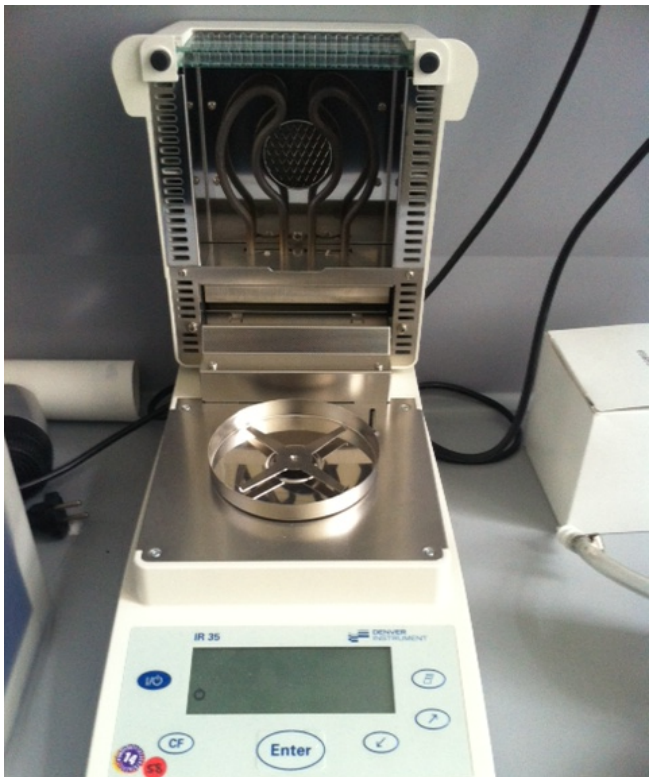

Figure 3.6. *IR35 Denver Instrument for the determination of the hydrogels dry weight*

3.2.2 Pores and permeation in hydrogels

The total amount of water in a hydrogel, for instance, the volume fraction of water, and its free or bound water character will determine the absorption and the diffusion of solutes through the hydrogel. The process of formation of pores in hydrogels might be by phase separation during synthesis or the pores might exist as smaller pores within the network.

The pore size distribution, the average pore size and the pore inter-connections are the most important factors of a hydrogel matrix that are often difficult to evaluate. So the

effective diffusion of a drug system through the hydrogel structures is mostly influenced by the composition and crosslink density of the hydrogel polymer network.

The uniformity of a protein/peptide drug loaded within a hydrogel will depend on the protein/peptide size, shape and net charge; the ionic, polar, apolar groups of the polymer, total available free water within the hydrogel; the addition of partition enhancers to the solution; temperature, pH and ionic strength and as well the drying method, if the hydrogel has been dried, since that often leaves a higher concentration of the drug at the outer regions of the hydrogel. It has been reported that there will always be a portion of the imbibed water in a hydrogel that is not available for drug permeation due to pore “dead ends”, small pores that are less than the diameter of the drug molecule, hydrogen bonded or hydrophobically bound water and drug-matrix polymer interactions (Kuo & Ma, 2000).

So, the release of a macromolecular drug from a hydrogel will be controlled by the pore volume fraction, the pore sizes and their interconnections, the size of the drug molecules and the type and strength of interactions of the drug with the polymer chains that make up the hydrogel network. On the other hand, the key factors that control the pore volume fraction, the pore sizes and their interconnections are the composition of the network polymer chains and the crosslink density. While the interactions of the drug molecules with the network chains will be determined by their respective compositions.

Then it is necessary to take into account the following situation when designing a hydrogel network for controlled release of a drug; it would be necessary to match the polymer composition and crosslink density with the size and composition of the drug molecule to be delivered. It has been mentioned earlier that, with the increase in crosslinking density there is an increase in the hydrophobicity and a decrease in the stretchability of the polymer network structure. Thus increase in crosslinking density results in decreased swelling of the hydrogel and reduced diffusion of the solute. Other factors which affect the diffusion of a solute include polymer molecular weight and solubility, and molecular weight of the solute itself. In general, the lower the molecular weight of the polymer chain, the higher is the diffusion of the solute; polar solutes of low molecular weight show higher diffusion coefficients.

However, it has been reported that for PEO hydrogels, the size and the molecular weight of molecules that are able to diffuse and the rate at which they diffuse both increase as functions of increasing polymer molecular weight and hydrolysable linkages. But in some cases; for alginate and likely other charged polymers, the diffusion rates of charged molecules

are not solely size-dependent, but they are also affected by charge interactions with the negatively charged alginate chains (Lu & Anseth, 2000).

3.3 Hydrogel biocompatibility properties

Hydrogels biocompatibility and non-toxicity is another important issue in the application of hydrogels in the biomedical field. Most polymers that were used for the synthesis of hydrogels in the biomedical field must pass the cytotoxicity and in-vivo toxicity tests.

Biocompatibility is the ability of a material to perform with an appropriate host, responses in a specific application. And it consists mainly of two elements: Bio-safety, a process through which an appropriate host, response not only systematic but also local (the surrounding tissue), the absence of cytotoxicity, mutagenesis and also carcinogenesis. The other element is the Bio-functionality, which means the ability of a material to perform the specific task for which it is intended.

Since the nature of tissue construct is to continuously interact with the body through the healing and cellular regeneration process, as well as during scaffold degradation, the above definition for biocompatibility is of great importance and particularly relevant in tissue engineering field. If these requirements are not reached, the hydrogel can be fouled or there may be damage and scarring to connected tissues. Toxic chemicals that may be used in the polymerization of synthetic hydrogels present challenge for in vivo biocompatibility if conversion is not 100%, because furthermore initiators, organic solvents, stabilizers, unreacted monomers and cross-linkers used in polymerization and hydrogel synthesis may be toxic to host cells if they seep out to tissues or encapsulated cells (Nilimanka, 2013).

In order to remove hazardous chemicals from preformed gels, various purification processes can be followed, such as solvent washing or dialysis. In situ gelation of scaffolds, usually with oligomers and pre-polymers, presents a special challenge since reactants used to synthesize the gel are injected into the body while still in a pre-polymer solution. Utilization of this technique is ideal for its minimal invasiveness, but requires special attention to ensure all components used are safe and reasonably nontoxic. Though natural polymers are frequently regarded to have superior biocompatibility over synthetic one, but the presence of synthetic cross-linkers and initiators used in the polymerizations of naturally derived

monomers and pre-polymers are subjected to the same toxicity as those of purely synthetic hydrogels (Nilimanka, 2013).

The evaluation of hydrogel biocompatibility test can be done both in vitro cell Culture medium, and in vivo. The in vitro test for biocompatibility is based on the evaluation of the cytotoxicity aspect of the material in the presence of live host cells and can usually be done in two ways. In the first method, also known as the “direct contact method” the material whose biocompatibility has to be tested or determined is placed in direct contact with the host environmental cells and is subsequently incubated for a specific period of time at 37°C. While in the second method, called Elution (extract dilution) method, the material is placed in a suitable physiological solution and is incubated for a specified period of time at 37°C, in order to allow leaching from the material. The leachates, so obtained are used to carry out the biocompatibility tests in the presence of the cells (Nilimanka, 2013).

The majority of the problems associated with hydrogel regarding the toxicity, arises from the presence of unreacted monomers, oligomers and initiators that leach out during application. So it is important to evaluate the toxicity of the hydrogel components like monomer, initiators and other building blocks used for its synthesis. Modifying the kinetics of polymerization and extensive washing of the hydrogels can reduce the toxicity. Another solution for reducing the toxicity can be that of producing the hydrogel without any initiators and using alternate path like radiation for the process of crosslinking, might eliminate the problem of the residual initiator.

The in vivo assessment of tissue biocompatibility of hydrogel is the knowledge of chemical composition of the biomaterial and the conditions of tissue exposure: nature, degree of exposure, frequency and the duration of exposure.

So, in order to avoid the growth of bacteria or any other substances that might be toxic in the hydrogel environment, we have decided to prepare most of the hydrogels samples in a phosphate saline buffer solution.

Chapter 4

Results and discussions

4.1 The importance of hydrogels mechanical and viscoelastic properties

The mechanical and viscoelastic properties of the hydrogels are of great interest, relative to their uses in the new technologies or application in the biomedical and tissue engineering field. Observation of the sol-gel transition mechanisms, the behavior of hydrogels during and after flow are fundamental principle needed for judging the feasibility of a hydrogel for a specific biological application. For instance, the gel has to be rigid enough to sustain itself as a scaffold for cell growth, while appropriate mechanical stiffness is essential for regulation of cell adhesion and cell gene expression. So, proper flow properties allow hydrogels to be excellent candidates for injectable therapeutic delivery vehicles if they shear-upon the application of a proper shear stress and rapidly self-heal into solids once the stress is removed.

To access these mechanical properties quantitatively, we have performed some deformation rheology on the nanocomposite hydrogels, and the measurement is meant to be carried out within linear viscoelastic region of the material, ensuring that the measured hydrogel properties are independent of the magnitude of imposed strain or stress. Some typical deformation tests we have performed are: “Dynamic deformation method” also known as small amplitude oscillatory shear measurement and “Static deformation method” or simply creep and creep recovery tests.

4.1.1 Dynamic method for the measurement of the mechanical properties of the hydrogels.

Dynamic method for the measurement of the mechanical properties of the hydrogels provides quantitative information on the viscoelastic and rheological properties of a material by measuring the mechanical response of a sample as it is deformed under a periodic strain or stress.

The applied shear strain in the sinusoidal oscillation can be described as $\gamma(t) = \gamma_o(\sin \omega t)$, and the resulting shear stress in output is a phase shifted sine wave with $\tau(t) = \tau_o(\sin \omega t + \delta)$ in which ω is the applied angular frequency and δ is the phase difference between the two waves. So, for a purely elastic material, the strain and stress waves are in phase, so $\delta = 0°$, while for a purely viscous response the two waves are out of phase by 90°, meaning $\delta = 90°$. And the viscoelastic materials give rise to a phase-angle somewhere in between the response of the elastic and viscous materials.

For small amplitude oscillatory shear measurements, the shear storage modulus, G´, loss modulus, G", and loss factor; tan δ, are critical hydrogel properties that are monitored against time, strain and frequency.

Using a complex notation to describe the applied sinusoidal strain, $\gamma^* = \gamma_o \exp(i\omega t)$, then the complex modulus of the tested material is $G^*(\omega) = \sigma^*/\gamma^* = G´ + iG"$, with G´ and G" as the real (elastic or in phase) and imaginary (viscous or loss or out of phase) components of G*, respectively. Whereas, σ^* is the complex stress of the material.

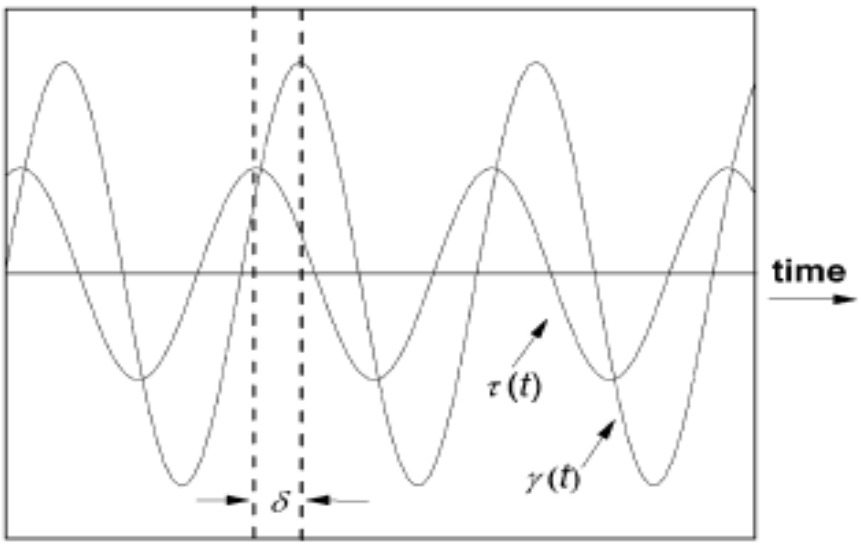

Figure 4.1. *Graphical representation of the amplitude oscillatory shear measurement (Yan & Darrin, 2010).*

The above graph shows the graphical representation of the amplitude oscillatory shear measurement, in which we can see the typical response of the viscoelastic material, when it is subjected to a shear strain deformation in input, and the resulting response of the shear stress in output and the phase angle.

Thus, we can define the total shear rate of the material as:

$$\acute{y} = \acute{y}_E + \acute{y}_V, \tag{4.1}$$

The equation above gives an idea of the viscoelastic property, as a summation of the elastic shear rate and the viscous shear rate of the material, which can also be defined as the ratio of the shear strain or deformation with respect to the time of deformation. So we have the following equation deriving from the Maxwell model of viscoelasticity, with respect to the complex terms:

$$\frac{d\gamma*}{dt} = \frac{1}{G}\frac{d\sigma*}{dt} + \frac{\sigma*}{G\theta} \tag{4.2}$$

Where $\gamma*$ is the complex shear strain, $\sigma*$ correspond to the complex shear stress, and $\boldsymbol{\theta}$ is the characteristic time constant and is equal to the ratio between the viscosity and the shear modulus of the material: $\boldsymbol{\theta} = \eta/G$. in this definition, G has no meaning related to the dynamics of the hydrogel polymer, but describes the modulus of the analogue. The same is true for η, which has no physical meaning related to the viscosity dynamics of the hydrogel polymer, but again describes the viscosity of analogue.

So, by substituting the complex shear strain: $\gamma^* = \gamma_{o*}\exp(i\omega t)$ into that of equation 4.2, it is possible to solve the linear differential equation, which allow us to obtain the following definitions:

$$\frac{\sigma*}{\gamma*} = \frac{G\,\omega^2\theta^2}{1+\omega^2\theta^2} + i\frac{\omega\theta G}{1+\omega^2\theta^2} \tag{4.3}$$

Thus, comparing the equation 4.3 to the earlier definition we gave regarding to the complex modulus of the material: $G^*(\omega) = \sigma^*/\gamma^* = G' + iG''$, we can conclude that:

$$G' = \frac{G\,\omega^2\theta^2}{1+\omega^2\theta^2} \tag{4.4}$$

And

$$G'' = \frac{\omega\theta G}{1+\omega^2\theta^2} \tag{4.5}$$

The storage or elastic modulus G´ in the equation 4.4, gives an idea of the deformation energy stored in the hydrogel during its shear process or deformation; for instance the stiffness of the material. While loss or viscous modulus G" in the equation 4.5, is a representative of the amount of energy dissipated by the material under shear or deformation; the flow or liquid-like response of the material.

Then we can finally define the damping or loss or dissipation factor of a material: tan δ, as a ratio of the loss to the storage modulus of the material:

$$\frac{G''}{G'} = \frac{1}{\omega\theta} = \tan\delta \tag{4.6}$$

The damping factor in equation in equation 4.6 is a measure of the ratio of the energy dissipated as heat to the maximum energy stored in the material during one cycle of oscillation. So, with G" > G´, then the damping factor tan δ > 1, showing that the sample behave more like a viscous liquid. While for G´ > G", the damping factor tan δ < 1, which implies that the sample behave more like an elastic solid.

These parameters are often measured as a function of strain, time and frequency and by monitoring the temporal evolution of the storage and the loss modulus one can easily observe the gelation process of hydrogels. While monitoring the resulting moduli of the hydrogel against strain, one can determine the linear viscoelastic region of the gel, within which G´ and G" are independent of the strain, and at the same time, one can easily determine the region in function of the strain, where the gels are likely to behave as a solid-like material (G´ >> G"), and the region where the gels are likely to behave as a liquid-like material (G" > G´).

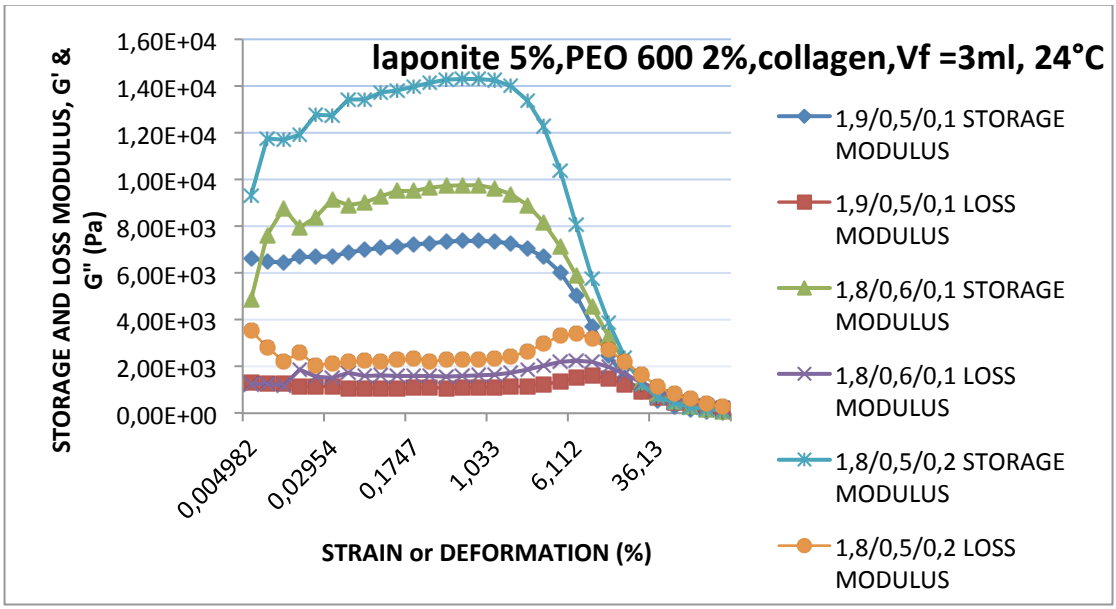

Figure 4.2. *Graphical representations of the hydrogels storage modulus and loss modulus as a function the strain (deformation) for some samples with different volumetric fractions.*

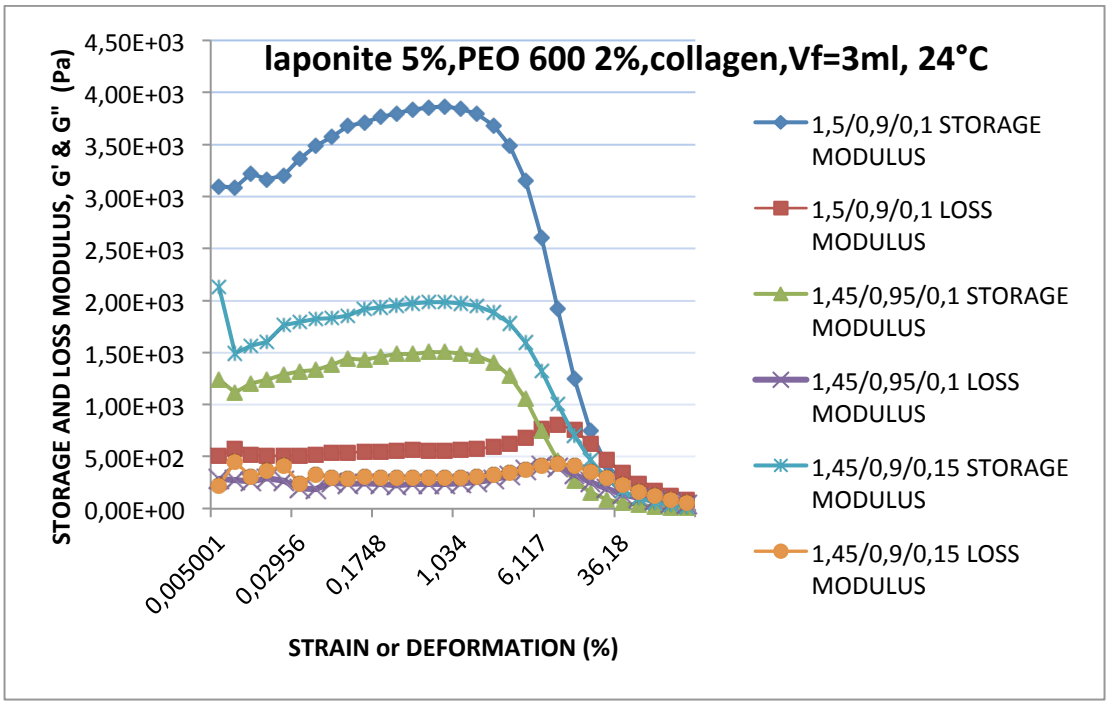

Figure 4.3. *Graphical representations of the hydrogels storage modulus and loss modulus as a function of the strain (deformation), for samples with different volumetric fractions.*

The hydrogels viscoelastic properties can be easily determined from the graphs in figure 4.2 and 4.3, where the variation of the gels storage modulus and loss modulus are plotted against the strain (deformation). The rheological properties were measured with a strain sweep experiment, with the deformation varying between the value of 0,005-150% in logarithmic scale, and a constant angular frequency of 1 rad/s at constant temperature of 24°C.

In both graphs we can observed that at different volumetric fractions, the gels storage modulus; the deformation energy that is stored in the hydrogels increases with increasing crosslinking agent (Laponite) concentrations and with the polymer concentrations. And at low values of deformation, typically bellow the yield point, the elastic properties of the material dominate over the viscous properties, corresponding to a viscoelastic solid behavior, with G´ > G", while with increasing deformation, the viscous properties increases, showing that the hydrogel has moved to the viscoelastic liquid region, where G" > G´.

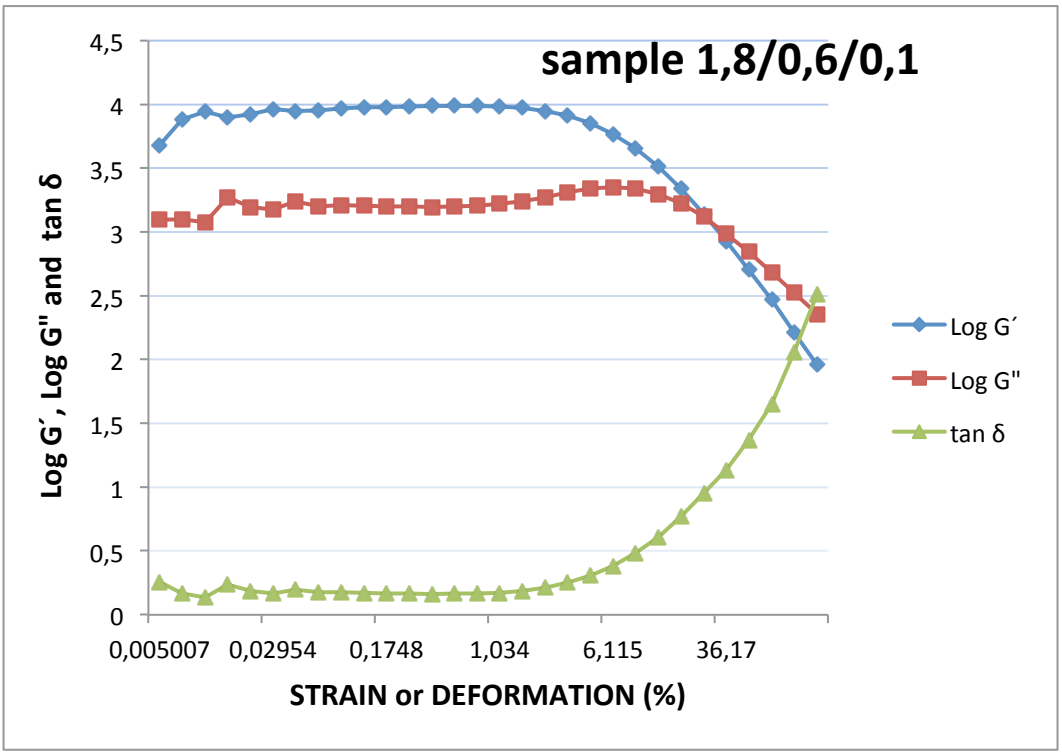

Figure 4.4. *Logarithmic scales of the hydrogels storage modulus, loss modulus and the damping factor (tan δ) as a function of the strain (deformation).*

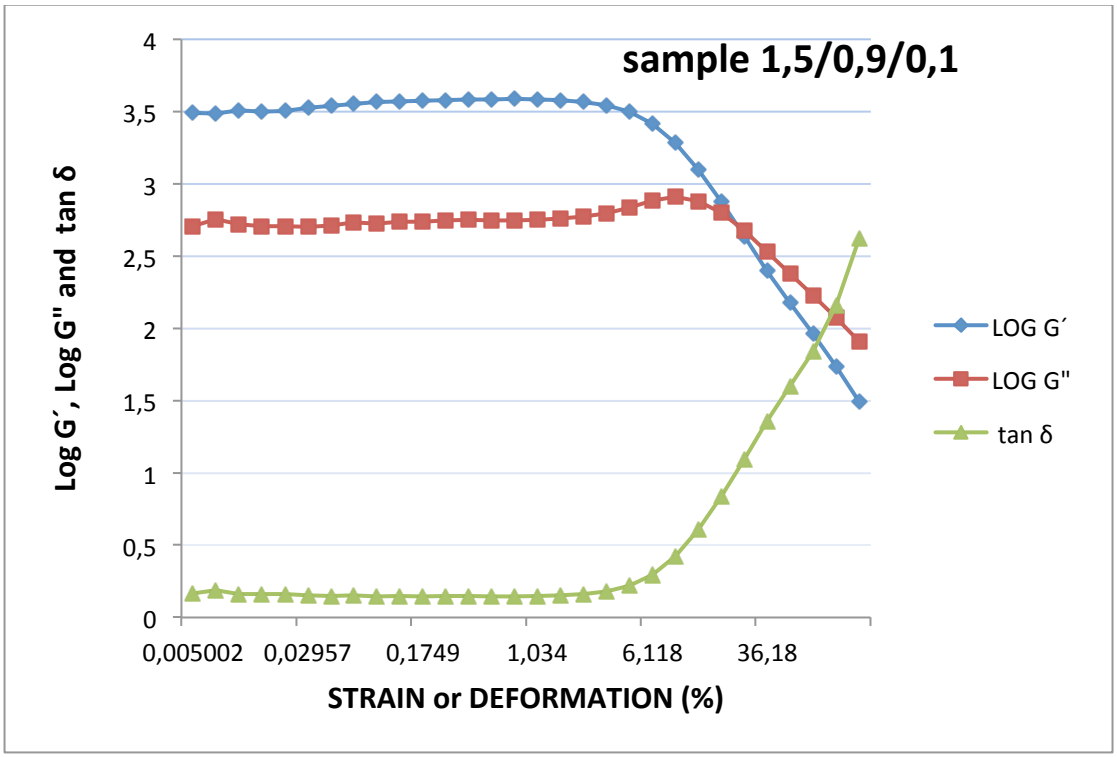

Figure 4.5. *Logarithmic scales of the hydrogels storage modulus, loss modulus and the damping factor (tan δ) as a function of the strain (deformation).*

The graphical representation of figure 4.4 and 4.5 in logarithmic scales of the storage modulus, loss modulus and the damping factor, confirms the viscoelastic properties of the hydrogels, whereby, at low deformations the hydrogels stiffness is high and the storage modulus G´ is in an unrelaxed state. As the deformation is increased, the viscoelastic nature of the material is seen and a transition region in G´ is observed. At still higher deformation, a new plateau in G´ occurs as the hydrogels is now in a relaxed and rubbery state. In contrast, the loss modulus G" goes through a maximum as the deformation is increased, leading to the formation of a viscoelastic liquid or simply viscous liquid. The viscous contribution is at maximum near the inflection point or the yield point of the transition region, where the values of G´ and G" are equal, but with a further increase in that of the loss modulus G".

Considering the damping or loss factor (tan δ), in both figure 4.4 and 4.5, we can easily observe that for values of (tan δ) in both cases are lower than 1; tan $\delta < 1$ at very low values of the deformation, in which Log G´ > Log G" and this correspond clearly to the viscoelastic solid behavior of the hydrogels. While with increasing value of the deformation, we observe the transition to the yield point, where Log G´ = Log G", and furthermore, the

value of G" increases, and this also lead to an increase in the value of the damping factor; tan $\delta > 1$, indicating a transition to the viscous liquid region.

Another method that we have used for the characterization of the hydrogels viscoelastic properties is the "Dynamic viscosity and shear stress method", where we able to emphasize properly the non-Newtonian behavior of the gels and the shear-thinning property. In vast majority of the cases, the viscosity was found to decrease with increase in shear rate, giving rise to what is generally known as "shear-thinning" behavior, although the terms temporary viscosity loss and "pseudo-plasticity" have also been employed for this description.

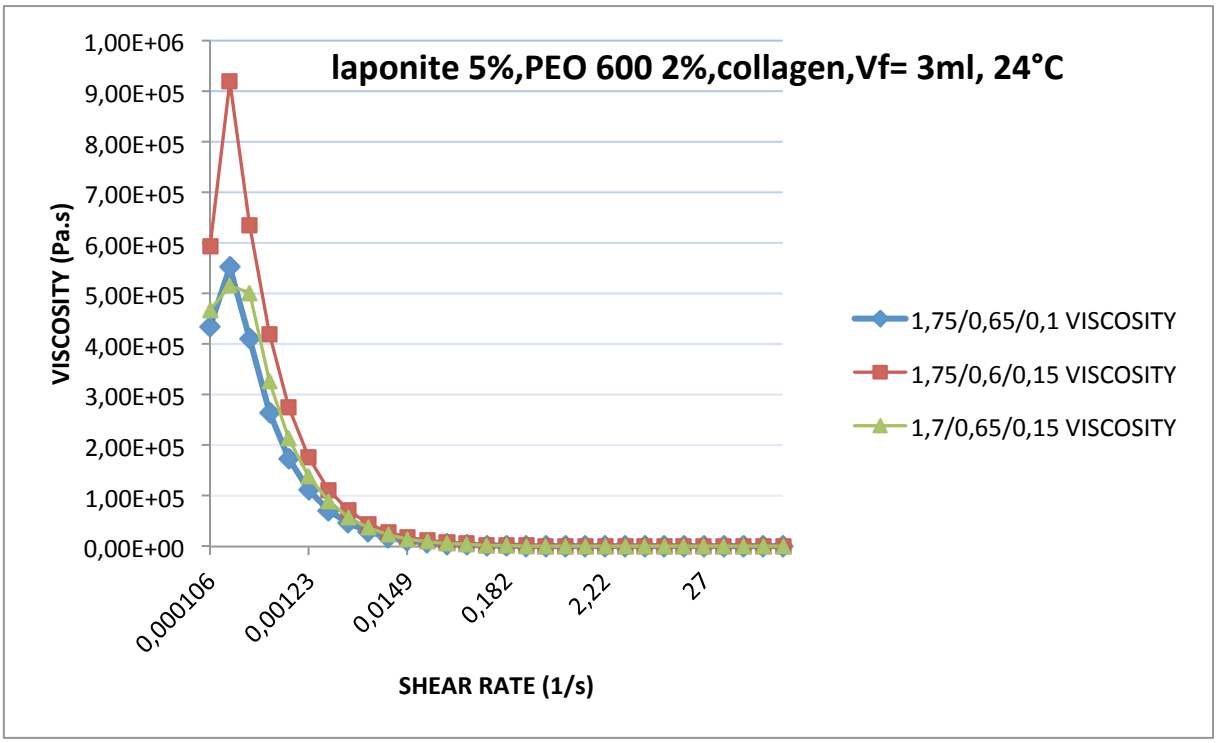

Figure 4.6. *Graphical representations of the hydrogels viscosities as a function of the applied shear rate, for different volumetric fractions.*

The graphical representation in figure 4.6 shows us that in the limit of very low shear rate, the viscosity increases, while a further increase in the shear rate, leads to a decrease in the value of the viscosity, until it finally reached a region of constant value. The initial increase in the value of the viscosity at lower shear rate, implies a shear-thickening non-Newtonian behavior of the hydrogel, for a very few range of shear rate. A subsequent decrease in the viscosity with increasing shear rate implies the typical shear-thinning or thixotropic behavior of the gels, which means a transition to an almost liquid-like state.

The final region of the curves, correspond to that of the Newtonian region, where the viscosity is constant with respect to the time of shearing and the stress in the liquid falls to zero immediately the shearing is stopped.

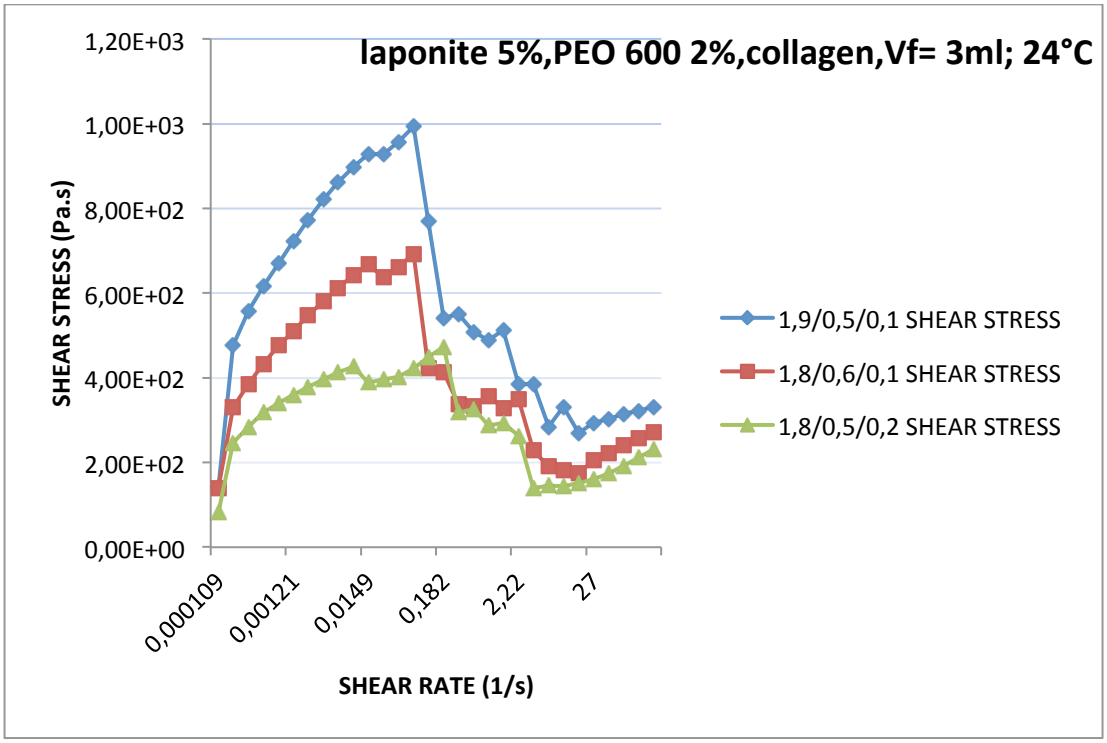

Figure 4.7. *Graphical representations of the variations of the hydrogels shear stress as a function of the applied shear rate, for different volumetric fractions.*

The aim of the graphical representation in figure 4.7 is to describe the minimum stress required to make a material flow. This minimum stress is also known as the yield stress or the yield point, and it is a measure of the strength of the material structure and below this critical value no flow takes place. Also, the yield stress materials are typically thixotropic, which means that they are dependent on the shear history of the sample and susceptible to ageing.

The initial increase in stress represent the elastic response of the material, while a decrease of the stress value, indicates the gradual structure breakdown of the system. The transition from viscoelastic to viscous flow is manifested as a peak in shear stress response which corresponds to the yield point. At low shear rate, the material is slow to respond and the stress increases to a relatively constant value. At the higher shear rates, the peak in the stress occurs followed by stress decay towards a relatively constant stress value.

4.1.2 Static method for the measurement of the mechanical properties of the hydrogels.

In addition to the dynamic method of measurements described in the previous paragraph, it is important to assess the properties of hydrogels during flow as well as their abilities to retain or recover their solid form morphology and rigidity after experiencing shear flow or large strain. With respect to the previous definitions, shear thinning and self-healing hydrogels are excellent candidates for injectable therapeutic delivery vehicles.

So, monitoring rheological behavior and structural evolution of these gels during and after flow can help evaluate encapsulated therapy retention and delivery during syringe injection and the ability of the material to stay localized after injection against possible biological forces in vivo (Engler & Sen, 2006).

The theory of viscoelasticity takes into account the relationships between elasticity, flow, and molecular motion in polymeric materials, and the mechanical behavior of all materials exhibit some degree of elasticity and flow, the concentrations and the size components in the gel; Laponite, polymers concentrations, often leads to different viscoelastic responses. The magnitude of the viscoelastic response is strongly dependent on the nature of the imposed mechanical motion, which is also a time scale dependent. Thus, the time dependence of the applied stress or strain is as important as the magnitude in predicting the material's mechanical response or properties.

Then the time dependency of the material on the strain or stress after an application of a mechanical stress or strain can be determine by these two experimental procedures: "creep and creep recovery test" and "relaxation test", at constant stress and strain respectively.

In the creep and creep recovery test, the material is subjected to a constant shear stress σ, and the shear strain of the material as e response to the applied shear stress is observed, indicating also that the shear strain of the material is time dependent, when it moves from the deformed state, to the partially or totally recovered state. The ratio of the time dependent shear strain to the applied shear stress is defined as the creep compliance, $j(t) = \gamma(t)/\sigma$, which is reciprocal to the elastic modulus of the material.

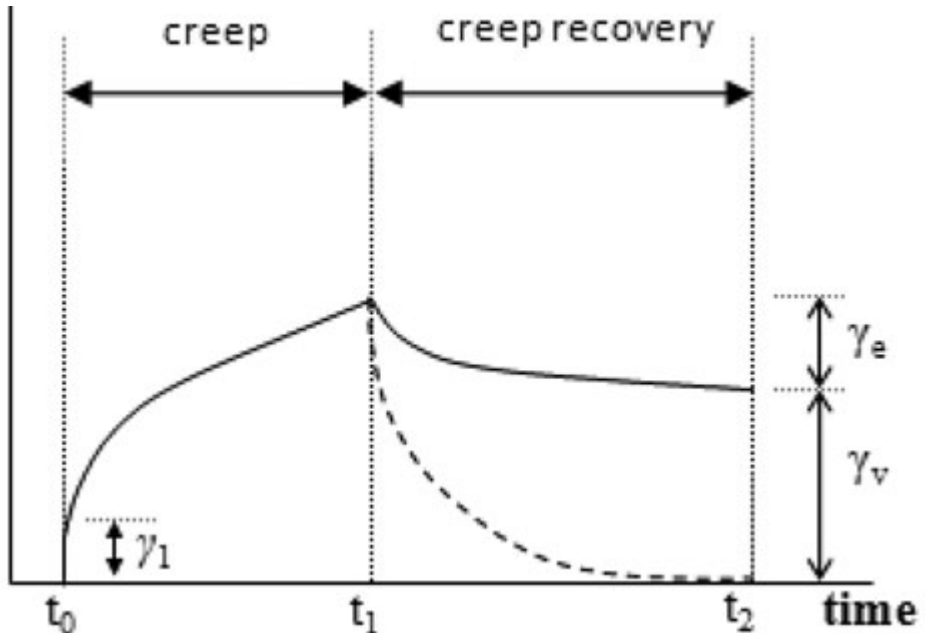

Figure 4.8. *Representation of creep and creep recovery of a polymeric material, as a function of time (Yan & Darrin, 2010)..*

The representation of creep and creep recovery of a polymeric material shown in figure 4.8, is necessary for describing the temporal evolution of strains for a viscoelastic liquid material and viscoelastic solid material. In the creep phase, the strain curves basically overlap initially, displaying an immediate strain jump of γ_1 due to a pure elastic response to a sudden application of σ_1, at t_0 and a subsequent time-dependent increase of strain. However, in the following recovery phase, the elastic solid manages to recover completely, (indicated in figure 4.8 with the dash lines), while the viscoelastic liquid material only recovers part of the deformation (indicated in the figure with a continuous line). It is also important to notice that the recovering phase of the material is also a function of time, so it depends if the time is long enough or not. Hence, the permanent deformation γ_v, represents the viscous portion of a material and γ_e the elastic portion.

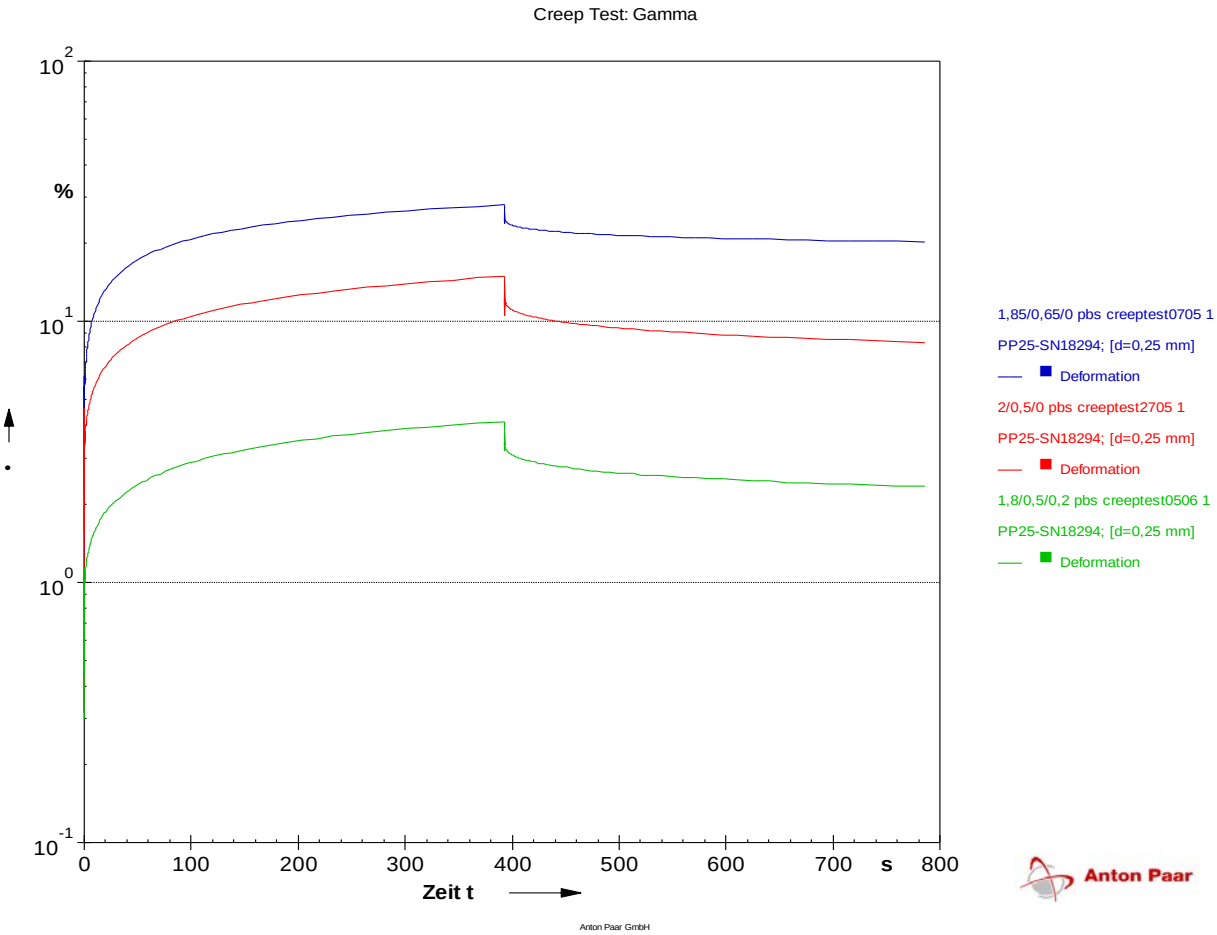

Figure 4.9. *Graphical representation of creep and creep recovery processes for hydrogels with different compositions.*

The creep and creep recovery tests for the hydrogel samples shown in figure 4.9 are conducted with the same experimental conditions, but it is also necessary to take into account the ageing conditions or effects of the hydrogels samples that go from the day of the preparation of the samples to the day the mechanical properties were measured. The following graphical representations in figure 4.10 takes into account the ageing effect of the hydrogels samples.

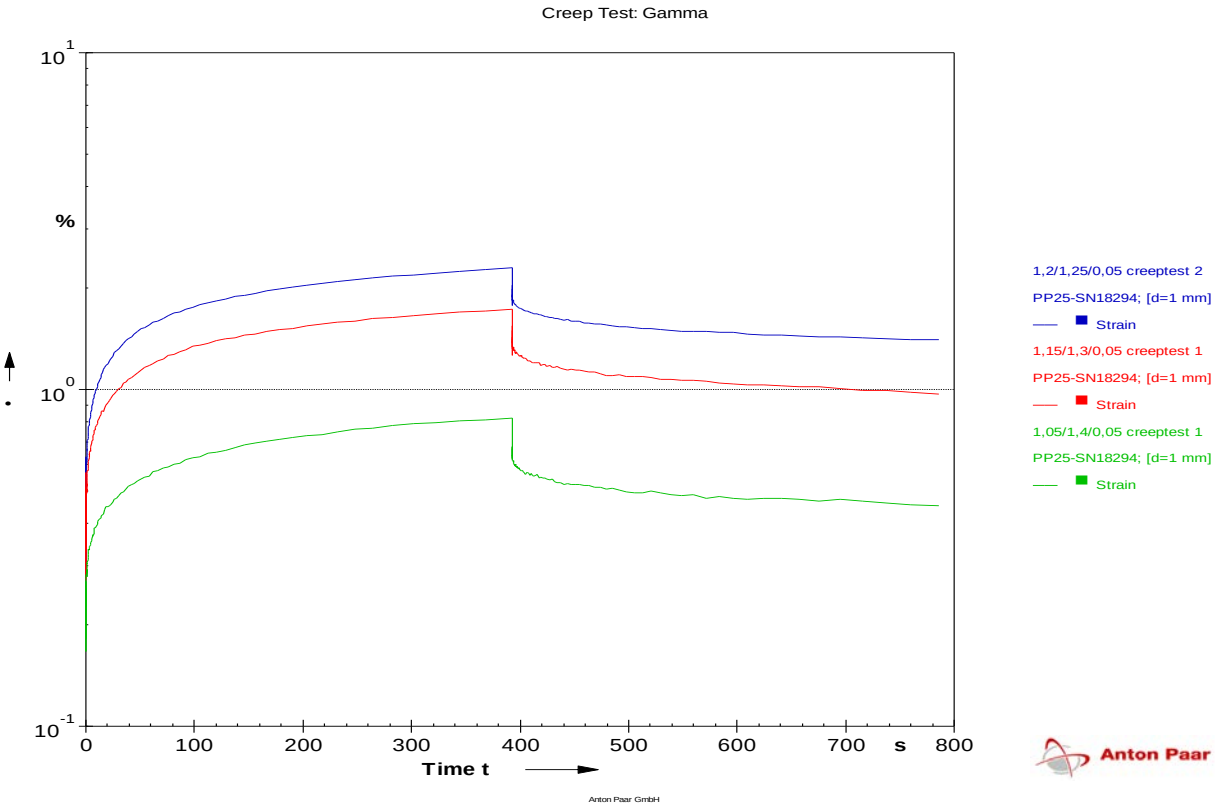

Figure 4.10. *Graphical representation of creep and creep recovery processes for hydrogels with different compositions and ageing effects.*

All samples were tested under the same experimental conditions, by subjecting the samples initially to a constant shear stress of 50 Pa for the creep region, and this shear stress was later brought to a constant value of zero for the creep recovery phase. And the total period of the applied shear stress is approximately 800 seconds.

The measurement of the creep and creep recovery of three of these samples: 1,2/1,25/0,05, 1,15/1,3/0,05 and 1,05/1,4/0,05 were made after almost three months from the date of preparation, a situation during which the hydrogel is at a stable condition or at rest, increases it stiffness, and this was also verified through the rheological measurement of the gels storage modulus, which increases with time. While, the other three samples were measured at the same period of time from the day they were prepared approximately a day.

Considering the definition of the creep compliance $j(t) = \gamma(t)/\sigma$, which is basically the reciprocal of the elastic modulus of the hydrogel G', with a unit of (1/Pa), we can observe that the creep compliance decreases with increasing elastic modulus, and this corresponds to the case of the hydrogel samples with high stiffness and storage modulus, particularly for

samples 1,2/1,25/0,05, 1,15/1,3/0,05, 1,05/1,4/0,05 and 1,8/0,5/0,2, indicating an “almost viscoelastic solid behavior”. These materials are said to be an “almost viscoelastic solid” because the shear strain of these material, after the cessation of the applied shear stress, were not able to recover completely back to the initial state they were before the application of a shear stress. This also depends on the time available for the recovery phase.

While the other two samples 2,0/0,5/0,0 and 1,85/0,65/0,0 are showing a typical viscoelastic liquid behavior, because they are characterized by a high value of creep compliance, and with very low value of the elastic strain γ_e. Within the linear viscoelastic region, the creep compliance is independent of the applied shear stress and therefore all j(t) curves obtained under various stresses should overlap with each other.

In most cases, creep compliance is compared to the reciprocal of the shear modulus that is measured in a small amplitude oscillatory shear tests in order to judge if the material or the samples display a pure elastic behavior (Kavanagh & Clark, 2002).

So, from the creep and creep recovery test, it is also possible to identify the nature of some hydrogels sample, just by considering the creep compliances, the range of the elastic strain and finally the range of the viscous strain, all of these as a function of time of the applied shear stress. The higher the storage modulus of a hydrogel, the lower is his creep compliance, and the more the material can be compared to a viscoelastic solid material. While the lower the storage modulus of a hydrogel, the higher is his creep compliance, and the more the material can be compared to a viscoelastic liquid material.

With the stress relaxation experiment, a constant shear strain is applied and the time dependence of the shear stress required to maintain that strain is measured. Also in this case, we can define the stress relaxation modulus, G(t), which is defined as the ratio of shear stress to shear strain, at constant deformation, $G(t) = \sigma(t)/\gamma$. A comparison of this definition can be made with that of the creep compliance, whereby, a high value of shear relaxation modulus corresponds to a high value of the yield shear or yield point of the material; that is the minimum stress required to make a material flow, and it is also a measure the strength of the material structure.

As we can observe in the graphical representation of the hydrogels in figure 4.10, the higher the elastic property or storage modulus of the hydrogel sample, the higher is the resulting stress relaxation modulus G(t), implicating a higher value of the initial shear stress

or yield stress needed for the material to be deformed, and the relaxation spectrum or the degradation process of the material to a constant or permanent phase is slower.

$$\tau = \tau_0 + K\acute{y}^n \tag{4.7}$$

Where τ is the total shear stress of the material, τ_0 is the yield stress or the yield point of the material, $\acute{y}$ is the shear rate and K, n, are the parameters of flow equations (function of the material viscosity).

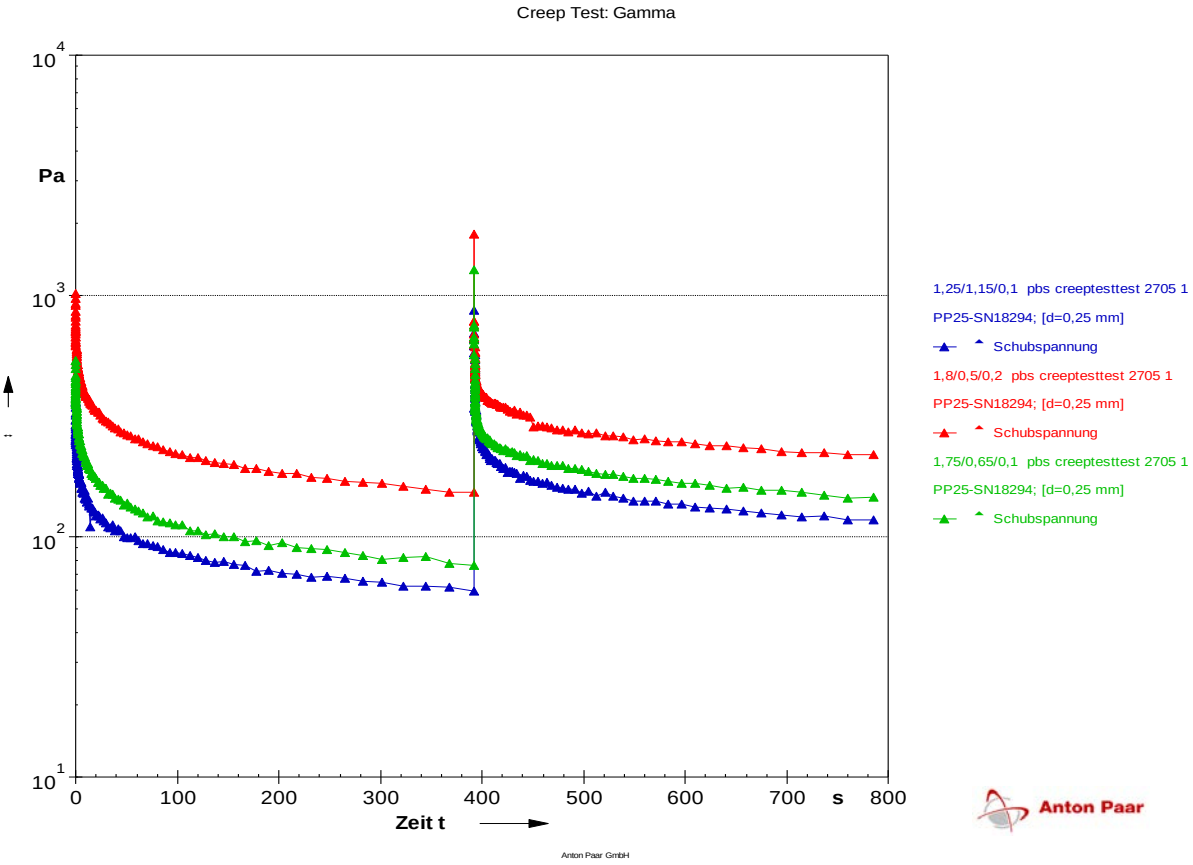

Figure 4.10. *Graphical representation of the shear relaxation behavior for different samples of hydrogels as a function of time.*

All samples in this experiment, were subjected to two stages of deformation, the initial stage was conducted at a constant deformation rate of 10% for a period of almost 400s, and after the material has reached a relaxed state, it was left for a few period of seconds, after which the second stage of deformation starts, and a constant deformation of 50% is once more

applied to the material, and the material is finally brought to a relax state after some period of time. As we can also observe in the figure 4.10, the relaxation process or the degradation to a constant linear shear for the sample with lower elasticity or storage modulus is faster, with respect to the other samples with higher storage modulus, and this indicate a typical higher tendency to viscoelastic liquid behavior for samples with lower elasticity.

4.1.3 Control of the hydrogels mechanical properties and the ternary diagram representation.

After the studies of the dynamic and static method for the measurement of the hydrogels mechanical properties, it is of great importance to improve these mechanical properties, in order to make them suitable for the desired applications. We have explored three different ways for controlling these properties: by altering the polymers composition (both the synthetic and the natural polymer), by increasing or decreasing the cross-linker density (Laponite concentration), and finally, by changing the conditions under which the hydrogels are formed. It is necessary to consider the fact that the variation of these three points, not only affect the mechanical properties of the hydrogels, but also affect other behavior of the material, such as the swelling behavior.

The variation or the control of the hydrogels mechanical properties depends strongly on the alteration of the composition of the polymers used in the synthesis of the hydrogel, so varying the composition of the physically stronger components: poly(ethylene oxide) and the collagen, will lead to an increase or decrease in the mechanical strength of the final product, either by increasing the stiffness or the elastic modulus of the hydrogel, or by decreasing its stiffness, so leading to a very low viscous material. Varying the composition of the poly (ethylene oxide) in the preparation of the hydrogel samples, can lead to the formation of the so called: sol gel system and a fixed or permanent gel structure, which properties varies based on the fact that a low viscous structure and a very high viscous structure are formed respectively.

The formation of the sol and permanent gel structure depends on the process of the saturation of the Laponite surface particles, where at very low concentration of the polymer, the surface saturation of the silicate particles is incomplete, and this result in the formation of a sol phase. While the formation of the shake or fixed gel occurred when there is an equilibrium state among the particles in the gel, with enough polymers available for coverage

of the silicate nanoparticles surface. A further increase in the concentration of the polymer (PEO) with respect to the Laponite concentration, lead once again to the formation of the sol gel phase, because the Laponite particles become completely saturated with PEO, and the polymer segments on the surface of the Laponite repel each other.

However, it is important to emphasize the fact that the incorporation of the natural polymer: collagen I, to the other two components; Laponite and PEO, in the synthesis of the hydrogels is necessary to improve the mechanical properties of these materials. Higher mechanical properties are obtained when the amount of the collagen in the system is increased.

The mechanical strength of the hydrogels is often due mostly to the presence or to the amount of crosslinking agent in the system, particularly in swollen state, where the physical entanglements are nearly nonexistent, the strength of the material increases dramatically with increasing cross-linking density. An increase in the cross-linking density can simply be obtained just by the addition of larger amount or concentration of the crosslinking agent: Laponite in the stock solution. However, a significantly increase in the crosslinking agent concentration, can easily enhance the material strength or mechanical properties, but at the same time, changes to other properties are likely to occur, other than the improvement of the mechanical properties. Higher crosslinking density can affect the hydrogels diffusivities, and hence the release of drugs and the swelling rates are likely to be reduced, and the maximum degree of swelling is also likely to be reduced.

The conditions under which the hydrogels are prepared is another interesting issue to be considered, these conditions may be refer to the type of solvents that were used in the preparation of the hydrogels. When the type of solvent or the nature of the solvent: the pH or the ionic strength of the aqueous solution is altered, the hydrogel structured obtained can easily change. So, preparing the hydrogels in de-ionized water or in a phosphate buffer saline solution, can lead to the formation of hydrogels with different mechanical properties and swelling degree, based on the solvent used.

Majority of the hydrogels samples that were prepared during these studies are listed in the table 4.1, showing the different composition of the hydrogels prepared, and their maximum storage modulus.

Table 4.1. *Hydrogels samples with their normalized concentrations and maximum storage modulus.*

Hydrogel volumetric fractions	Normalized Laponite compositions	Normalized PEO 600, compositions	Normalized collagen compositions	Maximum storage modulus (Pa)
1,9/0,5/0.1	0,76	0,2	0,04	7379
1,8/0,6/0,1	0,72	0,24	0,04	9752
1,8/0,5/0,2	0,72	0,2	0,08	14270
1,75/0,65/0,1	0,7	0,26	0,04	5860
1,75/0,6/0,15	0,7	0,24	0,06	7031
1,7/0,65/0,15	0,68	0,26	0,06	3963
1,65/0,65/0,2	0,66	0,26	0,08	2968
1,6/0,75/0,15	0,64	0,3	0,06	1729
1,55/0,8/0,15	0,62	0,32	0,06	1435
1,5/0,9/0,1	0,6	0,36	0,04	3865
1,45/0,95/0,1	0,58	0,38	0,04	1503
1,45/0,9/0,15	0,58	0,36	0,06	2133
1,4/1,0/0,1	0,56	0,4	0,04	2115
1,35/1,05/0,1	0,54	0,42	0,04	2751
1,25/1,15/0,1	0,5	0,46	0,04	3907
1,2/1,25/0,05	0,48	0,5	0,02	778
1,15/1,3/0,05	0,46	0,52	0,02	693
1,05/1,4/0,05	0,42	0,56	0,02	708
2/0,5/0,00	0,8	0,2	0,0	4966
1,85/0,65/0,00	0,74	0,26	0,0	6401
1,5/1,0/0,00	0,6	0,4	0,0	1129
1/1,5/0,00	0,4	0,6	0,0	1509
1,775/0,5/0,225	0,71	0,2	0,09	11400
0,85/1,65/0,0	0,34	0,66	0,0	3980
2,2/0,0/0,3	0,88	0,0	0,12	924
0,95/1,55/0,0	0,38	0,62	0,0	2210
1,0/0,5/1,0	0,4	0,2	0,4	3470
0,1/1,2/1,2	0,04	0,48	0,48	554
0,2/0,8/1,5	0,08	0,32	0,6	328
0,0/0,0/1,0	0,0	0,0	1,0	37

All the samples in the above table 4.1 were prepared with a total volumetric fraction of 2,5%, in order to have the same condition when the fractions will be normalized to a total value of one. All samples are prepared and brought to a final volume of 3 ml in phosphate buffer saline solution, and their mechanical properties were measured at a constant temperature of 24°C, with a parallel plate-plate system of 25 mm in diameter, and a constant distance of 0,25 mm between the two plates.

The following diagram in figure 4.11, is a ternary diagram for the system: Laponite, PEO and collagen, this mixture was prepared in a total volumetric fraction of 2,5%. The diagram is needed to explain the distribution, the type and the elasticity of the hydrogels that were formed after the mixture of the ternary components.

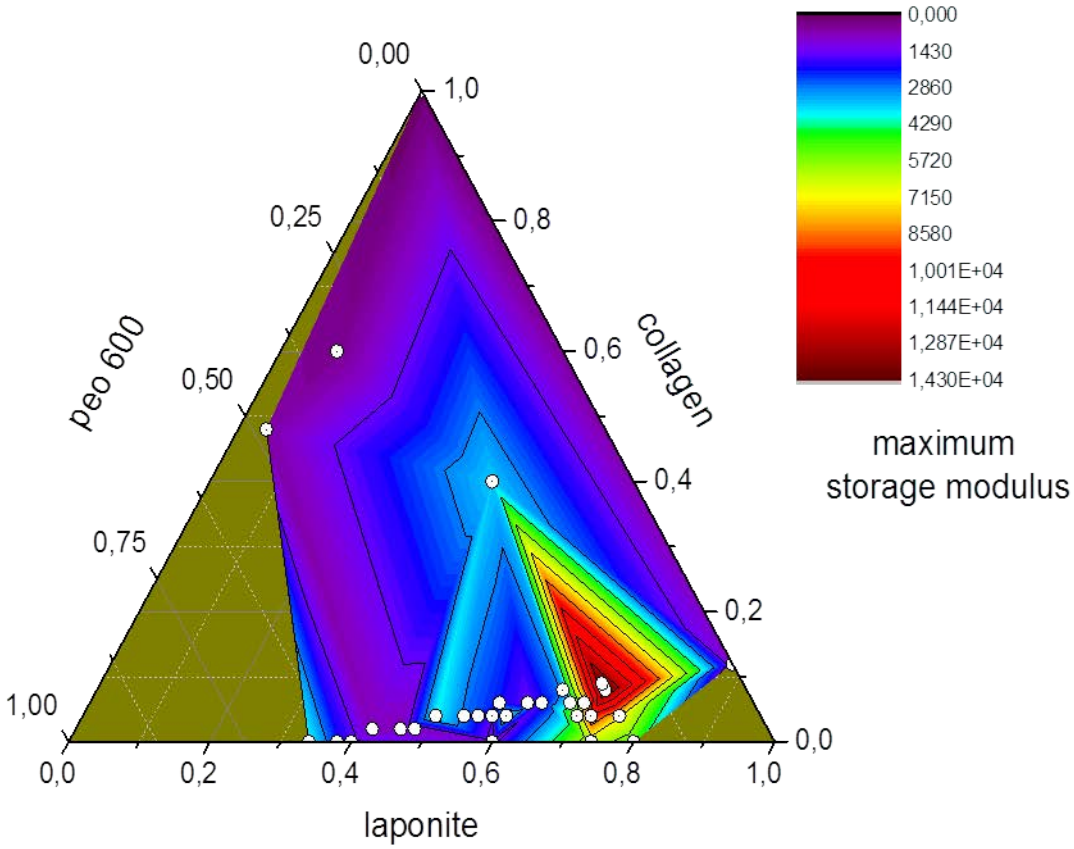

Figure 4.11. *Ternary diagram for the system Laponite-PEO-Collagen, with the respective maximum storage modulus of the hydrogels samples.*

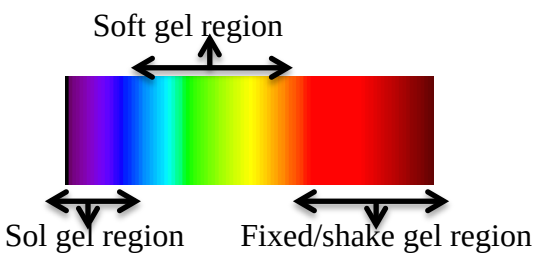

Figure 4.12. *Hydrogels phase distribution with respect to the ternary diagram.*

In the previous two figures, both figure 4.11 and 4.12, we can easily understand the phase distribution of the hydrogels samples, based on their maximum storage modulus distribution and their physical behavior. The highest storage or elastic moduli were obtained with samples containing higher value of the crosslinking agent; Laponite, combined with an adequate value of the polymers.

The phase distribution of the hydrogels; sol, soft and fixed or shake gels in the ternary diagram, are described based on their storage or elastic modulus, because the higher the storage modulus of the hydrogel sample, the more they can be categorized in the fixed or shake gel region.

4.2 Comparison of the swelling behavior of hydrogels prepared in phosphate buffer saline solution and in de-ionized water, and the effect of crosslinking agent on the swelling behavior.

As discussed earlier in the description of the factors affecting the mechanical properties of the hydrogels system, the degree of swelling is directly related to the material strength or elasticity. Most of the methods for improving the hydrogels strength; such as changing the composition of the hydrogel components, increasing the crosslinking density and changing the reaction conditions are basically designed to increase or decrease the hydrogel degree of swelling.

The swelling behavior in hydrogel is another important aspect in the design of hydrogel structures, because it allows us to determine the amount or the quantity of water imbibed in a gel, and this is really useful for characterizing the hydrogels for biomedical applications. The total amount of water in a hydrogel, for instance, the volume fraction of water, and its free or bound water character will determine the absorption and the diffusion of solutes through the hydrogels.

We have studied the swelling behavior of the hydrogels system in two different mediums; in the phosphate buffer saline solution and in a de-ionized water, in order to quantify the swelling capacity of these two mediums and to understand the effect of salt on the degree of swelling. The effect of the crosslinking agent; Laponite on the degree of swelling was also determined, and this was done just by varying the concentrations of the crosslinking agent both in the initial stock solutions and in every hydrogels samples.

The following two tables below shows the procedure for the calculation of the degree of swelling of some hydrogels samples, prepared from a stock solution of Laponite 2,5%, PEO 600 1,5%. The stock solutions were prepared in a de-ionized water and phosphate buffer saline solution, in order to be able to study the differences in the degree of swelling of the two mediums.

Table 4.2. *Swelling degree for hydrogels samples prepared in de-ionized water, containing Laponite 2,5%, PEO 600 1,5%, and taking all samples to a final volume of 3ml with PBS.*

Volumetric fractions	Initial weight of swollen gel (g)	Weight dry gel + salt (g)	Salt weight (g)	Final weight dry gel (g)	Swelling degree
1,9/0,25	2,57	0,058	0,0036	0,0544	4620
1,6/0,25	2,79	0,058	0,00031	0,0577	4740
1,3/0,25	1,38	0,039	0,01	0,0290	4660

Table 4.3. *Swelling degree for hydrogels samples prepared in phosphate buffer saline solution, containing Laponite 2,5%, PEO 600 1,5%, and taking all samples to a final volume of 3ml with PBS.*

Volumetric fractions	Initial weight of swollen gel (g)	Weight dry gel + salt (g)	Salt weight (g)	Final weight dry gel (g)	Swelling degree
1,9/0,25	2,46	0,117	0,031	0,086	2760
1,6/0,25	2,98	0,102	0,031	0,071	4090
1,3/0,25	2,89	0,104	0,031	0,073	3830

All samples listed in table 4.2 and 4.3 are prepared in the same physiological conditions, the main differences in these samples, beside from the volumetric fractions, is the concentrations of salt in the system. From comparing these two tables, we can observe that the degrees of swelling of the samples that were prepared mainly in de-ionized water (with very few amount of salt) are higher than those of the samples that were completely prepared in a phosphate buffer saline solution.

Other degrees of swelling of some hydrogels samples were studied by increasing the concentration of the stock solutions of both Laponite and PEO 600 to a value of 5% and 2% respectively. The stock solutions were prepared in a de-ionized water and phosphate buffer saline solution, in order to be able to study the differences in the degree of swelling of the two mediums.

Table 4.4. *Swelling degree for hydrogels samples prepared in de-ionized water, containing Laponite 5%, PEO 600 2%, and taking all samples to a final volume of 3ml with PBS.*

Volumetric fractions	Initial weight of swollen gel (g)	Weight dry gel + salt (g)	Salt weight (g)	Final weight dry gel (g)	Swelling degree
2,0/0,5	2,897	0,083	0,0110	0,0740	3800
1,85/0,65	2,884	0,080	0,0094	0,0706	4000
1,75/0,75	2,903	0,083	0,0085	0,0745	3800

Table 4.5. *Swelling degree for hydrogels samples prepared in phosphate buffer saline solution, containing Laponite 5%, PEO 600 2%, and taking all samples to a final volume of 3ml with PBS.*

Volumetric fractions	Initial weight of swollen gel (g)	Weight dry gel + salt (g)	Salt weight (g)	Final weight dry gel (g)	Swelling degree
2,0/0,5	2,859	0,112	0,031	0,081	3400
1,85/0,65	2,933	0,114	0,031	0,083	3400
1,75/0,75	2,827	0,104	0,031	0,073	3800

As we can observe in the above two tables, table 4.4 and 4.5, we can see that even with an increase in the Laponite concentrations, both in the stock solutions and in the volume fraction of the final hydrogels samples, the degrees of swelling of the samples that were mainly prepared in a de-ionized water are higher than those of the samples that were completely prepared with a phosphate buffer saline solution. And also, comparing the swelling degree of all samples that were prepared with Laponite stock solution of 5% with that of 2%, we can also observe that an increase in the concentration of the Laponite content, decreases the hydrogels swelling degree. Meanwhile, this also prove that increasing the concentration of the crosslinking agent, increases the mechanical properties of the resulting hydrogel, but also lead to a decrease in it degree of swelling.

Table 4.6. *Comparison of some hydrogels swelling degrees and elastic moduli for samples prepared in PBS and de-ionized water.*

Volumetric fractions	Swelling degree in PBS	Maximum storage modulus in PBS (Pa)	Swelling degree in de-ionized water	Maximum storage modulus in de-ionized water (Pa)
2,0/0,5	3400	4966	3800	3170
1,85/0,65	3400	6401	4000	1830
1,75/0,75	3800	2050	3800	212

The table in figure 4.6 is used to prove that increasing the salt concentration in a hydrogel system increases it storage or elastic modulus, meanwhile, the resulting swelling degree decreases, the inverse behavior is verified in the case of the samples with less salt concentration.

Normally, the response of hydrogel during the swelling phase is dependent on the presence of hydrophilic functional groups such as –OH, -COOH. These groups make the hydrogel hydrophilic and due to the capillary action and the difference in the osmotic pressure, water diffuses into the hydrogel. So, the increased swelling degree observed at low concentration of Laponite nanoparticles (2,5% with respect to the stock solution) could be attributed to an increase in the osmotic pressure within the polymer network. An increase in the osmotic pressure promotes water influx in the system, resulting in higher degree of swelling of the samples that were prepared with 2,5% stock solution of Laponite, compared to that of 5%.

Increasing the concentration of the silicate particles in the hydrogel samples, increases the density of the resulting structure and this can easily affect the water uptake of the system by decreasing it swelling degree. Water uptake represents the migration of water molecules into preformed gaps between the polymer chains, and denser hydrogel structure diminish the accessibility of water molecules to the hydrophilic parts of the polymer molecules, thus less water can penetrate into the hydrogel structure.

As mentioned earlier, the swelling ability of anionic hydrogels in various salt solutions is relatively lesser than the swelling degree of the samples prepared mainly in de-ionized water. This process of undesired swelling loss is often attributed to a “charge screening

effect" of the additional cations, which cause a non-perfect anion-anion electrostatic repulsion.

And also, in salt solution, the osmotic pressure resulting from the difference in the mobile ion concentration between the gel and the aqueous phase decreases, and consequently, the absorbency amounts of the systems are diminished.

So, considering all the above phenomena, at higher concentration of silicate particles, the strong physical interaction between the polymer chains and Laponite increases the overall crosslink density of the hydrogel network, which dominates the osmotic effect of Laponite and limits the swelling of the hydrogels by preventing water influx, so leading to a decrease in the degree of swelling of the system.

4.3 Hydrogel reproducibility and ageing effect

Another interesting factor affecting the characterization of the hydrogel rheological properties is the reproducibility of these properties, whereby, for reproducibility we meant the process of obtaining the same mechanical or rheological properties of the same hydrogel sample, when measured at different range of time, but in the same physiological conditions.

The inhomogeneity of the hydrogels system might be one of the factors affecting the reproducibility of the hydrogels. In most cases, the inhomogeneity is caused by the process of mixing the resulting hydrogel sample, the ambient conditions and also by the degree of interactions between the silicate nanoparticles and the polymers. The presence of inhomogeneity in the hydrogel system, can easily affect it reproducibility, leading to the situation of obtaining different rheological properties (storage/elastic and loss modulus) of the same sample when measured at different range of time.

It is also important to consider the period between which the hydrogel sample was prepared and the period when it mechanical properties were measured. This is necessary to underline the fact the rheological properties of the hydrogels system often changes with time. A better understanding of the fundamental physics leading to the interactions between the hydrogel particles is necessary, but this remains a difficult challenge despite the presence of the advance techniques for characterizing materials. Laponite clay is one of the materials, where the interactions between particles are enigmatic despite the presence of a wide range literature on the material (Schmidt & Nakatani, 2002).

Four hydrogels samples have been selected for the analysis of the hydrogel reproducibility and ageing effect: 1,8/0,5/0,2. 1,75/0,65/0,1. 1,25/1,15/0,1 and 2,0/0,5/0,0. In order to quantify the reproducibility and the ageing effect, each sample was prepared for a number of time equal to three (n=3), in order to be able to make a proper statistical evaluation of the behavior of the hydrogel system, meanwhile the rheological evaluation of every each sample was measured with a time scale, varying between one, six and nine days. And the resulting behaviors are reported in the following graphical representations where we can observe the variation of the rheological properties (storage or elastic modulus of the hydrogels against the resulting strain or deformation).

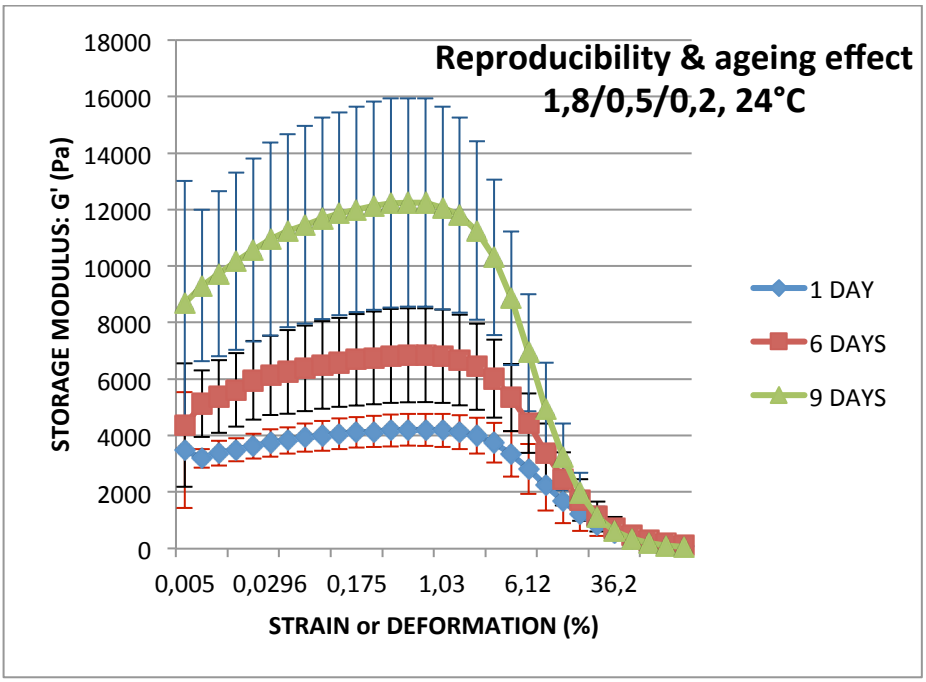

Figure 4.13. *Graphical representation of the hydrogel reproducibility and ageing effect for sample 1,8/0,5/0,2 measured at a constant temperature of 24°C.*

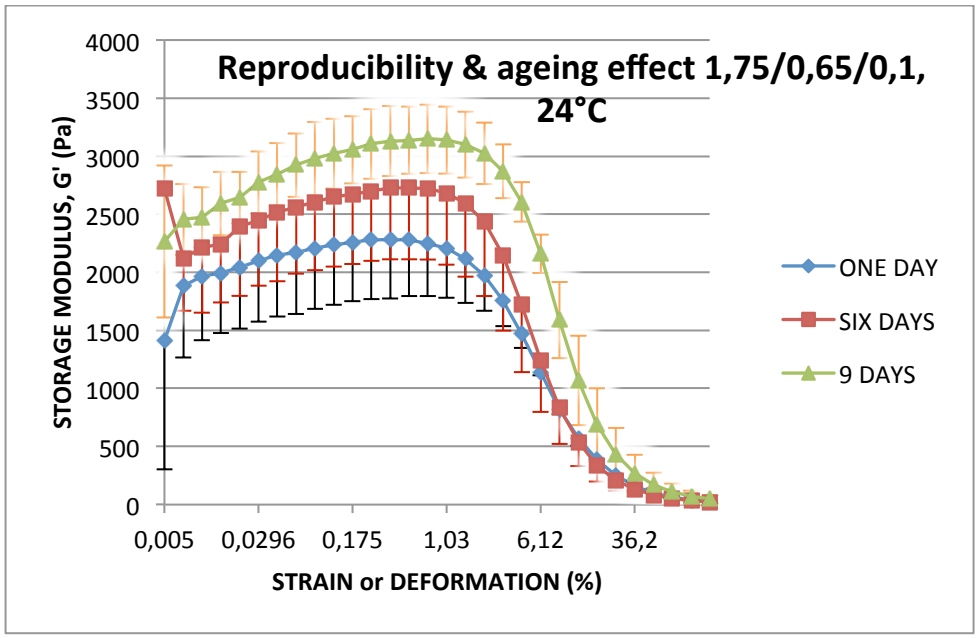

Figure 4.14. *Graphical representation of the hydrogel reproducibility and ageing effect for sample 1,75/0,65/0,1 measured at a constant temperature of 24°C.*

As we can observe from the graphical representations in figure 4.13 and 4.14, the storage or the elastic modulus of the two samples increases with time, indicating that when the hydrogel samples are left at rest, at room temperature and in the absence of any other external factors that might influence the structure of the system, the gel exhibit an increase in the storage modulus, which means that the gel structure acquire more stiffness with time. However, the reproducibility effect of these two hydrogels samples cannot be clearly explained with those graphical representations, due to the fact the standard deviation of each measurement almost overlap one another.

But if we consider the storage modulus in the case of one day measurement for sample 1,8/0,5/0,2 or that of the nine days measurement for sample 1,75/0,65/0,1 we can easily observe that the standard deviations of these two measurements are not so elevated, and thus, we can conclude the two systems are reproducible in those cases. A better graphical representation for the description of the hydrogel reproducibility and ageing effect will be discuss later in this paragraph.

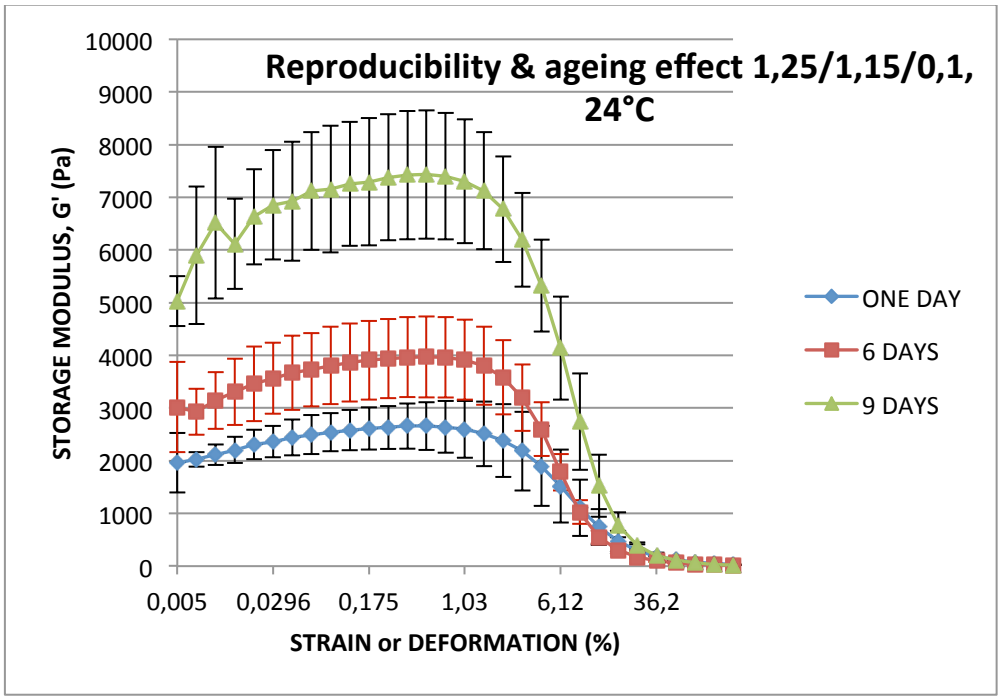

Figure 4.15. *Graphical representation of the hydrogel reproducibility and ageing effect for sample 1,25/1,15/0,1 measured at a constant temperature of 24°C.*

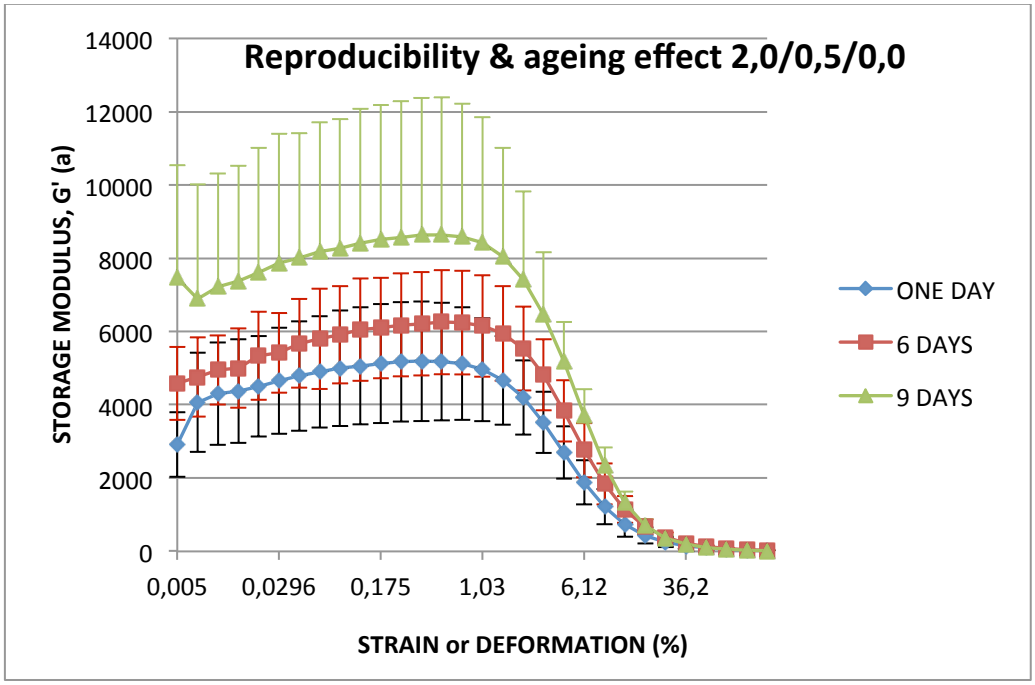

Figure 4.16. *Graphical representation of the hydrogel reproducibility and ageing effect for sample 2,0/0,5/0,0 measured at a constant temperature of 24°C.*

Also in these two recent graphical representations in figure 4.15 and 4.16, we can observe that the storage modulus of the two samples increases with time, indicating the same exact behavior as those of the samples describe in figure 4.13 and 4.14. These hydrogels samples exhibit an increase in the material stiffness with time, however also in these two cases, the reproducibility effect of these two samples cannot be easily proven with those graphical representations, because the standard deviation of some of the measurements overlap one another.

But, if we consider the case of the sample 1,25/1,15/0,1, we can clearly observe that the elastic modulus of this sample increases with time, and also, the reproducibility effect is properly outlined, because none of the standard deviations of the three measurements overlapped each other, and the variations of these values are also very low.

In order to reproduce clearly the reproducibility and ageing effect of the hydrogels samples stated above, it is necessary to give some definitions regarding to the mathematical expression we needed for describing these behaviors.

With respect to the four hydrogels samples and to the time scale the rheological properties were measured (one, six and nine days), we can define the following equilibrium modulus of the samples.

We have defined the equilibrium modulus (EM) as the ratio between two storage moduli of the same sample that were measured in different time scales. And in order to be able to describe correctly these behaviors, all samples were measured for a number of time equal to 3 (n=3), then the statistical approach was followed by calculating the standard deviations of every sample.

Then with respect to the one day measurements, we defined the equilibrium moduli as the following:

For t = 1,

$$EM_1 = \frac{G'1,1}{G'1,1} \;.\; EM_1 = \frac{G'1,2}{G'1,2} \;.\; EM_1 = \frac{G'1,3}{G'1,3} \tag{4.8}$$

While for the six days measurements, the equilibrium moduli are as follows:

For t = 6

$$EM_6 = \frac{G'6,1}{G'1,1} \ . \ EM_6 = \frac{G'6,2}{G'1,2} \ . \ EM_6 = \frac{G'6,3}{G'1,3} \tag{4.9}$$

And finally, for the nine days measurements, the resulting equilibrium moduli are as follows:

For t = 9

$$EM_9 = \frac{G'9,1}{G'1,1} \ . \ EM_9 = \frac{G'9,2}{G'1,2} \ . \ EM_9 = \frac{G'9,3}{G'1,3} \tag{4.10}$$

So, considering for instance the one day measurements, the average value of the equilibrium modulus was calculated and the resulting standard deviation was also calculated. The graphical representations for these calculations are plotted below for the four hydrogels samples that we are considering for the reproducibility and ageing effect.

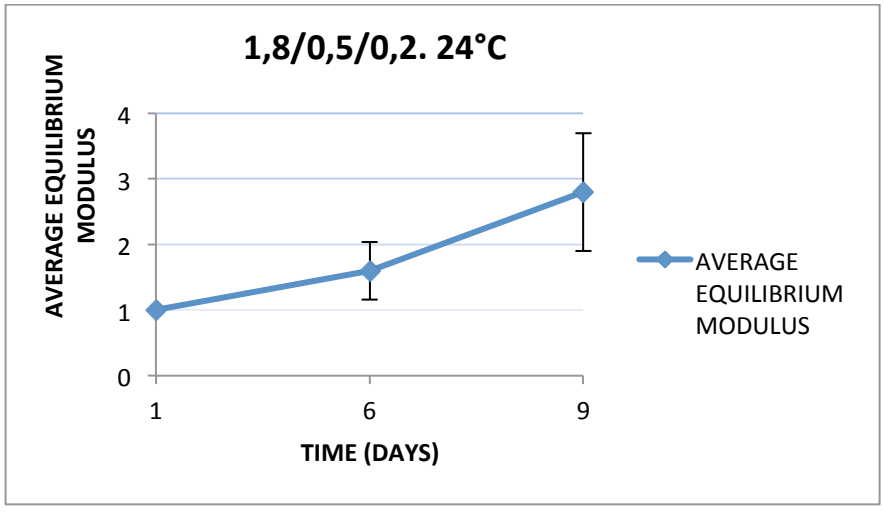

Figure 4.17. *Graphical representations for the average equilibrium modulus for sample 1,8/0,5/0.2, measurement made at 24°C.*

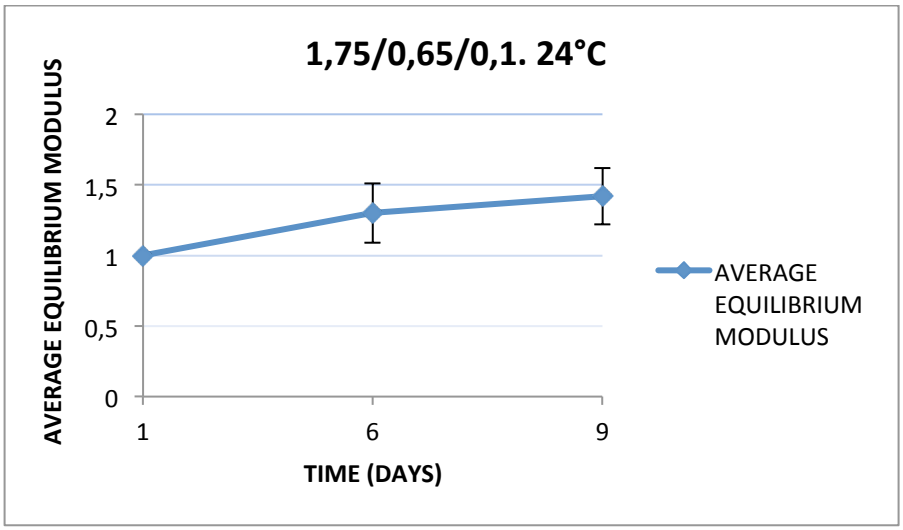

Figure 4.18. *Graphical representations for the average equilibrium modulus for sample 1,75/0,65/0,1, measurement made at 24°C.*

As we can observe from the graphical representations in figure 4.17 and 4.18, the average equilibrium modulus of the two samples increases with time, indicating an increase in the stiffness of the material with time. So with these representations, the ageing effect on the hydrogels systems is clearly shown. And also, the reproducibility of these two samples we considering in the figure 4.17 and 4.18 are also reliable, because the standard deviations of the two systems are relatively low.

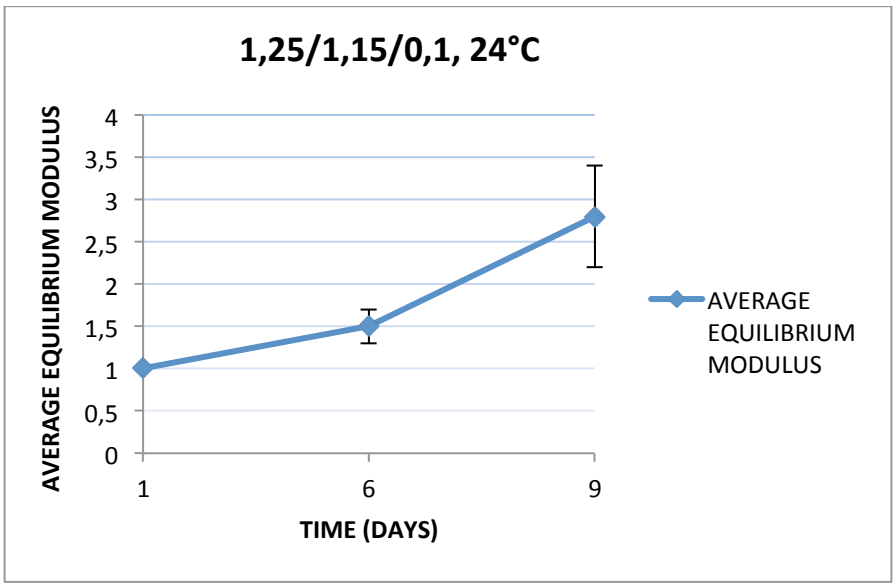

Figure 4.19. *Graphical representations for the average equilibrium modulus for sample 1,25/1,15/0,1, measurement made at 24°C.*

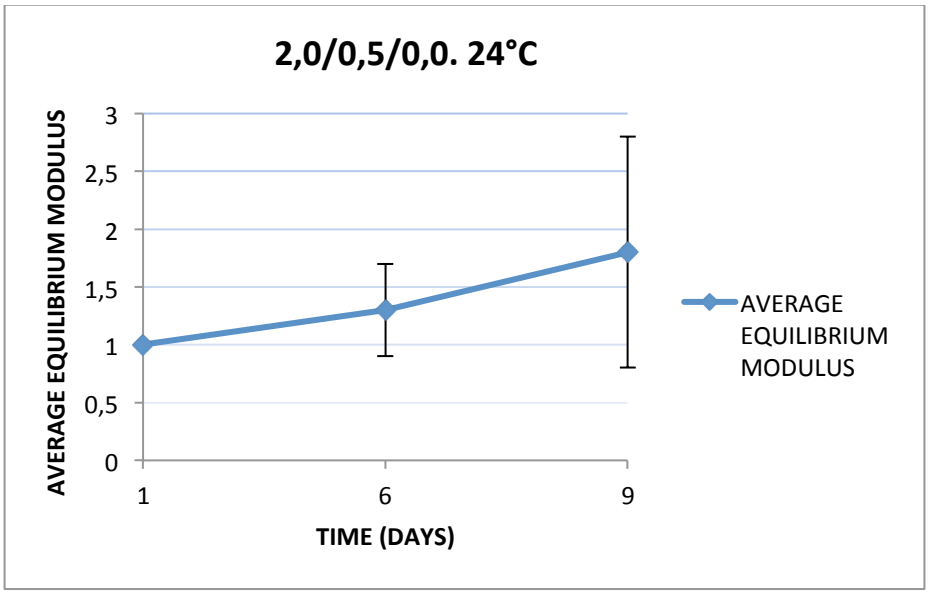

Figure 4.20. *Graphical representations for the average equilibrium modulus for sample 2,0/0,5/0,0, measurement made at 24°C.*

Also, in these two recent representations in figure 4.19 and 4.20, we can conclude that with the use of this type of representation, we can easily outline and define the reproducibility and ageing effect of the hydrogel system, however, the reproducibility of these hydrogels samples cannot be said to be 100% accurate, because in some cases, the errors are also high.

Meanwhile, the increase in the storage or elastic moduli of the hydrogels samples with time, is a very relevant point to be take into account, because it has been proven that the hydrogels systems will continue to have a growth in the storage modulus with time, however, there is a limit of time to this, when the storage moduli of these hydrogels systems will begin to show a dramatic decrease, due to the degradation of the polymers or a slow dissolution of the particles over time (Hossein & Sardinha, 2005).

4.4 Temperature effect on the hydrogel rheological properties

We have also made a study on the effect of temperature on the rheological properties of the hydrogels systems, just by varying the temperature at which these properties were measured.

It is very important to outline the fact that temperature has a considerable impact on the process of gelation or degradation of the hydrogels system. So, the variation of temperature during the rheological measurement of the hydrogels systems was made on the

following samples: 1,8/0,5/0,2. 1,75/0,65/0,1 and 2,0/0,5/0,0, all these samples were measured at both 24°C and 37°C, and each measurement was made for a number of time equal to three (n = 3), in order to describe properly with statistical approach the temperature effect on the hydrogels rheological properties. And also, all the samples considered in this experiment are prepared and maintain in the same physiological conditions.

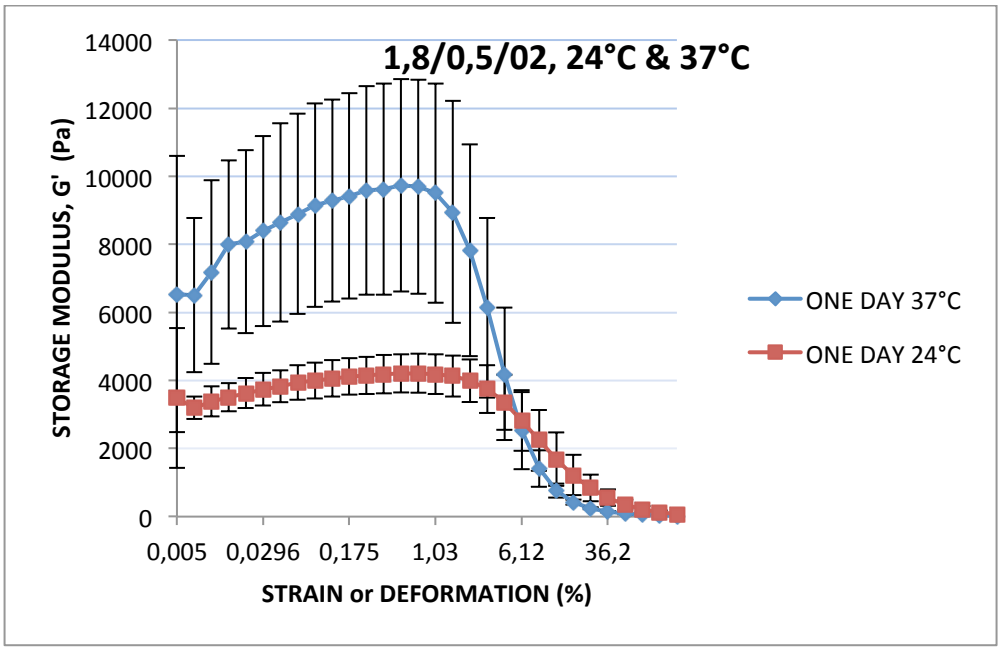

Figure 4.21. *Temperature effect on hydrogel sample 1,8/0,5/0,2, measurement made at 24°c and 37°C.*

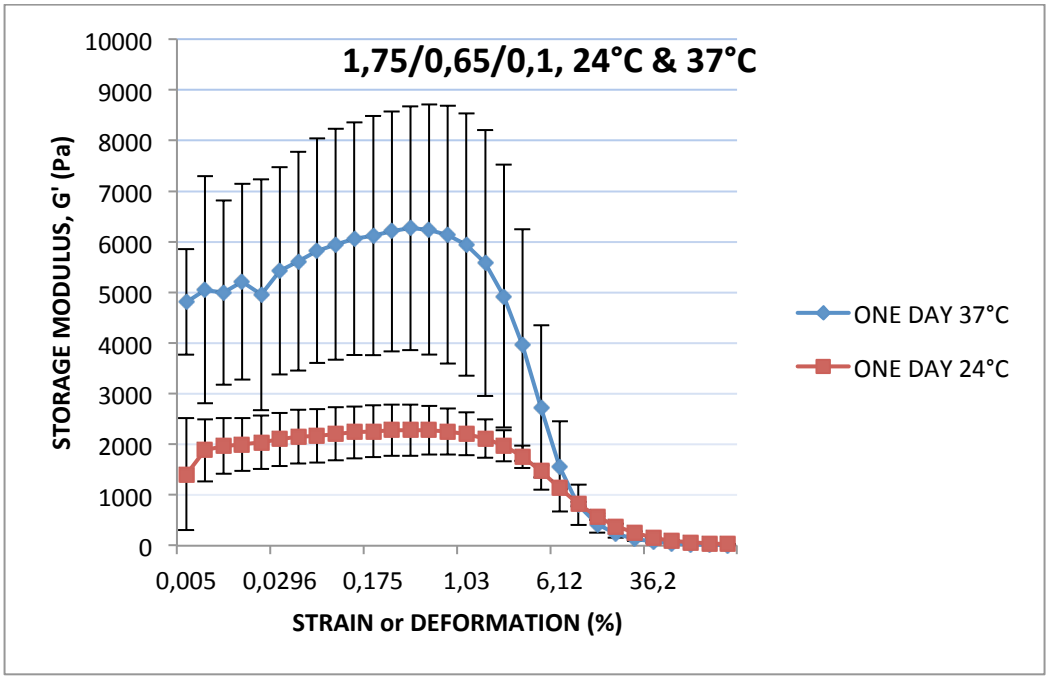

Figure 4.22. *Temperature effect on hydrogel sample 1,75/0,65/0,1, measurement made at 24°c and 37°C.*

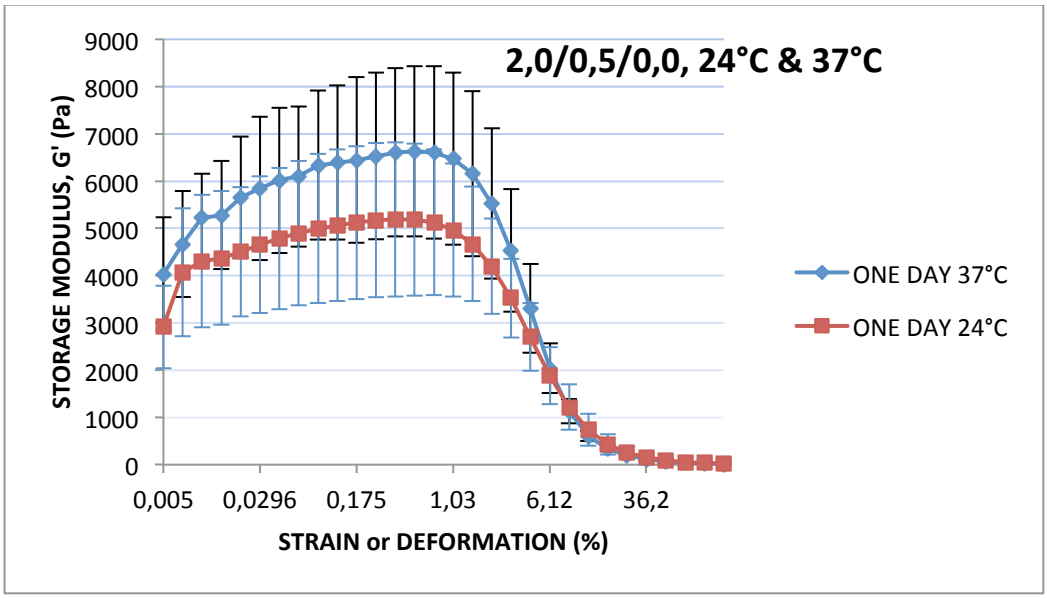

Figure 4.23. *Temperature effect on hydrogel sample 2,0/0,5/0,0, measurement made at 24°C and 37°C.*

As we can observe from the recent three graphical representations in figure 4.21, 4.22 and 4.23, that an increase in the temperature from 24°C to 37°C, at which the rheological properties of these hydrogels samples were measured, result in an increased of the resulting storage or elastic modulus of the samples. However, this is not the only effect that is observed with an increased in the temperature, we have found out that the increase in temperature also have an effect on the yield or cross-over point of the hydrogels systems, that is the point where the transition from the viscoelastic solid behavior to viscoelastic liquid behavior occurred. The following graphical representations below are used to show the differences in the yield points of the hydrogels samples, when subjected to two different range of temperature during the measurement of the rheological properties.

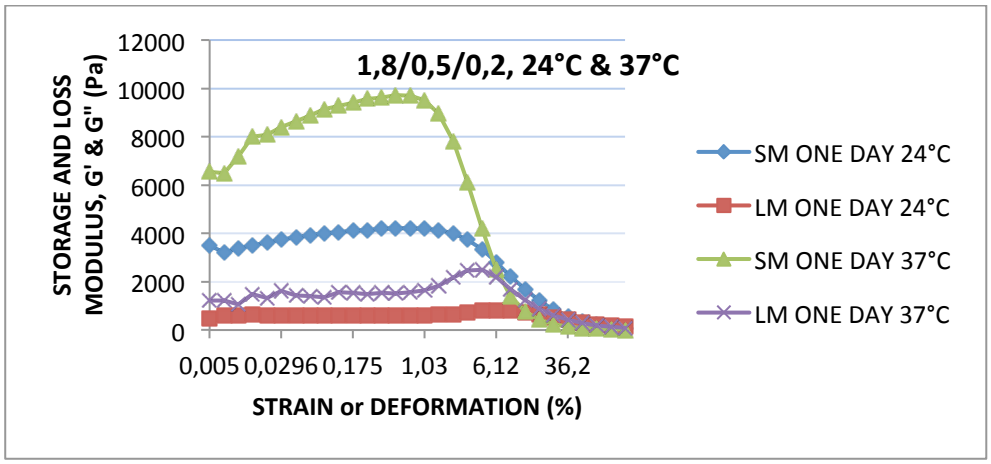

Figure 4.24. *Temperature effect on the yield point of sample 1,8/0,5/0,2, at 24°C and 37°C.*

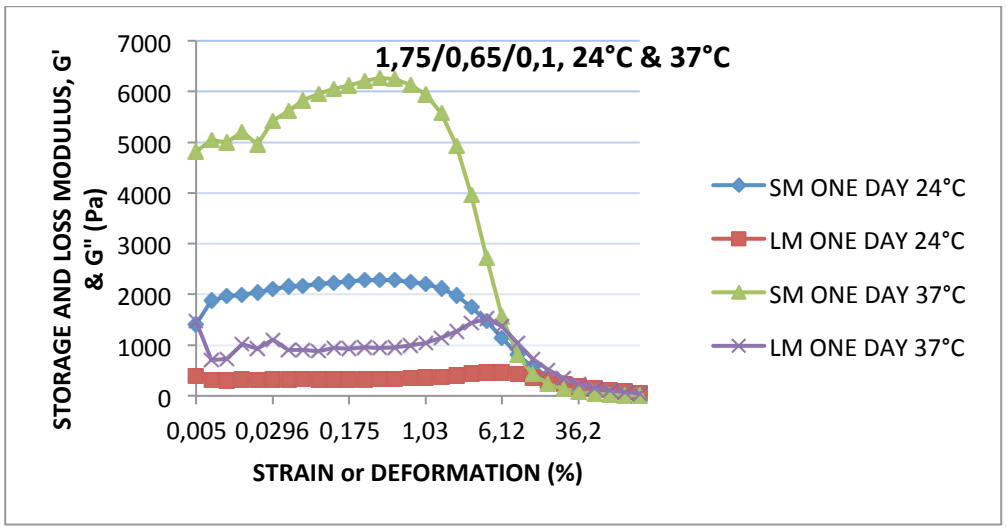

Figure 4.25. *Temperature effect on the yield point of sample 1,75/0,65/0,1, at 24°C and 37°C.*

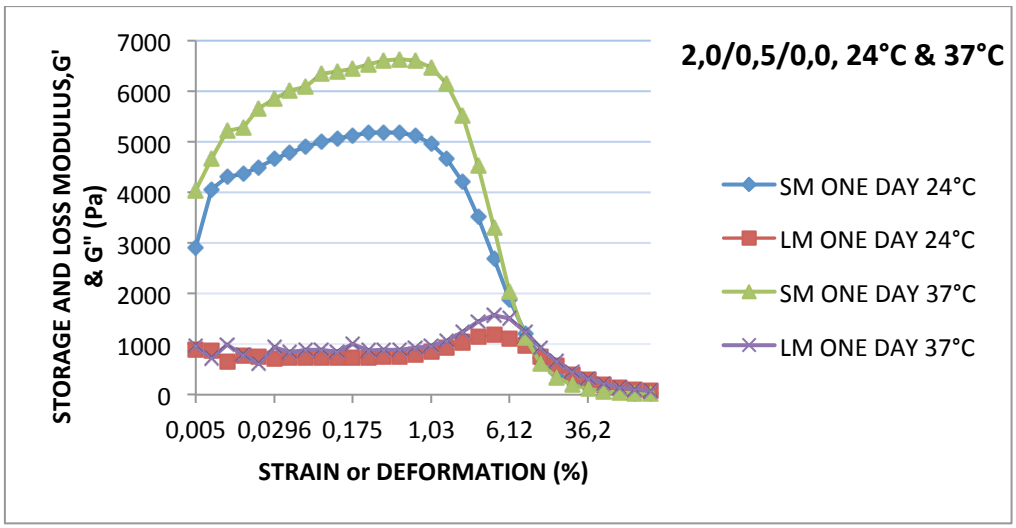

Figure 4.26. *Temperature effect on the yield point of sample 2,0/0,5/0,0, at 24°C and 37°C.*

As we can clearly observe from this recent representations in figure 4.24, 4.25 and 4.26, that increasing the temperature at which the rheological properties of the hydrogels samples were measured, lead to an increase in the elastic modulus of the sample that were measured at higher temperature, but also lower the yield point of the material, with respect to that of the sample that were measured at lower temperature.

So, the transition from the viscoelastic solid to viscoelastic liquid material, resulted to be faster with an increase in the temperature at which the rheological properties are measured.

Conclusion

Nanocomposite hydrogels are new generation materials useful for a wide range of applications, from stimuli-responsive sensors and actuators to microfluidics, pharmaceutical and biomedical devices. And large amount of recent reports have explained some of the physics and chemistry behind the unique properties of these hydrogels materials, making them to find a wide range of applications in the biomedical and tissue engineering field.

However, improvements in the mechanical properties and biocompatibility of the hydrogel systems will need to be considered before their application in the field. So, it is necessary to find out the procedure for obtaining hydrogels with greater mechanical properties, and that are able to withstand any physiological changes within the system. Application of hydrogels with a great mechanical properties and at the same time having an optimal degree of swelling, is of great importance, because the higher the swelling properties or pores or even the equilibrium water content of a hydrogel system, the higher is the diffusion rate of solutes through it, but also an easier control of nutrients permeation into the cells and of cellular products out of the hydrogels systems is obtained.

The necessity for having the best mechanical properties and swelling behavior simultaneously in the hydrogel system, has led us to the development of hydrogels with different compositions of the components and with different medium of growth. And with this, we were able to underline and define the viscoelastic properties of these gels, by using both the dynamic and static method for the rheological measurement. Beside, different hydrogels phase were described by using the ternary diagram, where we were able to show the various physical states of the gels, based on the fact that sol, soft and fixed or permanent gels were obtained.

Monitoring the rheological behavior and structural evolution of these gels during and after flow can help to understand the process of therapy retention and drug delivery during syringe injection and the ability of the material to stay localized after injection against possible biological forces in vivo. With the dynamic method of measurement, it was possible to describe the viscoelastic properties of the hydrogels, simply by showing the variation of the gel storage and loss modulus; that is the deformation energy stored in the hydrogel during its shear or deformation process, and the amount of energy dissipated by the material under shear of deformation, respectively. Basically, by increasing the concentration of the crosslinking

agent: laponite, and varying the composition of the polymers, both the synthetic and the natural polymer, fixed or permanent gels were obtained, indicating a system with a stronger mechanical properties, that is, with higher storage or elastic modulus. And this improvement in the storage modulus is only possible when the polymer concentration is large enough for the coverage of the laponite surface. And it was proven that with polymer concentrations higher than that of the laponite, sol and soft gels were formed, indicating a predominance of the viscous property of the gel. Dynamic viscosity and shear stress experiments, were also conducted on the hydrogels system, and these were necessary for emphasizing the non-Newtonian behavior of the gels and their shear-thinning property.

So, in order to outline the hydrogels thixotropic behavior during flow or their abilities to retain or recover their solid state morphology and rigidity after experiencing shear flow or strain, static method experiment was also conducted on them, which basically consist of "creep and creep recovery test measurement" and "relaxation test measurement". The creep and creep recovery test, allow us to explain the temporal evolution of gel viscoelastic solid or liquid-like behavior, by applying a constant shear stress to the material and measuring the corresponding shear strain as a function of time, which indicate the ability of the gel to recover or not to the initial state. And it has been proven that hydrogels with a higher value of stiffness or elastic modulus, exhibit a lower value of creep compliance, indicating a material that can be categorized as an "almost viscoelastic solid material", with an ability to recover almost completely to the initial state. The opposite behavior is observed in hydrogels exhibiting the so called "viscoelastic liquid material". While the relaxation test experiment was conducted in order to be able quantify the strength of the hydrogel structure; the higher the stress relaxation modulus of the gel, the higher is the stress needed for the material to be deformed and the relaxation spectrum or the degradation process of the material to a constant permanent phase is slower.

It has also been demonstrated that, increasing the crosslinking density, there is an increase in the hydrophobicity and a decrease in the stretchability of the hydrogel network structure, and thus, a decrease in the hydrogel swelling degree, a situation that correspond to a reduction of the diffusion rate of solute or drug through the system. And a higher swelling degree was also obtained in samples that were predominantly prepared in a deionized water with respect to those prepared mainly in phosphate buffered saline solution. Higher swelling degree corresponds to lower mechanical property of the hydrogel structure. So, depending on

the type of applications, it would be necessary to find a situation where both the mechanical properties and the swelling behavior of the gels are satisfied.

Another interesting study that was made, is on the hydrogel reproducibility and ageing effect, together with the variation of the temperature at which the rheological properties of the gels were measured. Whereby, hydrogel reproducibility is referred to the process of obtaining the same rheological properties of the same hydrogel sample, when measured at different range of time, and in the same physiological conditions. And based on the tests that have been performed on some hydrogel samples, the hydrogels reproducibility can be said to be almost impossible in most cases, due to the fact that there is a large variation in the statistical representations of the rheological data of some samples. The main factor affecting the hydrogel reproducibility can mainly be attributed to the gel inhomogeneity; which derived mostly from the process of mixing the resulting sample, the ambient conditions and also from the degree of interactions between the silicate nanoparticles and the polymers.

It is also important to take into account the fact that the hydrogel rheological properties varies with time, and with respect to some hydrogels samples that were prepared and measured in different time range, it was possible to prove that the gel stiffness (elastic or storage modulus) increases with time until a certain period, and then later followed by a decrease in the gel stiffness, after the degradation of the gel structure with time. The temperature effect was also considered when measuring the gel rheological properties, and it was proven that, increasing the measurement temperature of the rheometer from 24˚C and 37˚C, there is an increase in the material storage modulus, but at the same time, the yield point of the system is anticipated with respect to the measurement that was made at 24˚C.

Abbreviation

IPN = Interpenetrating polymeric network

PEO = Polyethylene oxide

PEG = Polyethylene glycol

PEGMA = Polyethylene glycol methacrylate

PEGDMA = Polyethylene glycol dimethacrylate

PEGDA = Polyethylene glycol diacrylate

Mw = Molecular weight

ECM = Extracellular matrix

PBS = Phosphate buffered saline

De = Deborah number

T = Characteristic time of deformation

τ = Characteristic time of the material

G´ = Storage or elastic modulus

G" = Loss modulus

%S = Swelling degree

W_s = Initial weight of the swollen gel

W_d = Final weight of the dry gel

EWC = Equilibrium water content

W_w = Weight of water in the gel

W_t = Total weight of the hydrated gel

ΔG_{mix} = Free energy of mixing

ΔG_{system} = Free energy of the system

$\Delta G_{elastic}$ = The elastic free energy contribution

m_{gel} = Mass of the initial swollen gel

m_{poly} = Mass of the polymer in the sample

m_{lapo} = Mass of the silicate particles

m_{H2O} = Mass of water in the gel

m_{salt} = Mass of the salt in the gel

$m_{dry\ gel}$ = Mass of the final dry gel

$\gamma(t)$ = Shear strain

$\tau(t)$ = Shear stress

tan δ = Loss factor

γ^* = Complex shear strain

$G^*(\omega)$ = Complex shear modulus, as a function of the angular frequency

σ^* = Complex shear stress

$\acute{y}$ = Shear rate

$\boldsymbol{\theta} =$ Characteristic time constant

η = Viscosity

ω = Angular frequency

j(t) = Creep compliance

G(t) = Stress relaxation modulus

τ_0 = Yield stress

EM = Equilibrium modulus

n_1 = moles of the swelling agent (water)

n_2 = moles of polymer

v_1 = volume fraction of the swelling agent

v_2 = volume fraction of polymer

K = Boltzmann constant

X = Flory polymer – solvent interaction parameter

$\Delta S_{elastic}$ = Entropy variation in the deformation process

v_e = Effective number of chains in the network

α_s = Expansion factor

$(\Delta\mu)_{elas}$ = Variation of the elastic chemical potential

$(\Delta\mu)_{mix}$ = Variation of the mixing process chemical potential

$(\Delta\mu)_I$ = Variation of the chemical potential due to the variation in the mobile ion concentration

$(\Delta\mu)_{elect}$ = Variation of the chemical potential due to the electrostatic repulsive force.

Idrogeli polimerici per le applicazioni biomedicali

La sintesi degli idrogeli a partire dall'interazione in soluzione acquosa, tra le particelle di silicati (Laponite) e polimeri, ha ottenuto una considerazione importante negli ultimi anni, grazie alla capacità di questi materiali di trovare un impiego fondamentale nel campo biomedicale e nelle applicazioni per l'ingegneria dei tessuti.

Fin dagli anni 60, gli idrogeli polimerici sono stati riconosciuti come sistemi più adatti per svolgere la funzione di rilascio "intelligente" di un farmaco, ed in particolare, grazie alla loro elevato grado di biocompatibilità, unito ad una particolare sensibilità alle variazioni di grandezze fisiche e chimiche, come per esempio: temperatura, forza ionica tra le catene, pH ed anche le condizioni ambientali, li rende particolarmente indicati alla soluzione di diverse problematiche in ambito medico, farmaceutico ed altri.

Un idrogel può essere definito quindi come un network polimerico, costituito da una struttura tridimensionale, avente una capacità elevata di trattenere grandi quantità di acqua e di rilasciarla oppure riassorbirla in risposta alle variazioni degli stimoli esterni. Questa capacità degli idrogeli di trattenere acqua è dovuta principalmente alla idrofilicità delle catene polimeriche costituenti la fase solida, e provocata dalla presenza di gruppi funzionali polari: OH^- e COO^- , in grado di formare con il liquido dei legami a ponte di idrogeno. La quantità di acqua presente negli idrogeli potrebbe variare tra un valore prossimo al 10% del peso del gel secco (zerogel) ad un valore pari a mille volte il peso del gel secco. Quindi, in pratica, un gel può essere considerato come una soluzione polimerica, costituito da una matrice solida (network) reticolata con interazione sia fisiche che chimiche (crosslinks) e completamente permeata da un liquido, avendo le proprietà coesive di un solido e le proprietà di trasporto diffusivo di un liquido. I gel vengono classificati in base alla natura della loro struttura, che può essere gel forti o deboli, e questo dipende principalmente dalle concentrazioni e dalle interazioni tra i componenti che costituiscono il gel: Laponite-polimero.

Oltre alla buona capacità degli idrogeli di trattenere acqua, essi possiedono anche delle buone proprietà meccaniche (per esempio la viscoelasticità), bassa tensione interfacciale con fluidi fisiologici, buona permeabilità con sostanze a basso peso molecolare (ossigeno). Queste proprietà appena elencate, fanno si che gli idrogeli trovino applicazioni per esempio nel settore del rivestimento (cateteri, fili di sutura), impianti (lenti a contatto, dischi intervertebrali), dispositivi biomedici (membrane per emodialisi, celle per elettroforesi),

sistemi di rilascio controllato (farmaci) ed anche nel campo dell'ingegneria tissutale (supporto per crescita cellulare).

L'obbiettivo principale di questo studio, è stato quello di sintetizzare degli idrogeli che abbiano proprietà sia meccaniche che strutturali simili a quelli di alcuni organi o tessuti del corpo umano, si parla quindi della necessità di rigenerazione di tessuti del corpo (epiteliale, tessuto nervoso, cartilagineo ed altri), attraverso la coltivazione di cellule su apposite strutture dette scaffold (strutture che fungono da matrice extracellulare ECM, e consentono la crescita e la formazione del tessuto desiderato), consentendo cosi di evitare il rincorso al trapianto di organi o tessuti danneggiati nel corpo. Inoltre, è necessario avere idrogeli che abbiano una buona proprietà di rigonfiamento, garantendo cosi un ottima proprietà di trasporto di soluto o medicinali.

Perciò, l'attenzione è stata focalizzata sullo studio del comportamento viscoelastico e di rigonfiamento dei gel. Il comportamento viscoelastico è stato determinato mediante la misura con il rheometro di alcune proprietà come il modulo elastico (quantità di energia assorbita da un materiale quando essa viene sottoposto ad una certa deformazione) ed il modulo di dissipazione (quantità di energia dissipata da un materiale, quando essa viene deformata), cosi come è stato possibile effettuare uno studio di “creep and creep recovery test”, “relaxation test experiment” e la possibilità di avere una reproducibilità di alcuni parametri rheologici con il tempo. Mentre per il rigonfiamento, sono stati studiati e determinati i fattori che influenzano il grado di rigonfiamento dei gel ed il contenuto di acqua di questi gel.

È stato possibile ottenere idrogeli con proprietà meccaniche diverse, semplicemente variando la concentrazione dell'agente di crosslinking: laponite, ed insieme alla variazione della composizione dei polimeri, sia quello naturale che sintetico. Facendo cosi, si è riuscito ad ottenere gel che possono essere definiti come “gel solidi oppure permanente” e quelli definiti come “sol gel”, in base al fatto che si riesce ad ottenere gel con elevato oppure basso valore del modulo elastico rispettivamente. Quindi, il fatto di ottenere idrogeli con buone proprietà meccaniche risulta essere possibile solo nel momento in cui la concentrazione del polimero è sufficientemente elevato per poter ricoprire la superficie delle particelle di laponite, e questo implica che prevale la proprietà elastica nel materiale. Mentre sono stati formati i “sol gel” quando la concentrazione del polimero risulta ad essere superiore rispetto al quella del laponite, dando luogo alla prevalenza della proprietà viscosa nel materiale.

Per poter delineare in modo appropriato il comportamento non-Newtoniano e tixotropico degli idrogeli, è stato condotto anche la prova di “creep and creep recovery”, dove si è dimostrato che la maggioranza dei campioni di gel preparati, risultano essere in grado di recuperare in maniera quasi completa, la loro forma oppure struttura iniziale, al seguito di un'applicazione di una deformazione costante. Questo ci ha consentito di classificare il gel come materiale di tipo “viscoelastic solid material” oppure “viscoelastic liquid material”, a seconda del fatto che si riesca ad ottenere un recupero completo o no della struttura iniziale del gel.

Con lo studio del rigonfiamento dei gel, si è dimostrato che idrogeli con elevato valore oppure concentrazione di laponite, presentano un grado di rigonfiamento più basso, e questo è dovuto principalmente all'aumento del grado di idrofobocità nelle catene, con l'aumento del modulo elastico o rigidezza del materiale, avendo come conseguenza la riduzione della velocità di diffusione del soluto oppure del medicinale attraverso il poro. Perciò, è stato necessario individuare le condizioni ottimali che garantiscono un grado di rigonfiamento elevato. E ciò viene fatto, variando non soltanto le composizioni dei gel, ma variando anche il tipo di solvente utilizzato nella preparazione di questi gel: acqua deionizzata ed una soluzione salina di fosfato (PBS). Facendo cosi, si è visto che il grado di rigonfiamento migliore si ottiene nei campioni di gel preparati principalmente in acqua deionizzata, e questo è da attribuire all'elevato valore della pressione osmotica ed assenza del “charge screening effect” nell’acqua deionizzata, diversamente rispetto a quanto avviene nella soluzione salina.

Un altro studio che è stato condotto, riguarda la riproducibilità dei dati oppure dei parametri reologici degli idrogeli, e la variazione di questi parametri con il tempo, cosi come è stato analizzato la variazione di questi parametri al variare della temperatura di misurazione con il reometro. Dove, per la riproducibilità dei parametri reologici, si intende una situazione in cui si riesce ad ottenere le stesse proprietà reologiche dello stesso campione di idrogelo, quando queste vengono misurate in istante di tempo diversi, e nelle stesse condizioni fisiologiche. Ed in base alle prove che sono state fatte, si potrebbe concludere che la riproducibilità dei parametri reologici dei gel è del tutto impossibile in molti casi, e questo è dovuto al fatto che vi è un'ampia variazione nelle rappresentazioni statistiche dei parametri reologici di molti campioni di gel. Il fattore principale che potrebbe influenzare la riproducibilità degli idrogeli può essere attribuito alla non-omogeneità della struttura dei gel di partenza, che al sua volta dipende dal processo di mescolamento, dalle condizioni ambientali ed dal grado di interazione tra le particelle di laponite ed i polimeri.

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