




POLITECNICO DI TORINO

MASTER'S THESIS

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# Shear-thinning biomaterial for birth control

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# Declaration of Authorship

We, Alessia Martiri and Maria Teresa Sposato, declare that this thesis titled "Shear-thinning biomaterial for birth control" and the work presented in it are our own. We confirm that:

- This work was done wholly or mainly while in candidature for a master degree at this University, Politecnico di Torino.
- Where we have consulted the published work of others, this is always clearly attributed.
- Where we have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely our own work.
- We have acknowledged all main sources of help.

Signed:

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"...reca la scritta, DON'T PANIC, niente panico, in grandi e rassicuranti caratteri sulla copertina."

*Douglas Adams, Guida Galattica per Autostoppisti*

# Sommario

Quello del controllo delle nascite è un problema globale, legato al controllo della dimensione della popolazione e dello sviluppo economico e sociale. Oggi sono disponibili diverse opzioni contraccettive, ognuna delle quali presenta degli effetti collaterali e spesso una probabilità di insuccesso elevata. Nonostante la grande varietà di birth control technologies, negli Stati Uniti il 49% delle gravidanze sono indesiderate [1]. Il bisogno di nuovi sicuri ed efficaci contraccettivi nasce poiché, benché nel campo degli anticoncezionali importanti cambiamenti siano avvenuti negli ultimi 50 anni, i metodi contraccettivi attualmente in commercio non aderiscono perfettamente alle condizioni sociali, economiche, religiose e di salute di molti. Nel seguente lavoro si propone un metodo di contraccezione innovativo che fa uso di un biomateriale con caratteristiche di shear-thinning grazie alle quali può essere facilmente iniettato attraverso un microcatetere, esibendo un comportamento simile a quello di un fluido, e capace di occludere le tube di Falloppio per prevenire la fecondazione. Questo tipo di approccio rientra nei metodi non chirurgici a lungo/medio termine ed è indicato anche per utilizzatori diabetici o con patologie cardiovascolari, per i quali i contraccettivi farmacologici sono fortemente sconsigliati. Il STB (Shear-Thinning Biomaterial) è progettato con l'intento di non indurre la crescita di tessuto fibrotico provocando una sterilizzazione permanente, ma al contempo, avere una velocità di degradazione molto bassa, che ne consenta l'impianto in vivo. Per di più, al fine di recuperare la fertilità, sono stati condotti degli esperimenti di inversione del processo di gelificazione, impiegando acqua e senza l'uso di additivi. Grazie al semplice meccanismo d'azione e di applicazione, l'efficacia di un anticoncezionale come quello sviluppato risulta indipendente dall'amplesso e dall'utilizzatore, per cui uso perfetto e uso tipico coincidono. Nel seguente lavoro il materiale, composto da Laponite e Polyethylene oxide, è stato prima sintetizzato secondo otto diverse formulazioni, e poi caratterizzato attraverso test di tipo meccanico, quali le misure di iniettabilità, occlusività, adesività ed elasticità, e chimico, per valutarne la stabilità in soluzione, lo swelling, la velocità di degradazione e l'aging. Infine, test cellulari su un ottimizzato set di gel hanno determinato l'effetto biologico sulle cellule messe a contatto con esso e messe a contatto con Laponite e PEO.

# Abstract

Birth control is a global problem, related to the management of the size of the population and to the economic and social development. Today, a huge number of contraceptive options are available, each of them has side effects and, often, a high probability of failure. Despite the wide variety of birth control technologies, 49% of pregnancies in the United States are unwanted [1]. The need for safe and effective new contraceptives arises because, even if in the field of family planning big changes have taken place in the last 50 years, the currently used contraceptive methods do not fit perfectly with the social, economic, religious and health conditions of many people.

In this work, we propose an innovative contraceptive method using a biomaterial with shear-thinning properties thanks to which it can be easily injected through a microcatheter, exhibiting a behavior similar to a fluid, and able to occlude the Fallopian tubes preventing fertilization. This type of approach is a long-acting non-surgical method and it is appropriate also for diabetic users or women with cardiovascular diseases, for whom hormonal contraceptives are not recommended.

The STB (Shear-Thinning Biomaterial) is designed with the aim of not inducing the growth of fibrotic tissue which leads to a permanent sterilization and, at the same time, it should have a very low degradation rate, for a future in vivo implantation. Moreover, in order to recover fertility, experiments have been carried out to reverse gelification, using water and without additives. Thanks to the simple mechanism of action and application, the efficacy of the developed contraceptive is independent from the intercourse and the user, thus perfect use and typical use coincide.

In the following work, the material, made of Laponite and Polyethylene oxide, was first synthesized in eight different formulations, then characterized by mechanical tests, such as injectability, occlusive tests, adhesiveness and elasticity tests, and chemical tests, to evaluate the stability in solution, swelling, degradation rate and aging. Finally, cytotoxicity studies on an optimized set of gels showed the biological effect on the cells in contact with STB and with Laponite and PEO.

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# List of Abbreviations

| | |
|---|---|
| **DMEM** | **D**ulbecco's **M**odified **E**agle **M**edium |
| **HTF** | **H**uman **T**ubal **F**luid |
| **PBS** | **P**hosphate-**B**uffered **S**aline |
| **PEO** | **P**oly **E**thylene **O**xide |
| **L/D** | **L**ive & **D**ead |
| **FBS** | **F**etal **B**ovine **S**erum |
| **DPBS** | **D**ulbecco's **P**hosphate-**B**uffered **S**aline |
| **Pen-Strep** | **Pen**nicilin-**Strep**tomycin-Glutamine |
| **PEG** | **P**oly **E**thylene **G**lycol |
| **DOPA** | **D**i **O**xi **P**henyl **A**lanine |
| **PMMA** | **P**oly **M**ethyl **M**eth **A**crylate |
| **STD** | **S**exually **T**rasmitted **D**iseases |
| **IUD** | **I**ntra **U**terine **D**evice |
| **STB** | **S**hear **T**hinning **B**iomaterial |
| **AIDS** | **A**cquired **I**mmune **D**eficiency **S**yndrome |
| **RT** | **R**oom **T**emperature |

# Chapter 1

# Introduction

## 1.1 Anatomical and Physiological Background

The reproductive system includes all the organs involved in mating, gametogenesis or other functions related to the propagation of the species, which ensure their survival [2], such as gonads, which are the primary reproductive organs, released hormones and gametes they produce (seminal cells and egg cells) but also external genitals, ducts and glands.

The male gonads are testicles, which, working together with genital ducts, glands and supporting structures including scrotum and penis, produce and transport spermatozoa, through ejaculation, to the female genital tract where fertilization occurs.

Anatomically, the female reproductive system includes, from outside to inside:

- vulva or external genitals including mons pubis, a central relief of skin and adipose tissue, labia majora and labia minora that are a set of external and internal bending of the skin surrounding the opening of the vagina, the clitoris, a small erectile organ, the vaginal orifice and the vestibular glands which secrete a lubricating liquid;
- vagina, about 8-10 cm long and of considerable stretching, which goes from the vulva to the cervix and receives the seminal fluid of the male;
- uterus, which is a hollow organ located in the centre of the pelvic cavity, in shape and size similar to a pear which has the function of housing and feeding the fetus. There are three layers in the wall of the uterus: the perimetrium is the outermost layer of epithelial cells and connective tissue, the myometrium

or the layer of smooth muscle whose contractions help expel the fetus in childbirth and the endometrium that includes an epithelial part and a spongy connective part that during menstruation exfoliate. The lower part, narrower than uterus, which is in direct connection with the vagina, is called cervix.

- Fallopian tubes;
- ovaries, or female gonads, that are glands located in the pelvic cavity, on both sides of the uterus, under and behind the uterine tubes. The distal portion of the tuba bends around the ovary so that it is covered up by fimbria without really being attached to it. The ovaries perform two functions: ovulation and secretion of hormones (estrogen, progesterone and small amounts of testosterone). The follicle is the structure inside the ovary that provides the environment for the development and maturation of the egg cell until ovulation, when the oocyte is released and goes in the Fallopian tube, ready to meet a spermatozoon, starting the first stage of pregnancy, the fertilization, thus continuing with his natural consequence toward childbirth, the implantation of the fertilized egg to the uterine wall.

### 1.1.1 The Fallopian Tubes

As it was just mentioned, normally fertilization takes place inside the uterine tubes (or Fallopian tubes or oviducts or salpinxes), tubular seromuscular organs, placed on both sides of the upper part of the uterus. On average, each tube is about 11 cm long with an extended range from 8 to 15 cm [3] but the sizes are variable from one species to another: in pigs, each Fallopian tube can measure 28 cm [4] (Figure 1.1).

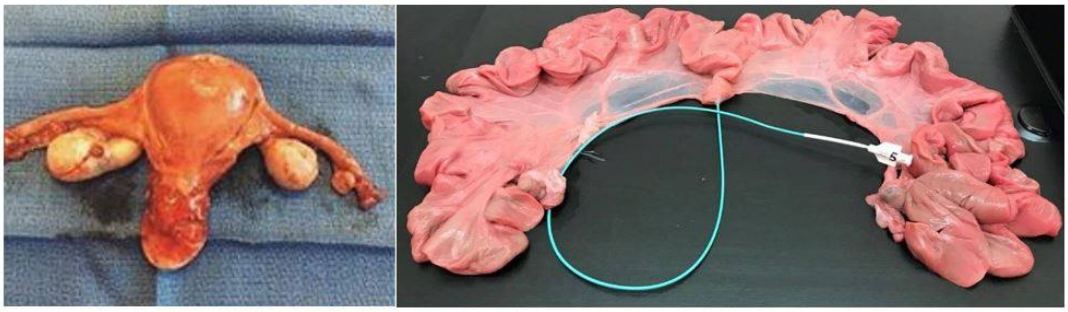

FIGURE 1.1: *Human vs porcine Fallopian tubes. The images show human internal genitalia [5] (on the left) and porcine internal genitalia (on the right). The differences in size are remarkable.*

In each Fallopian tube, four regions can easily be recognized because of distinctive histological characteristics (Figure 1.2): intramural segment, isthmus, ampulla, infundibulum.

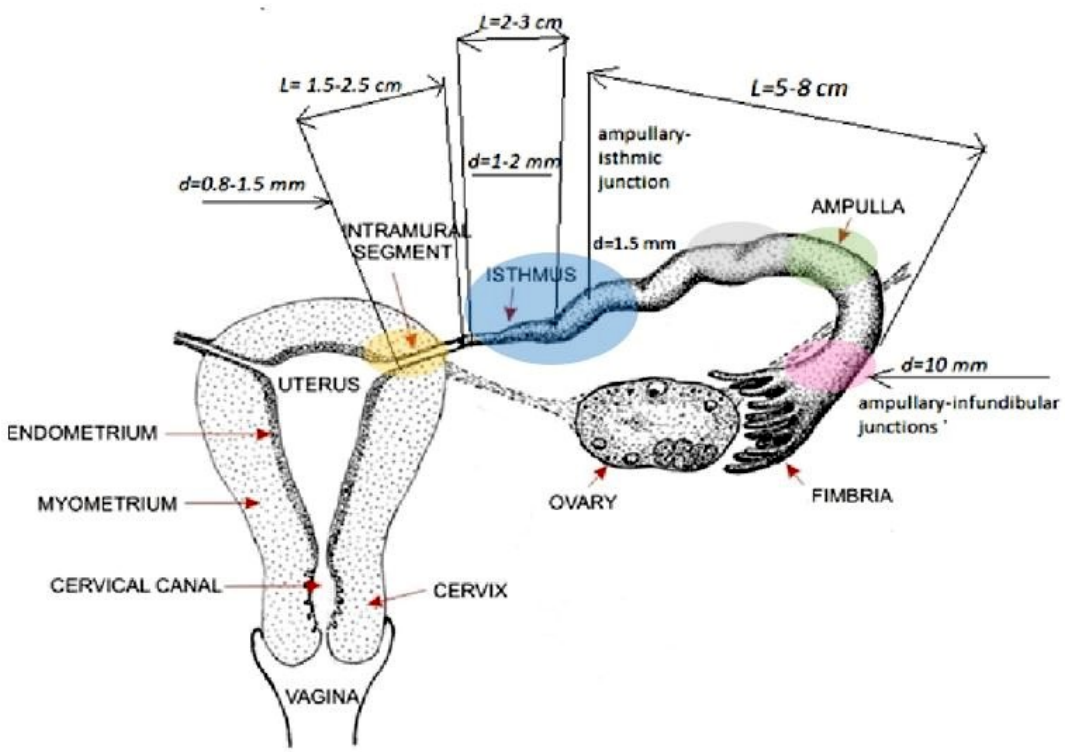


FIGURE 1.2: *Different regions of Fallopian tube. Photo taken and edited from [6]. In the figure, it is easy to recognize intramural segment, isthmus, ampulla and infundibulum. For each of them, dimensions were highlighted.*

Their common purpose is the transport of the oocyte from the ovary to the uterus:

when an egg cell is released from the ovary, it is pushed into the tube by the movement of the surrounding liquid, which is carried by the cilia on the inner surface of the tube; after ovulation, for few minutes, the peristaltic contractions of the uterine tube push the egg towards the uterus, then it continues going for 4 days thanks to the cilia movement until it reaches the uterus [1]. Thus there are two main mechanical helps to the transport of gametes: the beat of cilia and the peristaltic muscular activity [7] [3].

Cilia (Figure 1.3) are prominent and numerous at the end of the infundibulum and in the ampulla, which is also equipped with many secretory cells. The frequency of the cilia movement varies periodically and reaches a peak during the period of highest fertility. Simultaneously, for the same reason, the secretory activity of epithelial cells in the distal area of the oviduct is also maximum.

In general, every month the female reproductive system undergoes important changes in structure and in the secretion of hormones. The beginning of each cycle is marked by menstruation; the final phase, which lasts 14 days and ranges from ovulation to menstruation, is called luteal phase: in this phase, the right conditions for fertilization are created.

The physiology of the oviducts in women is closely related to the phase of menstrual cycle. The first things that are dependent on the cycle are the vascular support that they receive from the uterine and the ovarian arteries, the lymphatic system and the pH, which ranges from 7.3 to 7.7 [8]. The mucosal layers and the growth of ciliated epithelium too are extremely sensitive to the levels of hormones, estrogen in particular (the use of progesterone instead depresses its growth).

Regarding musculature, Fallopian tubes are surrounded by three layers of muscles: a circular central layer and two layers of smooth musculature. Proceeding from distal to medial regions of the oviduct, myosalpinx increases in thickness until it reaches the isthmus first and then the tubal-uterus junction which is wrapped in four bundles of twisted spiral fibers. It is assumed that this is functional to the storage of spermatozoa in this region and to prevent the passage of menstrual fluid. The innervation follows the same trend, which is poor in ampulla and infundibulum, and becomes dense starting from the isthmus which acts as a physiological sphincter.

While female gametes are descending toward the uterus, male gametes are ascending into the uterine tubes: after 2-4 ml of sperm (with 40-150 million/ml of spermatozoa) is deposited inside the vagina, most of it, entering the cervix, get lost because of the mucus-filled cervical canal that causes a gradient in number of spermatozoa from the external to the internal genitalia, up to the uterine tubes. Inside them is present a fluid of considerable importance during fertilization, which has a flow of 4 ml/24 h [9] and contains amino acids, electrolytes and energy substrates (like pyruvate, lactate and glucose, to support gametes [10]) in concentrations depending from the cyclic changes.

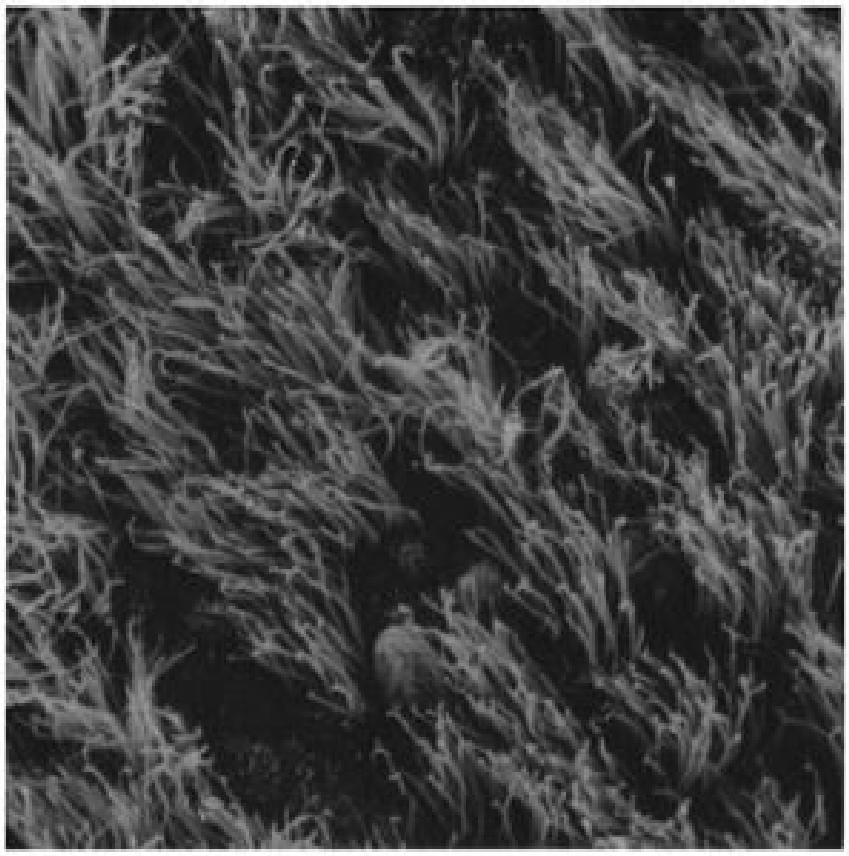

FIGURE 1.3: *A scanning electron micrograph of tubal cilia from the isthmus of a porcine Fallopian tube [11].*

## 1.2 Necessity of New Conctraceptives

Every couple needs to make a contraceptive choice. The chosen contraceptive method depends on the different physical characteristics, age and state of health. The three

main aspects of a contraceptive are:

1. efficacy, the ability to prevent certainly unintended pregnancy. Considering all the variables that can influence the failure of the contraceptive method, the efficacy is measured in terms of theoretical efficacy, the percentage of women that become pregnant in the first year when the use is consistent and correct, and typical efficacy [12], in which possible errors of use are taken into account.

2. safety, the lack of side effects.

3. reversibility, the possibility to be fertile again after the interruption or after the explantation of the contraceptive.

However, given the serious consequences of an unwanted pregnancy due to the failure of a contraceptive method, it is not necessarily true that methods that have few side effects are safer if they have a high probability of failure. The most common cost of an unplanned pregnancy is abortion which, especially in countries where is illegal, can lead to serious health problems, including death. According to the World Health Organization Guttmacher Institute, half of surgical abortions are dangerous [3]. Moreover, many studies on the social effects of unwanted children, demonstrate that women that completed an unwanted pregnancy have a high probability of developing addictions, like alcoholism, and illness, are less efficient at work and less significant in the family budget [13]. The cost of an unwanted pregnancy is also high for the economic and social development of the country, as it is related with the control of the size of the population. In a lot of less developed countries the use of contraceptives is encouraged and family planning programmes are supported [14]. Figure 1.4 summarizes all the advantages of family planning.

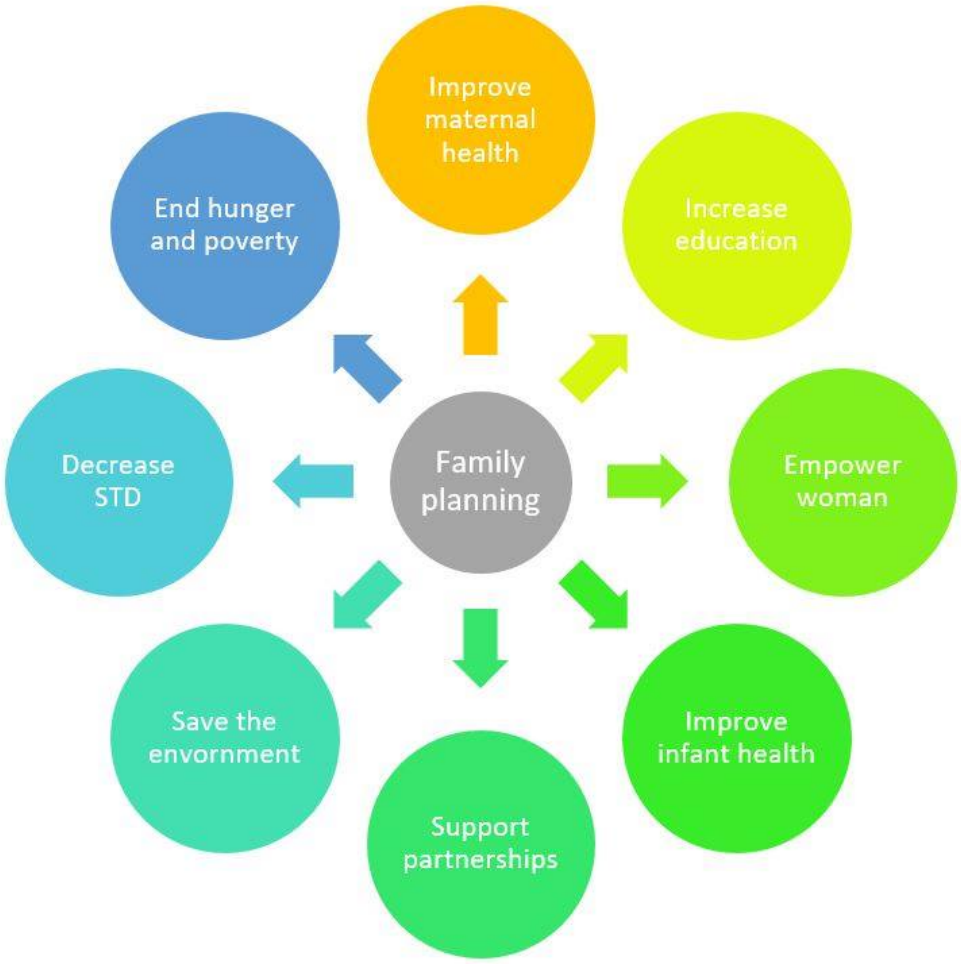


FIGURE 1.4: *Importance of family planning [15].*

These are some of the many reasons why the world contraceptive market, it is estimated, before the end of 2025, will reach the value of 37242.7 million dollars [**global**]. Nowadays a huge number of contraceptive options are available: hormonal contraceptives, mechanical barriers, intrauterine methods, natural methods of fertility recognition and surgical sterilization. Some of them can be considered short-acting birth control methods, some work on a long-term basis but they are still reversible, others are permanent sterilization techniques. Despite the wide variety of birth control technologies, what emerges from the statistics is that in developing countries more than a third of pregnancies are unwanted. And the portion increases if you consider high-income countries [3]: only in the United States, it is estimated that about half (49%) of all pregnancies are accidental [1].

The need for safe and effective new contraceptives arises because, although in the field of reproduction and pregnancy prevention important changes have taken place in the last 50 years, the contraceptive methods currently on the market clearly do not adhere perfectly to the social, economic, religious and health conditions of many people [16]. Starting from within the couple, the poor overview of birth control methods does not allow men to effectively share the responsibility for contraception with their partners; equity in terms of physical safety is not guaranteed: women are the main users of contraceptives and those who carry around the weight of side effects.

Moreover, no single contraceptive technology, at the same time, meets criteria such as discretion, ease of use, non-systemic approach, total protection against STD and lower Pearl index (indicative of pregnancies occurring in 100 women who use a certain method within a year [17]).

Fertility control is a complex and problematic process so before talking about innovative technologies, the difficulties linked to existing contraceptives should be identified, from the difficulty of predicting the effects after tens of years to the reconnaissance of risks for categories of people suffering from specific diseases, the not-healthy users.

The pill is undoubtedly the most popular contraceptive method, with 11.6 million users in the United States [18] and 100 million worldwide [19], and the most widely used systemic drug used by healthy women as a preventive measure [20]. It is a hormone-based, oral administered pharmaceutical product that interferes with cyclic hormonal changes by blocking ovulation and inhibiting the passage of sperm, thickening cervical mucus. There are two types of them: combined oral contraceptive pill (containing an oestrogen and a progestogen) and progestogen-only pill, and they have the advantage of being easy to use, regularise the menstrual cycle and protect against ectopic pregnancies (an ectopic pregnancy is a pregnancy in which the implantation happens outside the uterus, frequently in a Fallopian tube). Transdermal patches, vaginal rings and other hormone-based contraceptives have the same operating principle even though the delivery system and the dosage of the drug is different. All of them are not recommended for smokers and women suffering from certain cardiovascular or diabetic diseases because of the development of blood

clots, one of the most serious disadvantages resulting from the use of hormonal contraceptives. The main cause for women to stop using the contraceptive pill is side effects [21].

Another method of birth control very popular among young couples, who have sexual intercourse only occasionally, is the condom. It has the advantage of being economical, does not require prescription and is the only device that prevents Sexually Transmitted Diseases, AIDS included. However, its effectiveness is user dependent and the number of women who accidentally become pregnant within the first year is very high compared to the theoretical result.

The most effective alternative in the prevention of pregnancies is surgical sterilization (female and male) [12]. However, it has been shown that about 10% of women who use sterilization within the first 6 years regret it.

There are also other contraceptive methods used less frequently such as diaphragm, Intra Uterine Device, contraceptive sponges, spermicidal jelly or cream, injectable or implantable contraceptives and natural methods such as periodical abstinence and discontinued coitus. Actually, the first two are valid options for safety since they are reversible, they don't have risks associated with hormonal contraceptives and, for the second one, it is not required daily adherence or coital adherence. However, the diaphragm, that is a kind of cup that should be inserted inside the vagina, has a big gap between perfect use and typical use and the IUD, which is a plastic t-shaped device that is inserted into the uterine cavity in order to prevent fertilization and nesting of the embryo in the uterus, can cause inflammation, perforation of the uterus, expulsion, pain and bleeding [22].

## 1.3 Existing Technologies for Fallopian Tube Occlusion

The story of Fallopian tube occlusion has origin in the very early past, but the need of new safer and not surgical methods is still the principal aim of many recent researches. The introduction of new equipment and surgical procedures such as gynecological endoscopy that uses hysteroscopy and laparoscopy led to new techniques for intrauterine sterilization with many advantages in terms of reduced hospitalization, safety, cost and pain for the patient.

The most common and oldest method of Fallopian tube sterilization is tubal ligation, an abdominal operation that blocks the passage of sperm. This method presents many different variations: in 1880 the simplest version experimented of this technique consisted in a simple ligation, while after a decade to overcome the high failure rate of the previous method was proposed , although this kind of operation led to stagnation of fluid in the zone between the ligations (hydrosalpinx). Other types of ligations are followed from combination of surgery as resection of a part of the tube, division and burial in different section of the Fallopian tube. The Pomeroy technique is one of the most used in which a loop of tube is created in the midportion part and, after suturing the base edge of the loop, the top is resected. The US collaborative Review of Sterilization shows that the efficacy is high, although the percentage depends from the used technique and the age of the woman [23].

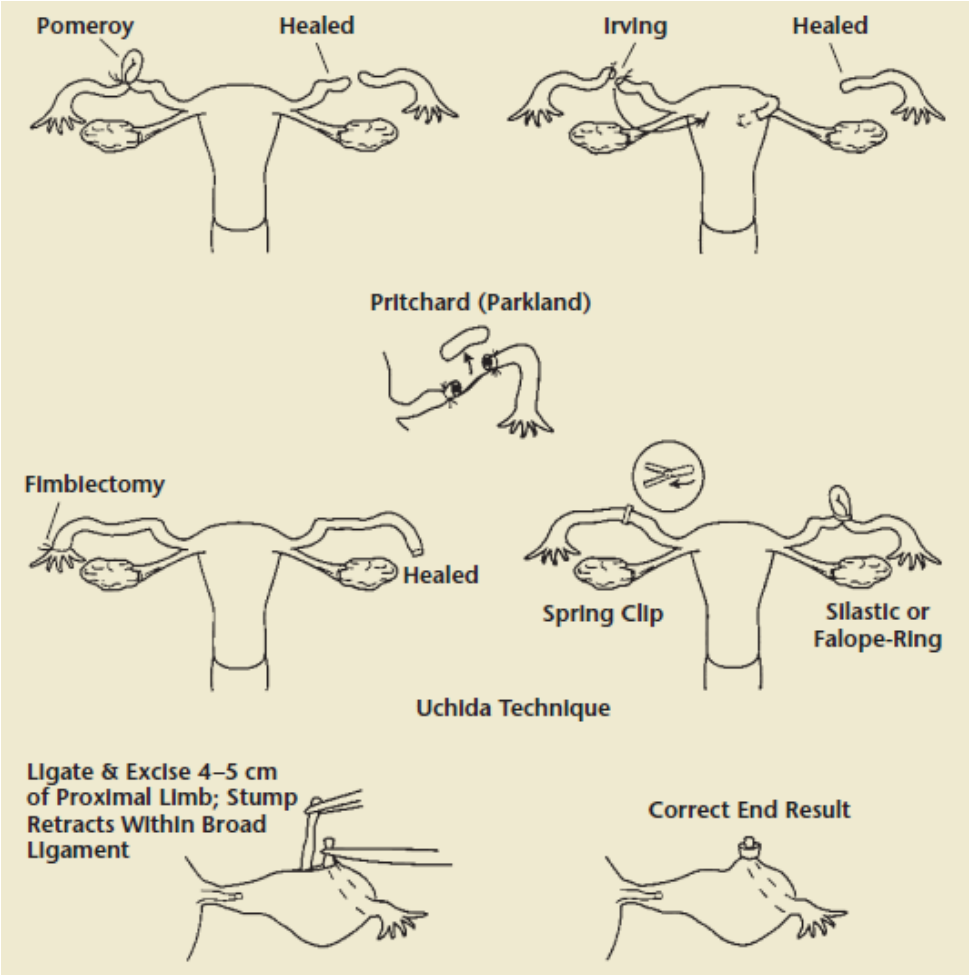


FIGURE 1.5: *Summary of tubal ligation techniques [23].*

Another solution is represented by the fulguration that consists in burning a segment of the tube by applying a current concentrated in a certain point: the severe heat generated damages in the tube by instantly dehydrated cells. It can be done in two ways: laparoscopically and hysteroscopically [24]. Traditionally electrocoagulation is laparoscopically operated, but the risk of attacking other organ is not trascurable. Lindeman et al. were the first to propose a transcervical electrocoagulation of the intramural part: the epithelium was cauterize by using the hysteroscopic channel and the $CO_2$ distension effect. Two differents probes were used for each tube to avoid the problem of the growing tissue on the probe, making the second tube sterilization less effective if the same probe was used. Lindeman used also a thermoprobe with less voltage instead of electroprobe; with this device it was possible to control better the release of current, but the efficacy was not improved [3]. Transcatheter radiofrequency electrocoagulation in rabbit Fallopian tubes led to successful occlusion by causing fibrosis of the mucosa without reaching the other organs, even if laparoscopic fulguration remains most used [25].

Since 150 years ago different chemical approaches were tested to occlude the Fallopian tubes, but these used pharmacologically agent presented high failure rate due to the toxicity of the agent that destroys not only the Fallopian epithelium, but also attack other part as the peritoneum and pelvic viscera.

Cytotoxic agents as quinacrine have been tested in animal and human via instillation through utero for a non-surgical sterilization [26]. The studies done in gynaecologically healthy woman by Zipper and al. showed very good results by using this simple low cost method, which needs repeated release over time of the agent in the intramural portion.In literature it is possible to find quinacrine injected through catheter or combined with IUD. However, the latter, as device to release quinacrine, was then replaced by the most efficient Hieu technique, showed in Figure1.6, where the pellets are inserted very close to the walls of uterine cavity [9]. The report of 2003 from different countries included India, China and USA evaluated this method as safe [27].

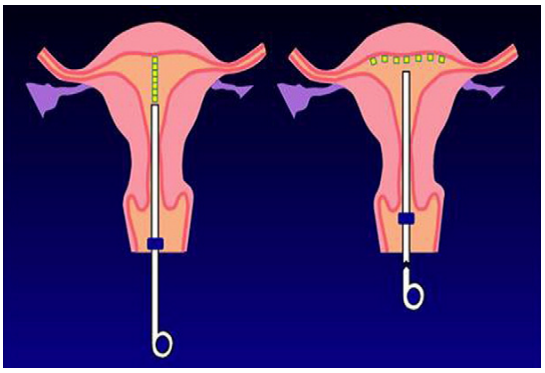

FIGURE 1.6: *Copper T technique vs Hieu technique. In the left Copper T technique the insertion of quinacrine pellets is not as close to the wall as in the case of the Hieu technique [9].*

Many patients were treated with derivates of cyanoacrylate: methycyanoacrylate (MCA) is a low viscosity tissue adhesive that polymerize when in contact with the ephitelium and release formaldhyede and hydrocyanic acid. The material, which can be delivered easily by using a device, stands in the isthmic tube where causes epithelium necrosis and does not reach the fimbria end. MCA degrades in 12 to 16 weeks replaced by the connective tissue [28]. This system was changed by using a iodine in a polymer carrier to provide a lower complication rate compared to MCA [29]. It is possible to find also solution of butyl-cyanoacrylate used to occlude in uterine tube for several months: the adhesion of this material seems a good propriety to occlude with almost no inflammation process [30].

Abdala et al. used 2 ml of Ethylene vinyl alcohol copolymer to occlude rabbit Fallopian tubes in the middle portion, injected through a 5F-catheter, with high sterilization rate in short-term window [31].
Rabbits are often used in these experiments due to the doubled uterus of their anatomy that leads to have a control and a test at the same time. After the injection of material, the histology shows the difference between control and the sterilization technique.

In another study an hydrogel that polymerizes in situ, made with acrylonitrile in a solvent of dimethyl sulfoxide with a contrast agent to make it visible, is injected through the uterus in the ampullary part of the Fallopian tube [32]. Later at the same type of hydrogel a sclerosing agent was added. Furthermore also the insertion of fibrin sealent, a two-material component very studied and approved by FDA

as hemostats and adhesive, was used after a bipolar electrocoagulation for achieve occlusion in the uterotubal junction [33].

Sterilization can also be achieved using clips of different types: tantalum, plastic or spring-loaded clips [34]. Initially this type of technique had many advantages such as the characteristic of a very easy insertion and the possibility of reversibility [35], then results showed high failure rate and cases of ectopic pregnancies. Filschie tubal ligation system [36] is a clip made with titanium and coated with silicone, approved by FDA that can be placed in the isthmic portion of the tubes.

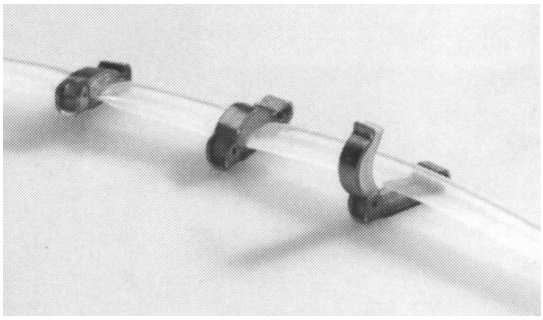

FIGURE 1.7: *Tubal clips example [37].*

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Another similar solution is the Falope ring, a silicone band that presents a lower pregnancy rate than clips techniques [38]. This safe and easy laparoscopy technique is comparable to the modified Pomeroy technique in terms of efficacy. However, this ring presents a lower duration of insertion, less pain and complications [39].

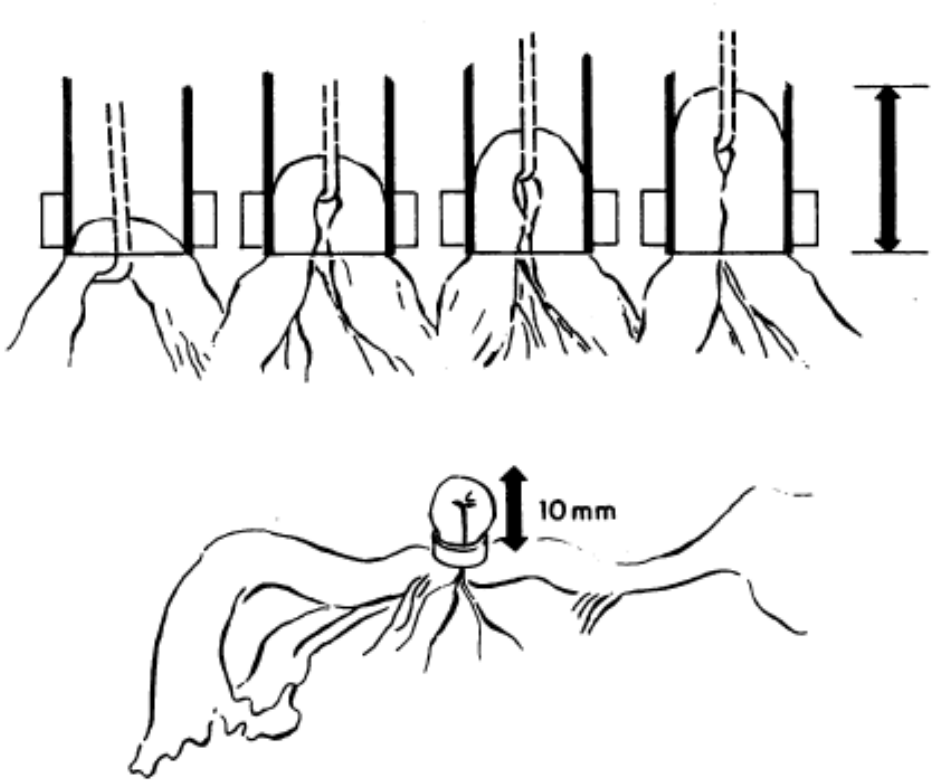


FIGURE 1.8: *Falope ring. In the upper part, procedure for the insertion of device and at the bottom, final result [38].*

The only occlusion device approved in US for transcervical sterilization is the Essure ®, a permanent hysteroscopic birth control solution made with an outer coil of shape memory alloy (Nitinol) and an inner one made with stainless steel and covered with Dacron fibers, which cause the obstruction of the tube for fibrosis reaction of epithelium. The efficacy of this device has been proven; it is a good option to avoid surgery with few side-effect and good response. Although one issue related to this device and to Adiana ® device too is that sterilization occurs after three months of the implantations, so the need for another contraceptive method in the meanwhile is required for women who choose Essure®.

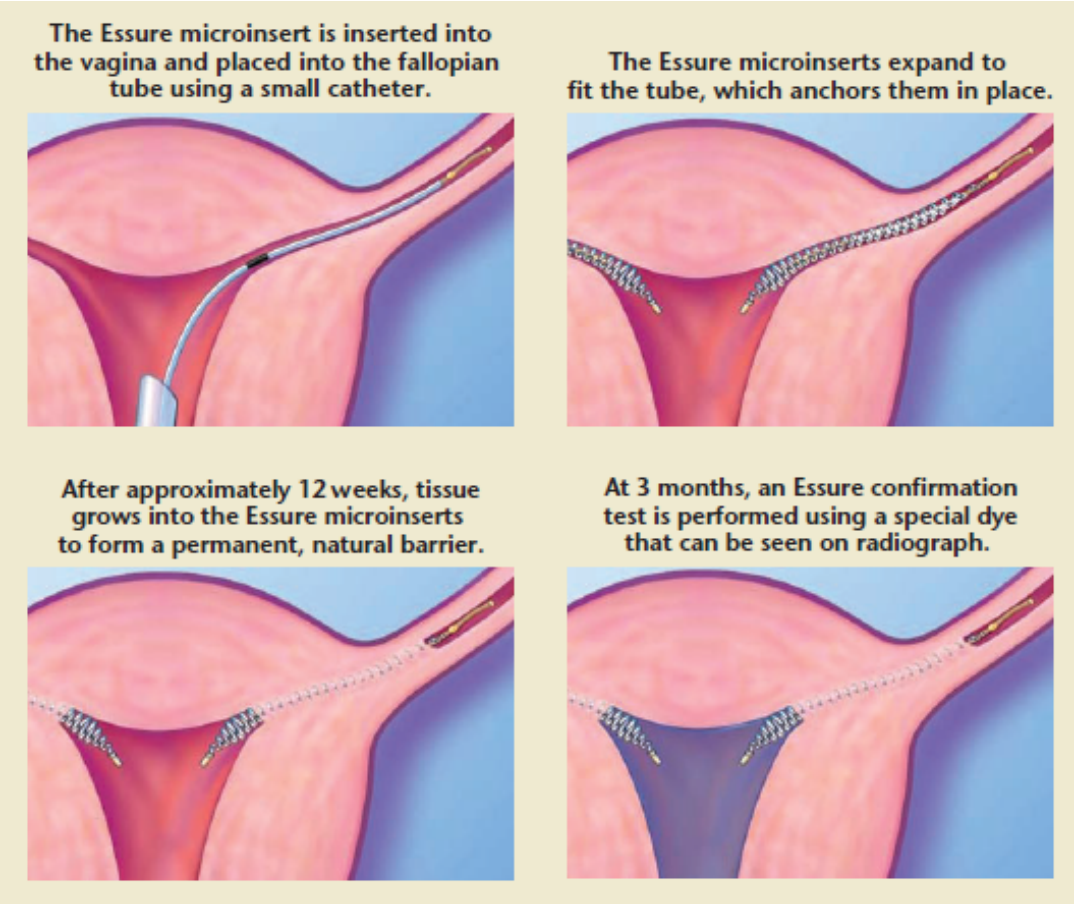


FIGURE 1.9: *Essure® device. Different steps of device insertion: first the Essure is placed with a catheter and until the tissue does not grow inside the device, the sterilization is not achieve.*

Adiana ® Permanent Contraception System, idea developed by Vancaille, has the purpose to interrupt the epithelium without causing inflammation. To achieve this aim, a biocompatible silicone implant was insert in the tubal lumen via hysteroscopy with a catheter, combined with a RF radiation that provoke a thermal lesion of the tissue [40].The failure rate in this case is higher than Essure device [41].

## 1.4 Our Technology

### 1.4.1 Laponite

Laponite is a commercial synthetic material composed by nanodiscs of a 1 nm of thickness and 25 nm of diameter [42].

The chemical composition is $Si_8[Mg_{5.5}Li_{0.4}H_4O24]^{0.7-}Na^{+}_{0.7}$. The products of degradation in physiological conditions are non toxic, similiar to bioactive glasses and the dissolution of some of its ions such as Mg2+ enhanced cell adhesion.

The charge is in total negative: the upper surface presents a negative charge while the lateral one presents a slight positive charge due to the sodium ions to counterbalance the negative charge. Although the charge in edge surface is pH dependent: negative charge is promoted at pH around 10 while at ph=7 a positive charge is present.

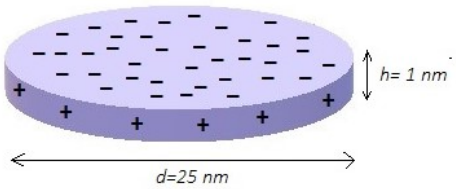


FIGURE 1.10: *Laponite ® Nanoclay.*

The presence of these positive charges on the lateral surface seems to induce gelation. One way to stop this mechanism is the addition of pyrophosphate, that can neutralised rim charges [42].

Many studies underlines that Laponite presents different phase states in relation to different parameters such as the presence of salt and its ionic force, used to measure the concentration of ions inside the solution, the presence of polymer, the clay concentration, the variation in pH and temperature[43]. Some well-defined phase charts are used; for example Mohurchid et Al proposed a phase state diagram of Laponite dispersion for two variable as shown in Figure 1.11: salt concentration and clay concentration.

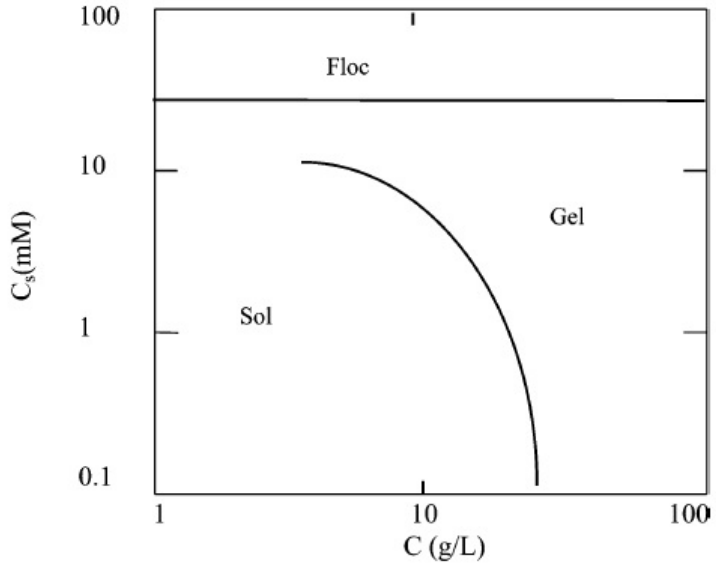


FIGURE 1.11: *An example of Laponite ®phase diagram proposed by Mohurcid et Al [42].*

In case of addition of salt to Laponite dispersion it is possible to notice that, for a established concentration of salt $C_s$ under a value where flocculation does not occurs, the higher concentration of Laponite C the higher the probability to form a gel.

### 1.4.2 PEO

PEO is a hydrophilic, safe and biologically inert biopolymer miscible in water because, compared to other polymers, has a distance oxygen-oxygen in its network similar to the distance that can be found in pure water chain, so the polymer fits well in water network, with a coiled shape of water around PEO [44]. Considering the different polymer chain lengths, PEO is used for polymers with a molecular mass above 20,000 g/mol.

The rheological proprieties showed that the viscosity of PEO depends on its concentration: so PEO exhibits a viscous behaviour for low concentrations and an elastic one for high concentrations.

### 1.4.3 Laponite-PEO mixture

In our material PEO is added to a solution of Laponite gel. The complicated interaction between the Laponite/PEO mixture was widely studied thanks to the good

biocompatibility of both the materials: the polymer can be added to nanoclay dispersion in order to promote cellular adhesion and proliferation. From mechanical point of view polymer is used to control the rheological proprieties of the suspension and to obtain the desired viscosity, however a critical concentration is necessary to obtain a proper polymer-clay network, with strong ion and hydrogen bonding.

Researches shows that PEO is absorbed in a very thin layer [45] on the surface of Laponite depending from polymer concentration as in Figure 1.12.

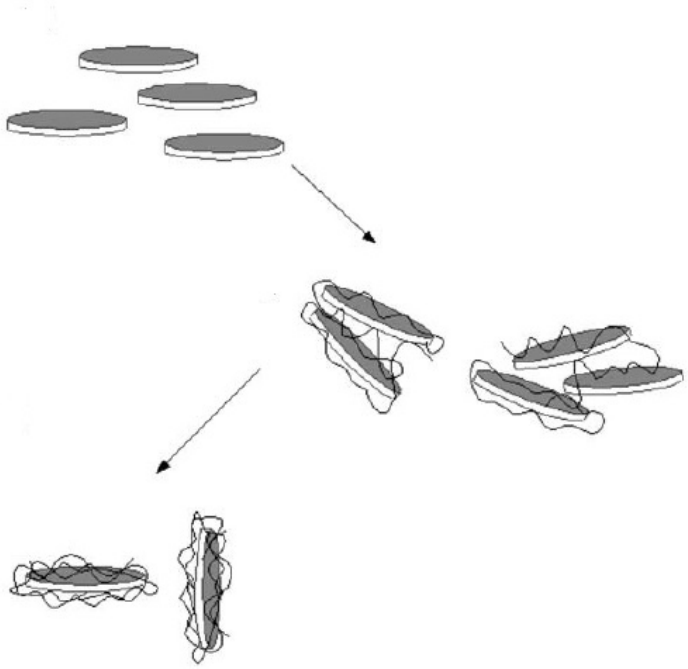

FIGURE 1.12: Different conformation in Laponite ®- PEO systems by increasing the amount of the polymer[46].

The mechanism described at rest shows that not all the concentrations of PEO have the same behaviour: supposing of having an excess of silica, at the beginning Laponite surface will be not saturated by PEO that leads to the formation of bridges between discs and so aggregation occurs. By increasing the amount of PEO the surface will be saturated by the polymer and the steric repulsion of chains so this will avoid aggregation of nanoclays. The situation changes when a shear is applied, because it deforms polymer chains and new Laponite® surface are now exposed to form polymer connections, aggregates grow very fast and gelation starts.

In accordance to the Laponite® and Peo concentrations, many phases can occur: shake gels, shear thickening phase and liquid phase as shown in Figure 1.13.

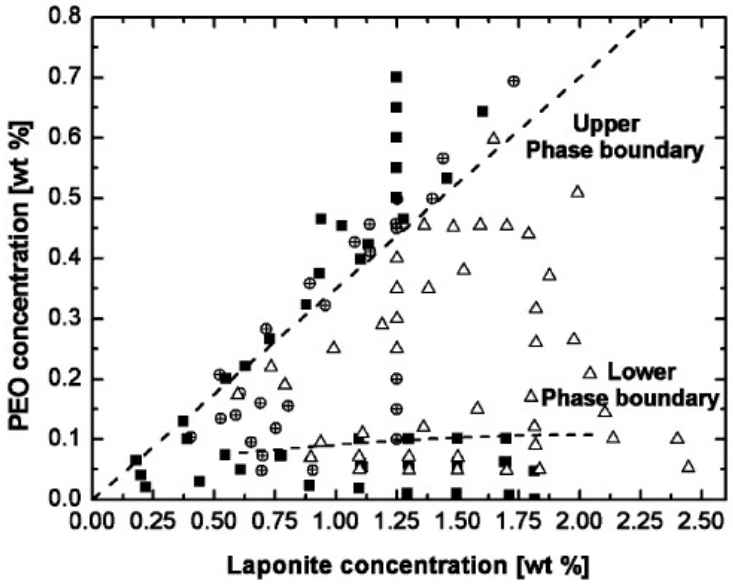


FIGURE 1.13: Different phase state of Laponite®- PEO systems depending on their concentrations. Triangles represent shake gel phase, circle shear-thickening phase and squares stay for liquid phase. [46].

Shake gels have the important propriety that can be reversible: elastic solids are created for a vigorous shaking, but they go back to the initial state when relaxing. The concentration of PEO for these gels is slight under the saturation concentration. If the saturation is not complete and the Laponite® surfaces are partially uncovered, the shear rate is lower and there is a behaviour of shear-thickening. In this case the viscosity increases and gels are stiffer [47].

# Chapter 2

# Experimental Section

## 2.1 Materials Preparation

In this work it is done an analysis of the effect of adding Poly(EthyleneOxide) (PEO) in Laponite to produce a material for a specific application: the Fallopian tube occlusion. The idea is to develop a contraceptive material easy to be injected though a catheter in order to avoid the use of more invasive surgical techniques and able to occlude the lumen of the oviduct. To prepare the gel in question, Laponite and PEO were used.

To prepare the nanocomposites, stock solutions of laponite [15% $(w/v)$; Laponite XLG-XR, BYK] and PEO [25% $(w/v)$; PEG, Sigma Aldrich] were made in Milli-Q water (see Appendix C for details).
For the sample preparation, the required amount of 15% laponite gel first and then the correct volume of water were added to the amount of PEO, in this order, according to the desired composition (the table below shows the evaluated gels). The mixture was vortexed vigorously for 15 $min$ at high speed and then put for 20 $min$ into the $80°C$ oven; finally, centrifuged to have a bubble-free material (for the complete protocol check Appendix C).

TABLE 2.1: **Evaluated gels** - *the table lists the prepared materials according to the design of the experiment to determine the optimal formulation of Laponite-PEO mixtures.*

| | **4% Laponite** | **5% Laponite** | **6% Laponite** | **6.5% Laponite** | **7% Laponite** | **8% Laponite** |
|---|---|---|---|---|---|---|
| **0% PEO** | • | | | | | |
| **1% PEO** | • | • | • | • | • | |
| **2% PEO** | | | | | | |
| **3% PEO** | | • | • | • | | |
| **4% PEO** | | | | | | |
| **5% PEO** | • | • | • | • | • | |
| **7% PEO** | | | | | | |
| **10% PEO** | • | • | | • | • | • |

## 2.2 Materials Characterization

### 2.2.1 Determination of the Lower and Upper Limit of Laponite/PEO Nanocomposites

To reduce the number of materials in the design of the study, two main characteristics were evaluated: the ability to form a gel and the injectability through a microcatheter. These preliminary experiments gave us the lower and upper limit in the range of compositions to investigate.

#### Tube Inversion

A gel is a material that exhibits no flow in the steady-state [48] and doesn't relax if a small mechanical stress is imposed for an infinite time. The existence of gel-like

properties can be qualitatively diagnosed by *tabletop* experiments. The tube inversion is the most common of these methods and permits to observe the sol-gel transition point without a rheometer, even if it has rheological basis [49].

In the tube inversion test, the material is placed inside a vial with cylindrical form. When the vial is turn upside-down the solution flows whereas if the gelation occurred, the material remains intact on top (Figure 2.1).

To be sure that the yield stress applied is the same, it is important to use the same vial size and the same mass sample, and to observe the outcomes after the same time [49].

For our materials, results are shown in Figure 2.2 and Table 2.2.


FIGURE 2.1: – *Sketch showing cylindrical vial with radius R, used for the test, containing a gel of length L.*

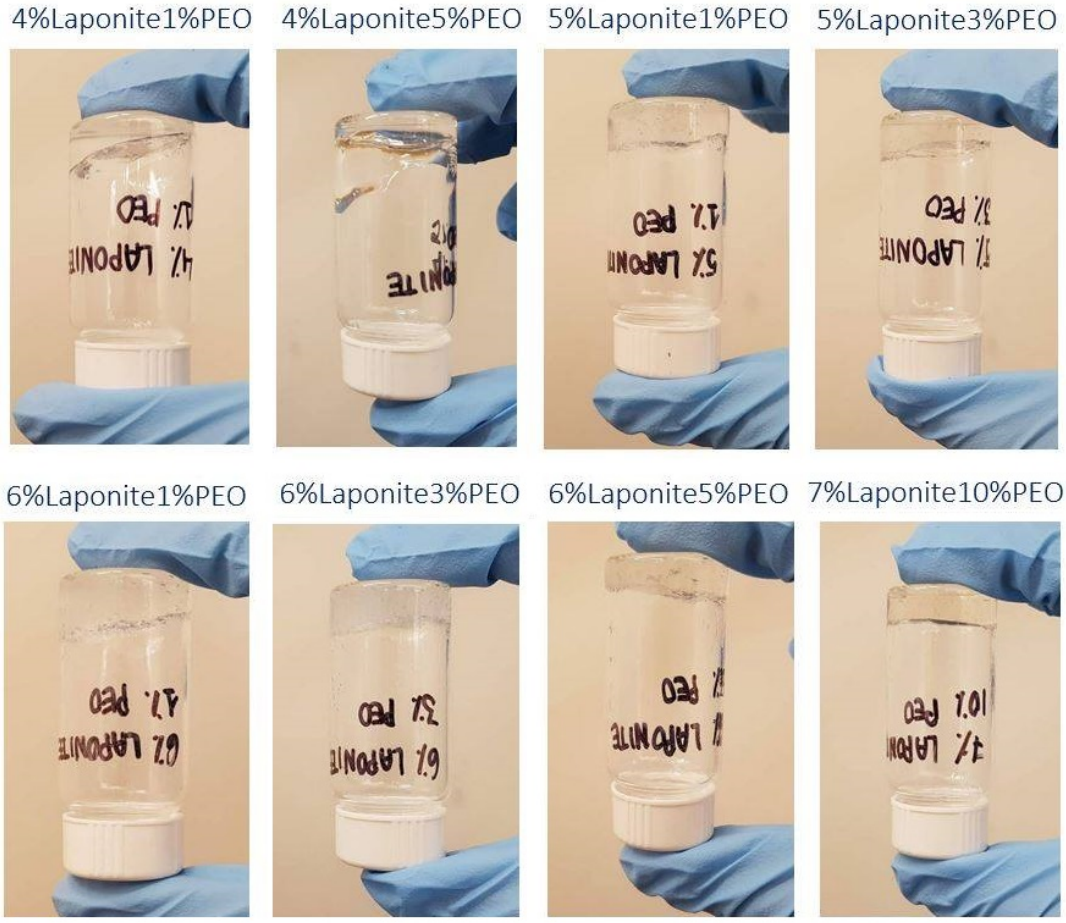


FIGURE 2.2: *Tube inversion test applied to determine if gelation occurred so to identify the lower limit of Laponite/PEO nanocomposites. After 30 min form the material synthesis, the* 4% *Laponite with* 0%, 5% *and* 10% *of polymer is not a gel whereas all the other materials were gels.*

From the literature, it is known that only for concentration of Laponite up to 3% by weight, the Laponite solution forms an irreversible gel [46]. So, for each combination of Laponite and PEO, starting from the 4% Laponite, to note gelation, a vial containing the sample was inverted to see if there is a flow or not.

This test showed that, after 30 *min* from the end of the material synthesis, the 4% Laponite with 0%, 5% and 10% of polymer is not a gel whereas all the other materials we considered were gels.

TABLE 2.2: **Gels formation** - *The probability to have a gel increases with an increase of the Laponite concentration and it decreases with an increase of PEO concentration.*

| | **4% Laponite** | **5% Laponite** | **6.5% Laponite** | **7% Laponite** | **8% Laponite** |
|---|---|---|---|---|---|
| **0% PEO** | N | Y | Y | Y | Y |
| **1% PEO** | Y | | | Y | |
| **2% PEO** | | | | | |
| **3% PEO** | | | | | |
| **4% PEO** | | | | | |
| **5% PEO** | N | | Y | Y | |
| **7% PEO** | | | | | |
| **10% PEO** | N | Y | Y | Y | Y |

**Preliminary Injectability**

The upper limit was decided evaluating the injectability, by hand. The material was transfer into a 3 $mL$ syringe that was later connected to a 4$F$ catheter, with a 0.035" inner diameter [Angio Dynamics Inc]; the extrusion force was applied manually. Three trials with the same material ensured that the results are reproducible.
Not all the materials are injectable: Table 2.3 summarises the results.

TABLE 2.3: **Gels Injectability** - *The upper limit of Laponite/PEO nanocomposites depends on how easily injectable are the gels. This table shows that from 6.5% Laponite gels start to be hardly injectable.*

| | **4% Laponite** | **5% Laponite** | **6% Laponite** | **6.5% Laponite** | **7% Laponite** | **8% Laponite** |
|---|---|---|---|---|---|---|
| **0% PEO** | *no gel* | | | | | |
| **1% PEO** | inj | inj | inj | *no gel* | not inj | |
| **2% PEO** | | | | | | |
| **3% PEO** | | inj | inj | inj | | |
| **4% PEO** | | | | | | |
| **5% PEO** | *no gel* | inj | inj | hardly inj | hardly inj | |
| **7% PEO** | | | | | | |
| **10% PEO** | *no gel* | hardly inj | | hardly inj | inj | hardly inj |

### 2.2.2 Injection Force Measurements

An essential characteristic of the shear-thinning material in study for its future use is to be injectable without difficulty through clinical catheters or needles. In our case the injection force test was performed also to evaluate the reproducibility, according to the idea that same materials have very similar injection forces, and the homogeneity of the produced gels. In fact, a not homogeneous material will have an irregular force versus extension curve. Another source of disturbance are air bubbles so, before starting the experiment, tha material was transfer into a 3 $mL$ syringe and then centrifuged at 3000 $RPM$ for 5 $min$ to clear bubbles (for the complete protocol, see Appendix C).

Each sample was analysed using INSTRON® 5943 as mechanical tester, a syringe and a 4$F$ catheter (65 $cm$ long) or a 18$G$ needle. The rate of the compression platen acting on the plunger is 33.96 $mm/min$. The data acquisition and processing were performed with BlueHill software and the test was considered started when it was

seen a first amount of gel coming out of the used delivery system (catheter or needle).

In our case, since some hydrogels have an injection force higher than 10 $N$, the 100 $N$ load cell of the INSTRON was used. The experimental setup is shown in Figure 2.3. If the catheter is used, the end of it should hang free without touching anything. Obviously to compare the injection forces, the samples should be tested at the same temperature, 25°$C$ for this experiment, using the same catheter, because length, diameters (inner and outer) and the material it is made of influence each measurement. The raw data should resemble the measurements shown in Figure 2.4.

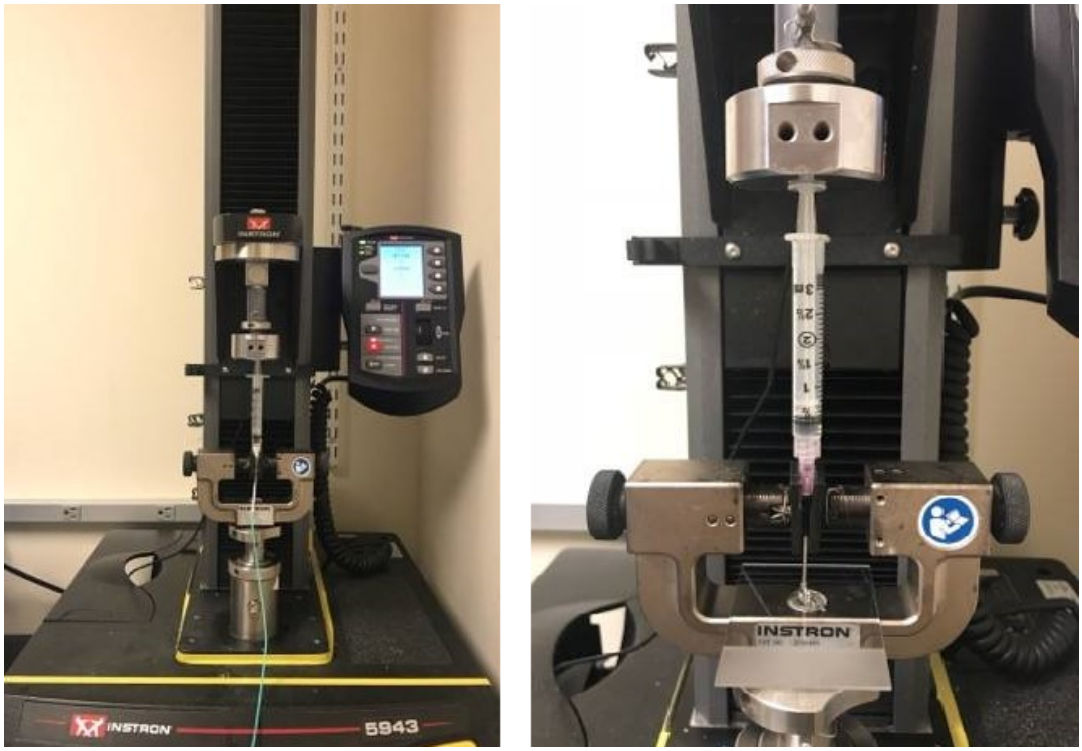


FIGURE 2.3: *Experimental setup for injection force measurements. In the left picture, a 4F catheter is used, in the right one, a 18G needle is used. The mechanical tester, its accessories and the catheter positioning are visible.*

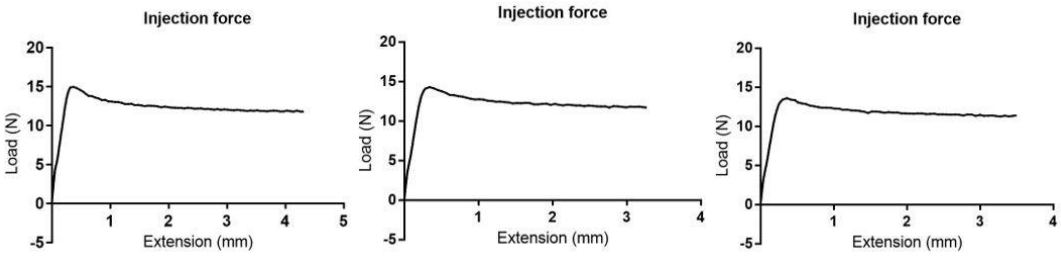


FIGURE 2.4: *Example of load versus extension curves obtained from the injection force experiment. The material in study was the* 5% *Laponite* 5% *PEO. As it can be seen, all the curves have a peak and reach a plateau around the same values.*

Once the load-extension curves have been obtained, to quantify injectability, the mean value of the plateau reached around 3 *mm* of extension was considered. The represented curves are the typical curves for an injection test: the force required to move the material inside the catheter at first is high, then, since the material is moving, to maintain the motion, it becomes constant. Results are shown in Figure 2.5. The results obtained with the 18*G* needle have a lower standard deviation compared to the catheter ones.

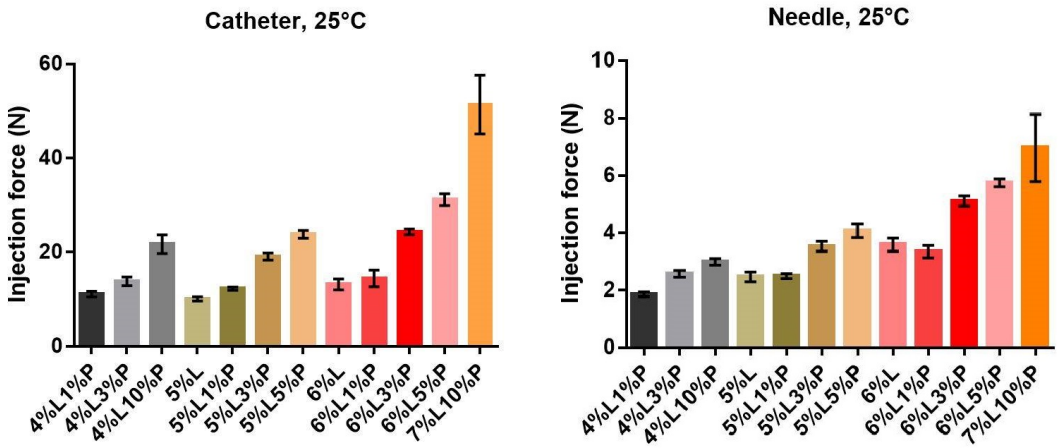


FIGURE 2.5: *Injection force versus composition. Both for catheter and needle the trend is almost the same: the higher the percentage of Laponite, the less easy to inject. Additionally, from low to higher PEO concentration: the higher the PEO concentration, the higher the injection force*

The 7% Laponite combinations were already known to be more difficult to inject. In all the three measurements, the graph showing the injection force revealed

also a low reproducibility due to the inhomogeneities inside the prepared gel: in fact, probably due to the high content of Laponite, slowly mixing the material with a spatula, the 7% Laponite 10% PEO seems not uniform. Moreover, during the washing step, was very difficult to get it out of the 4-French catheter. Therefore, the Laponite concentration should not exceed the 6% mark, mainly for measurements' reliability problems.
There is a visible trend among the synthesized materials: the higher the percentage of Laponite, the less easy to inject. Additionally, from low to higher PEO concentration: the higher the PEO concentration, the higher injection force, although the material is softer.
Among the most promising materials, besides the good injectability of the 5% Laponite combinations, the 6% Laponite and 6% Laponite 1% PEO seems the optimum materials, able to combine proper stiffness and good injectability.

### 2.2.3 Occlusion Pressure Measurements

The main purpose of this experiment is to find the best composition to block a Fallopian tube, modelled in vitro as Tygon tubes of different diameters (Figure 2.6). To test how obstructive is the blockage, while the gel was occluding and a pump was injecting medium against it, the pressure upstream of the tube was monitored.
In each human uterine tube, four parts can be recognized, from the medial to the lateral one:

- intramural segment, that is from 1.5 to 2.5 *cm* in length with a lumen of 0.8 to 1.4 mm;
- isthmus, whose length ranges from 2 to 3 *cm* with a diameter of 1 to 2 *mm*;
- ampulla; the ampullary segment is the most variable: it ranges from 5 to 8 *cm* in length, with a diameter of 1.5 *mm* at the ampullary-isthmic junction, to 10 *mm* at the ampullary-infundibular junction;
- infundibulum, close to the ovary.

From the natural sizes of the anatomical areas of a Fallopian tube, it was decided to use plastic tubes [Wall Tygon® 2375 Ultra Chemical Resistant Tubing, 57547, United States Plastic Corps] with the following dimensions: 1 $mm$, 1.8 $mm$, 3 $mm$, 4 $mm$ and 10 $mm$.

The selected tube is cut in a 10-$cm$ piece and it is filled with material until the desired length (15 $mm$ or 30 $mm$) using a 3-$mL$ syringe [BD Luer-Lok Tip, Becton, Dickinson and Company] with a specific needle [BD PrecisionGlide] (the used needles are from 18$G$ to 25$G$).

Subsequently, the tube was connected to a 60-$mL$ syringe placed on a syringe pump [Syringe pump, Harvard apparatus PHD 2000 Infuse/Withdraw] with a 3-way connector; the third entrance of it is connected to the PASCO pressure sensor [Pressure Sensor, PASCO Dual Pressure Sensor] and, with a USB link [USB Pasco linker], this one is connected to the computer. The flow rate was set at 1 $min/ml$ and constant. (Figure 2.6 shows the complete set up of the experiment). The experiment is performed at room temperature, 3 times per each displayed bar, to have statistical significance. After each test, the tube was washed and dried to not influence the next measurement.

The variables that were changed for the measurement of the occlusion pressure are: medium (air, water, PBS), diameter of the tube and length of the material deposited in the tube (15 $mm$, 30 $mm$). The latter was chosen to give an idea of the amount of material injected: the length was preferred to the injected volume in $mL$; the table below shows the relation between these two quantities (Table 2.4).

TABLE 2.4: **Injected volume of each length and diameter** - *It was decided to change the length of the material inside the tube instead of the injected volume to quantify the amount of material deposited. The table shows the relation between these two quantities.*

| | Length | |
|---|---|---|
| Diameter | 15 mm | 30 mm |
| 1 mm | 0.01 ml | 0.02 ml |
| 1.8 mm | 0.035 ml | 0.07 ml |
| 3 mm | 0.25 ml | 0.50 ml |
| 4 mm | 0.30 ml | 0.60 ml |
| 10 mm | 0.50 ml | 0.1 ml |

The software we used to observe the measured pressure was PASCO Capstone; whenever the pressure shows a peak and then a decrease in slope is because the material is not occluding anymore. Results are shown in Figure 2.7-2.11.

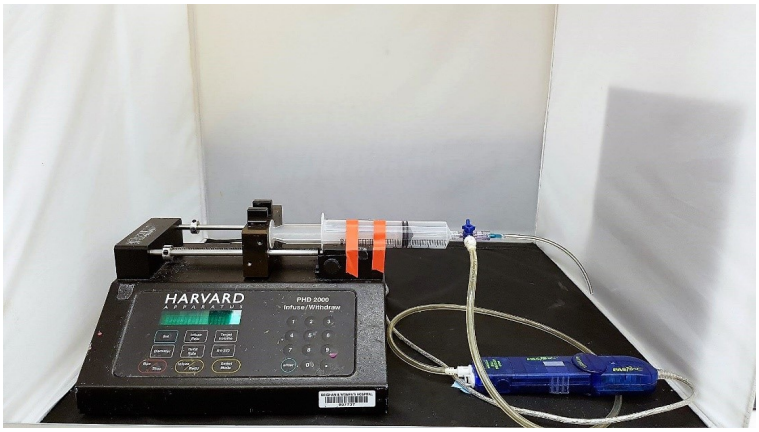


FIGURE 2.6: *Experimental set up for occlusion pressure measurements. Syringe pump, Pasco Dual pressure sensor, transparent tube with the material inside to cause a blockage and all the connections are shown. The chosen flow rate for the medium that will flow against the material is* $1\ mL/min$.

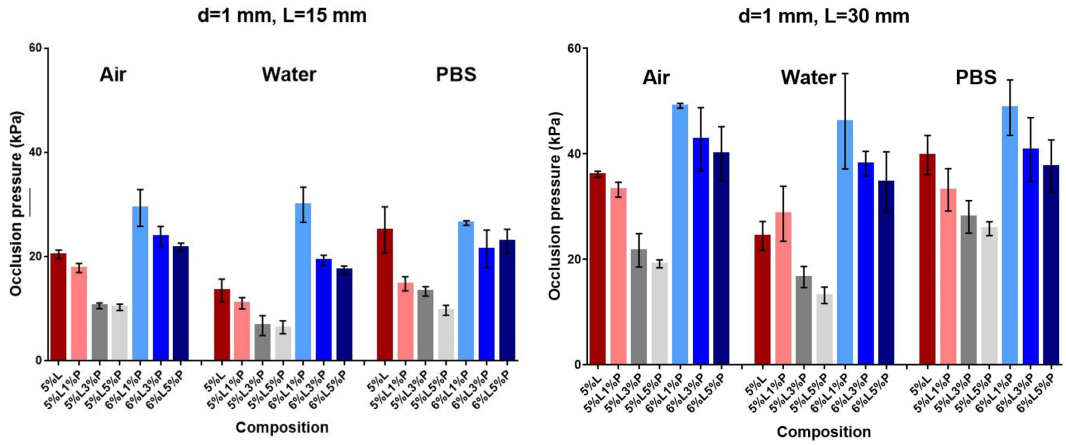


FIGURE 2.7: *Occlusion pressure measurements in* 1 *mm tube, putting* 15 *mm (left figure) and* 30 *mm (right figure) of material inside the tube. Air, water and PBS were spilled against the blockage.*

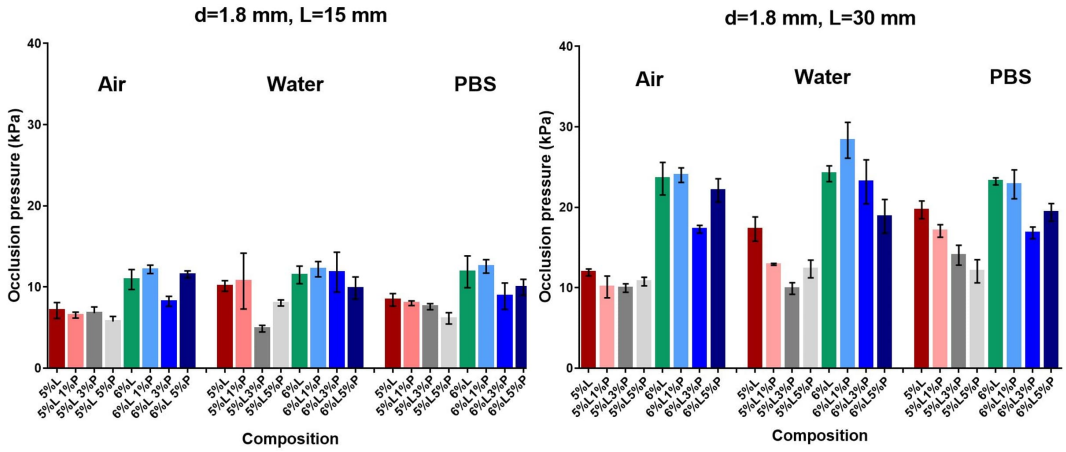


FIGURE 2.8: *Occlusion pressure measurements in* 1.8 *mm tube, putting* 15 *mm (left figure) and* 30 *mm (right figure) of material inside the tube. Air, water and PBS were spilled against the blockage.*

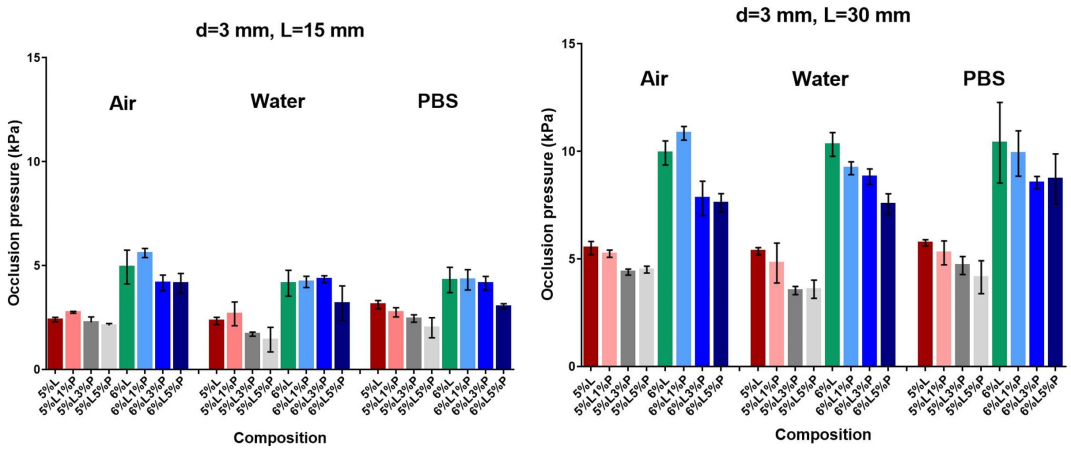


FIGURE 2.9: *Occlusion pressure measurements in* 3 *mm tube, putting* 15 *mm (left figure) and* 30 *mm (right figure) of material inside the tube. Air, water and PBS were spilled against the blockage.*

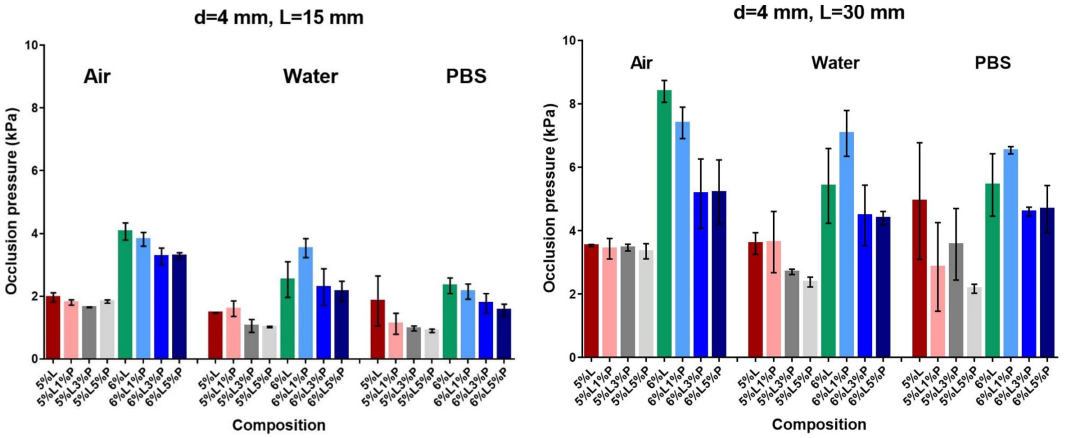


FIGURE 2.10: *Occlusion pressure measurements in* 4 *mm tube, putting* 15 *mm (left figure) and* 30 *mm (right figure) of material inside the tube. Air, water and PBS were spilled against the blockage.*

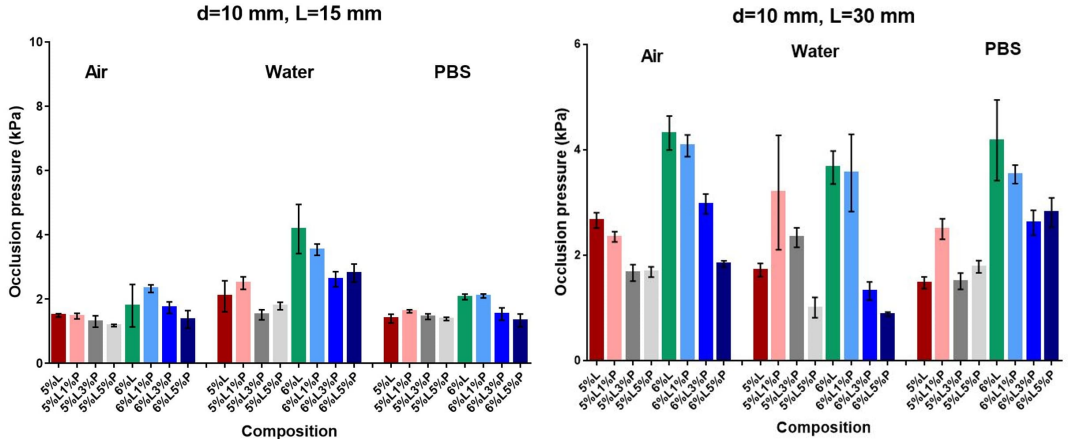


FIGURE 2.11: *Occlusion pressure measurements in* 10 *mm tube, putting* 15 *mm (left figure) and* 30 *mm (right figure) of material inside the tube. Air, water and PBS were spilled against the blockage.*

Obviously, considering the injected volume, the pressure is higher for bigger volume of nanocomposite, even if the difference between the 0.5 $mL$ and 1 $mL$ is of few kPa sometimes. The parameter that gives more considerable changes in pressure is the tube diameter.

All the bar charts show that the higher the concentration of Laponite the higher the occlusion pressure, so the less the occlusion fails, whereas, among the same concentration of Laponite, by increasing the concentration of PEO, the occlusion pressure decreases. For all the tube diameters and injected volumes, the same trend can be found, even when the medium changes.

For the smallest tube, the 6% Laponite is not indicate in figure because the material completely blocked the tube for many times, the absolute pressure rose above 200 $kPa$ and the sensor was not accurate anymore.

The most promising materials are 6% Laponite and 6% Laponite 1% PEO that can resist to a higher occlusion pressure and are easy to inject, as shown in the previous experiments. A summary of the values of occlusion pressure for the two different lengths of material inside the tubes is in Figure 2.12. The bar chart shows, as expected, that the occlusion pressure decreases for the larger tubes.

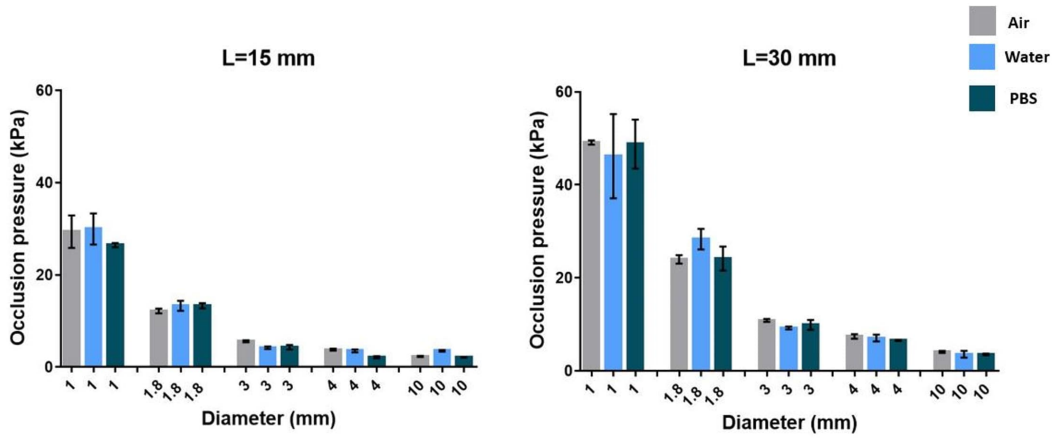


FIGURE 2.12: *A summary of the values of occlusion pressure for 6% Laponite 1% PEO injecting two different lengths of material inside the tubes. The bar chart shows, as expected, that the occlusion pressure decreases for the larger tubes.*

**Occlusion Pressure: Effect of the Temperature**

The monthly menstrual cycle in women matches some temperature changes in the body. Despite these temperature fluctuations, to have a more realistic in vitro model, the temperature effect on the strength of the occlusion was evaluated. The experiment was performed at 37°C but, since the set-up was a bit complex, it was chosen to study only one of our best materials: 6% Laponite 1% PEO.

A water bath in a Petri dish was used to reach 38°C in the 1 *mm* tube occluded with the material and filled with PBS at 37°C.The material was previously put inside the incubator at 37°C for 1 *h*, 3 *h*, 6 *h* and 24 *h*.

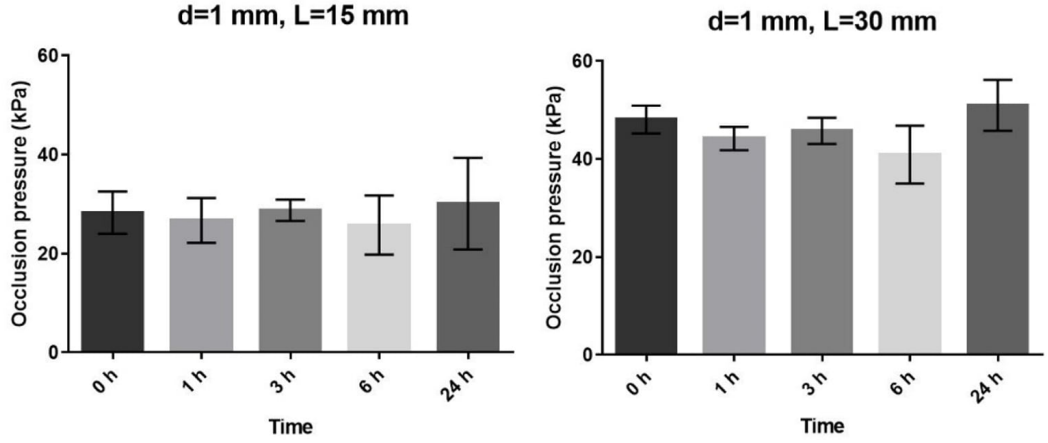


FIGURE 2.13: *Occlusion pressure measurements in* 1 *mm tube, putting* 15 *mm (left figure) and* 30 *mm (right figure) of material (*6% *Laponite* 1% *PEO) inside the tube. The syringe pump was making PBS flow against the occlusion with a constant flow rate of* 1 $mL/min$. *The PBS, the material and the tube were kept at* 37°C. *A very slight trend is visible but it is possible to say, comparing these results with those at* 25°C, *that occlusion pressure doesn't change because of the temperature and is stable over time.*

The results (Figure 2.13) shows that the occlusion pressure doesn't change because of the temperature. In fact, the obtained values, few kPa lower than 30 for 15 *mm* of inserted material and around 50 *kPa* for 30 *mm*, are the same of the ones at room temperature (in Figure 2.6).
Furthermore, the material occlusion property is stable over time, beside the fact that a very slight trend is visible.

**Occlusion Pressure: Effect of the Aging**

From the previous experiment was born the idea to see until which day the occlusion ability of the one of our two best materials was the same. The experiment of aging for the occlusion pressure is done by keeping some volumes of 6% Laponite 1% PEO for 1, 2 and 7 days at room temperature in a different 50 *mL* Falcon tube, well closed, for each time point. All the materials should come from the same batch to be comparable. The results are shown in Figure 2.14.

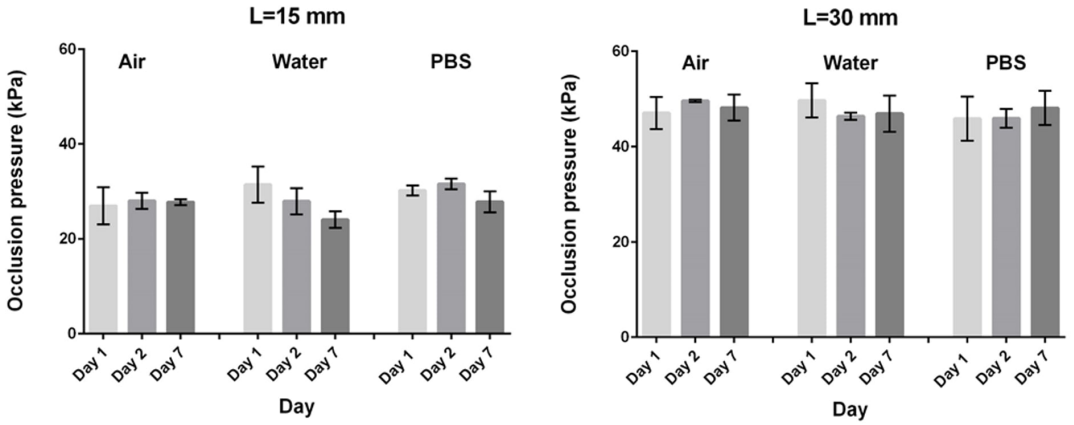


FIGURE 2.14: *Occlusion pressure measurements in* 1 *mm tube, putting* 15 *mm (left figure) and* 30 *mm (right figure) of material inside the tube. The used media were air, water and PBS, the flow rate was* 1 $mL/min$ *and the experiment was performed at* 25°$C$. *The occlusion pressure remained quite constant until day* 7, *except for the measurement in water, using* 15 *mm of* 6% *Laponite* 1% *PEO, for the last timepoint.*

The values in figure are obtained monitoring upstream the pressure inside the 1 *mm* tube, putting 15 *mm* and 30 *mm* of material inside the tube. The flow rate was 1 $mL/min$ and the experiment was performed at 25°$C$. The occlusion pressure remained quite constant for all the media until day 7, except for the measurement in water, using 15 *mm* of 6% Laponite 1% PEO, for the last timepoint, that is 7 days. However, the fact that for PBS and air the occlusion pressure is the almost the same might mean that the fall at day 7 is due to an outlier.

### *Ex-vivo* Experiment

An *ex vivo* experiment is a study that is carried out with a tissue or an organ isolated from a living body.

In our case, porcine Fallopian tubes were used: the diameter of these tubes is bigger than the human oviducts; for the intramural segment, the difference is 0.8-1.5 *mm* for women versus 15 *mm* for the porcine ones. Considering the huge size of the organs, this experiment is considered an extreme case.

6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO, 6% Laponite 5% PEO are the materials to compare in this experiment, since the composition with a percentage of Laponite of 5% are less strong as occlusive agents, because of the lower amount

of solid particles.

The procedure to estimate the occlusion pressure *ex vivo* is similar to the *in vitro* one. For the ex-vivo occlusion pressure, some parameters were changed: the flow rate was increased to 20 $mL/min$ since with lower values the sensor was not able to detect changes in pressure and the measurement was long-lasting. The quantity of material to use, this time, in view of the fact that the organ is not transparent, was chosen in mL: so, 1 $mL$ and 2 $mL$ were employed.

Obviously, using animal tissues, cold ice was needed to hydrate the tubes and prevent tissue to go waste.

Before starting it is important to check visually the entirety of the organ: a catheter is inserted inside it and, passing through cervix, the intramural section is reached. Connecting the catheter to a syringe, air can be insufflate inside the organ: this step is done for all the parts of the Fallopian tube, to be sure that there are no holes or anatomical problems in the tissue. Subsequently the initial part of the Fallopian tube is cut in a piece of 10 cm and the diameter is measured inserting in the lumen different tubes with a known outer diameter, to force the tissue to maintain a cylindrical aspect.

To connect the syringe and the Fallopian tube, a homemade connector was created taking off the plunger from a 10 $mL$ syringe and cutting the part closer to it, reducing the length of the syringe of 1 $cm$. This was then connected to a soft and flexible Tygon tube. After deposing the material, if water or PBS are used, the homemade connector should be filled too with them, turning on the syringe pump, lifting the free larger end of the connector and waiting until the it is completely full. During the measurement, the animal tube should be kept tied around the connector to not let the medium go back outside and falsify the result (the detailed protocol can be found in the Appendix C).

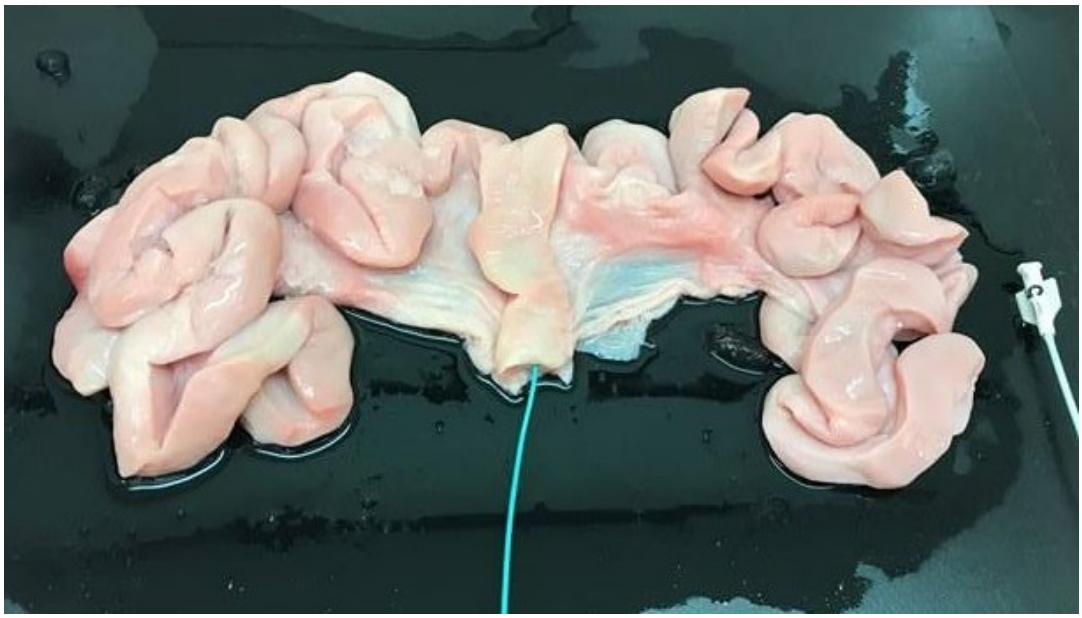

FIGURE 2.15: *Used porcine Fallopian tube and uterus. The intramural parts have a diameter of 1.5 cm, bigger than the human ones, that have a diameter varying in the range 0.8-1.5 mm. The inserted 5F catheter is visible.*

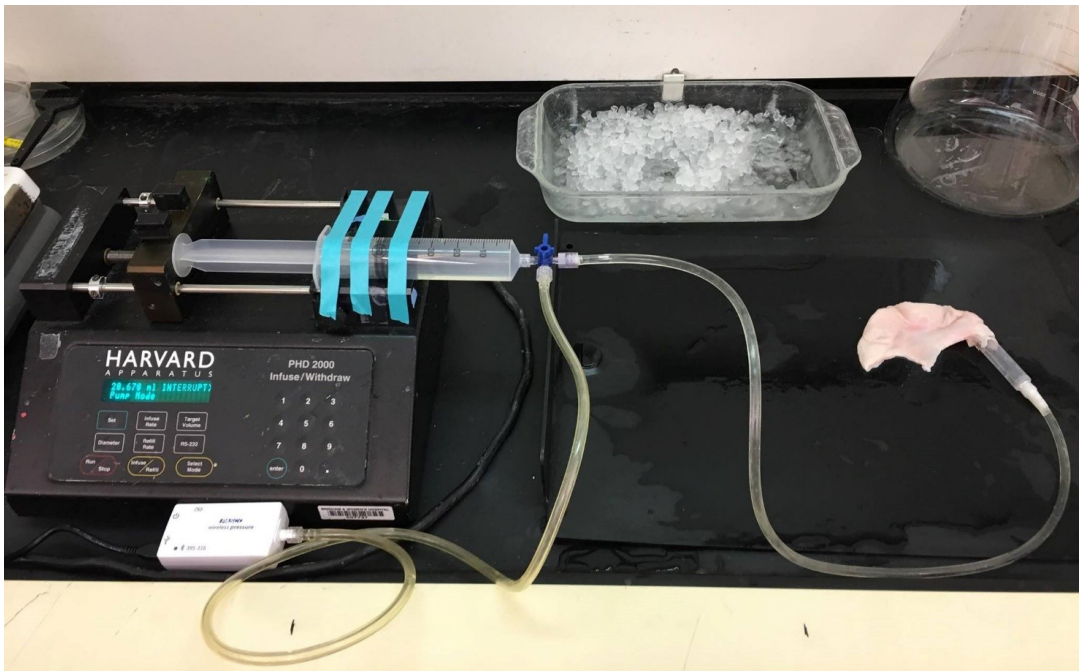


FIGURE 2.16: *Experimental setup for ex-vivo occlusion pressure measurements. Syringe pump, Pasco wireless pressure sensor, homemade connector, piece of porcine oviduct and all the connections are shown. The chosen flow rate for the medium that will flow against the material is 20 ml/min, higher than the one used in vitro.*

FIGURE 2.17: – *Homemade connector created taking off the plunger from a 10 mL syringe and cutting the part closer to it, reducing the length of the syringe of 1 cm. This was then connected to a soft and flexible Tygon tube.*

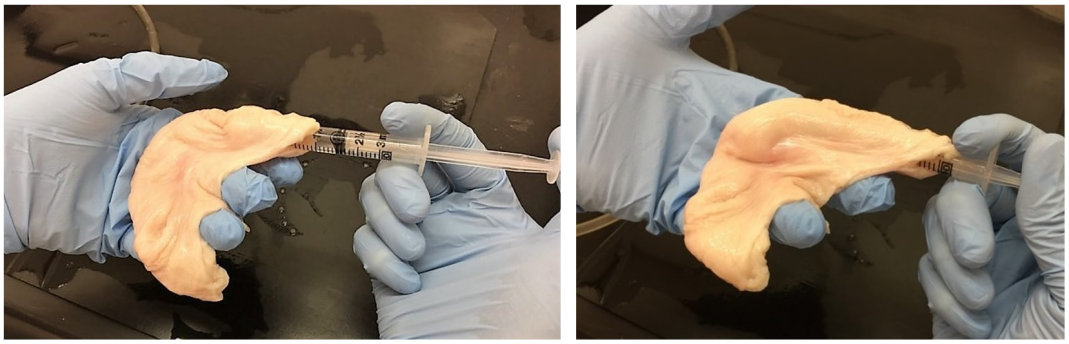

FIGURE 2.18: *Injecting the material inside a piece of Fallopian tube. The injection was done using a 3 mL syringe and pushing slowly the plunger to let the material be well distributed inside the tube.*

Results are shown in Figure 2.19.

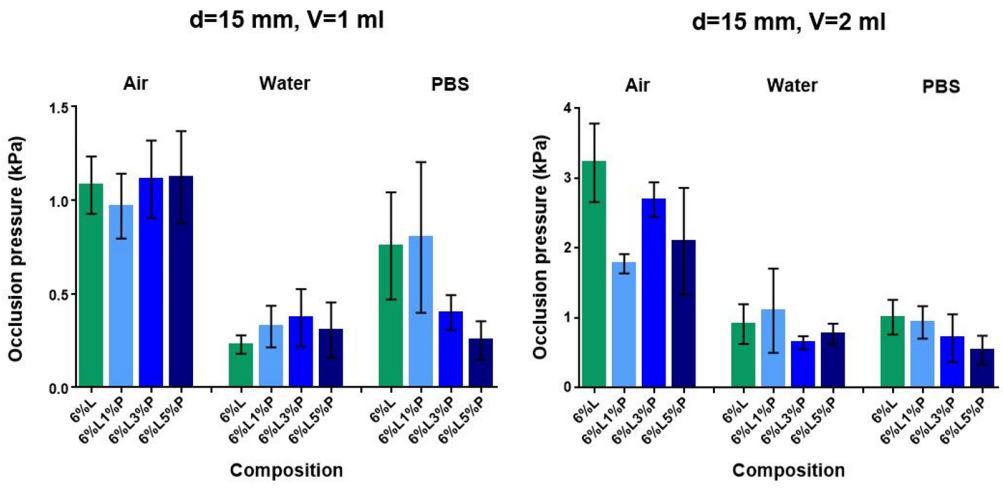


FIGURE 2.19: *Occlusion pressure measurements in Fallopian tube, putting 1 mL (left figure) and 2 mL (right figure) of material inside the organ. Air, water and PBS were spilled against the blockage. In all the tests 6% Laponite and 6% Laponite 1% PEO gave good results, except in one case. Anyway, excluding few bars, obtained pressures are very small and standard deviation is quite high.*

Predictably, injecting the doubled amount of material, the gel creates a stronger occlusion. Overall, the obtained pressures are very small; this is due the big diameter, the wet environment, and the elasticity of the tissue that might allow the medium to find its way at the interface between the material and the tube's wall. Naturally, the most comparable tests in vitro are the ones with 1 *cm* plastic tubes, because of the closer diameter. In all the groups of tests 6% Laponite and 6% Laponite 1% PEO gave good results. The exception is when air is pumped and 2 *mL* of 6% Laponite 1% PEO are injected. Anyway, excluding few cases, the standard deviation is quite high. It could be more interesting to repeat the same experiment with rabbit Fallopian tubes, that are smaller and very popular in this field but, for us, they were extremely difficult to buy.

### 2.2.4 Stability Test

To characterized the degradation properties of the optimised shear thinning biomaterial, a stability test in medium for all the compositions in study was performed at $37°C$. To simulate the environment were the material should stay, first PBS and then HTF were used. PBS (Phosphate-Buffered Saline) is a water-based solution, popular in biological research, because the ions concentrations match the one in the human body. HTF (Human Tubal Fluid) is a synthetic medium made to mimic the composition of the oviduct fluid in humans, generally used for in vitro fertilization. The one we used was fist formulated by Quinn et al. [50] and its components are: sodium chloride, potassium chloride, magnesium sulphate, potassium phosphate, calcium chloride, sodium bicarbonate, glucose, sodium pyruvate, sodium lactate, gentamicin. Anyway, the differences between the various HTF types are very small, and many of the ions concentrations depend on the phase of cycle.

Small Petri dishes ($35 \times 10$ *mm*), four for each material, were used to perform the experiment. After weighting them, a mold of PMMA was placed inside. This mold, made with the laser cutter from a PMMA sheet (thick 3.2 *mm*), was centred in the dish and used to give a cylindrical shape to the gel. At this stage, the dish with the material inside was again weighted and 4 *mL* of PBS or HTF were slowly injected around the cylinder of material. Before storing every dish inside the incubator at $37°C$, to prevent drying and evaporation, they should be well sealed (for the whole detailed protocol, check Appendix C).

For the PBS, the changes in weight were observed at the following timepoints (obviously once the medium is removed): 1 *h*, 5 *h*, 12 *h*, 72 *h*, 1 *week*, 2 *weeks*, 3 *weeks*, 4 *weeks*, 5 *weeks*, 6 *weeks*, 8 *weeks*; and at each timepoint pictures were taken (Figure 2.20). Results for PBS are shown below.

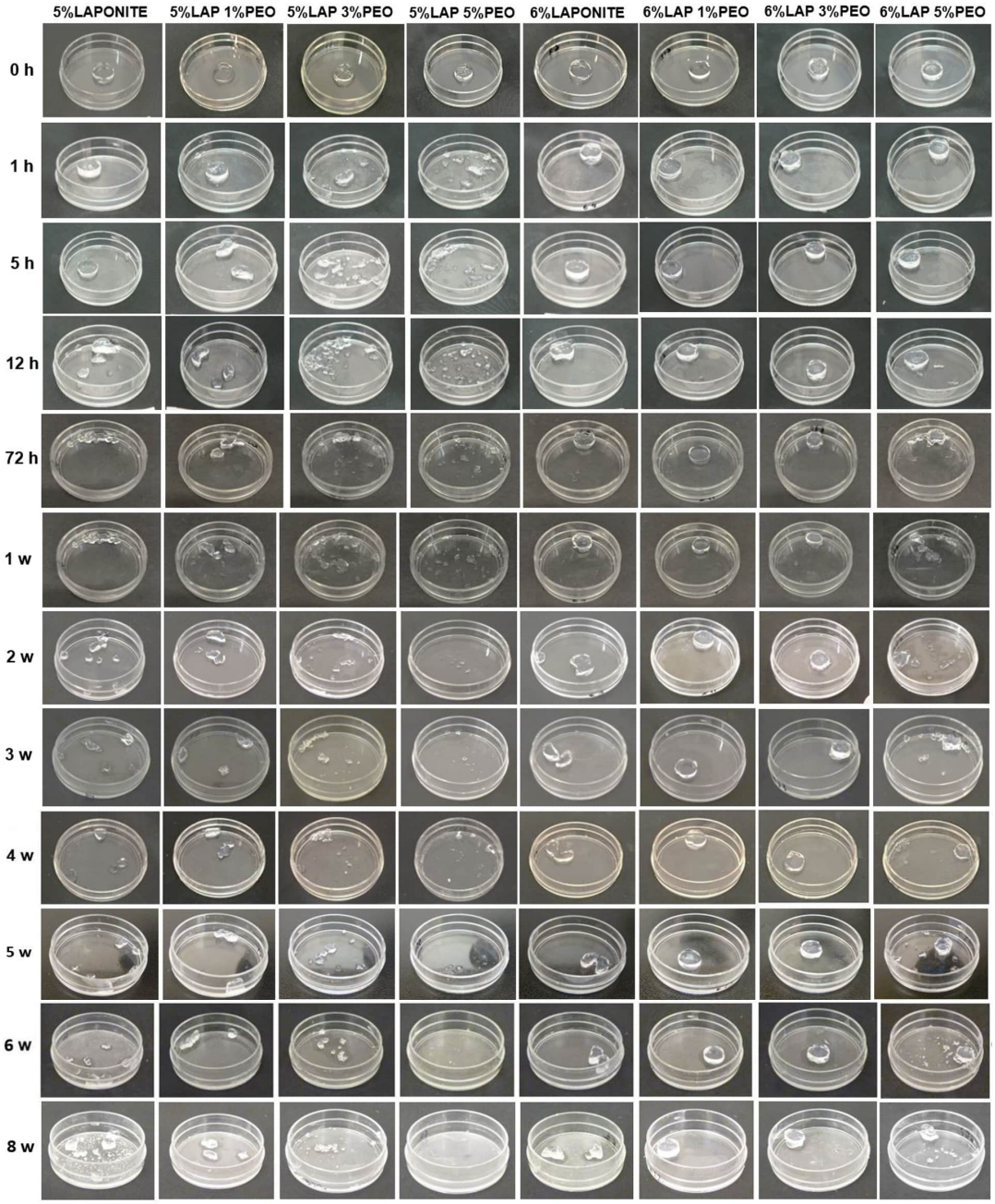


FIGURE 2.20: *Stability in PBS. Changes in weight were observed at the showed timepoints (obviously once the medium is removed) and at each timepoint pictures were taken. Four samples for each composition were prepared and between them the most representative was chosen to be plotted. There is a visible trend: the higher the PEO concentration, the less stable is the material over time.*

For HTF, the experiment was performed in the same way but the observed timepoints were: 1 *h*, 2 *h*, 4 *h*, 10 *h*, 1 *day*, 2 *days*, 4 *days*, 1 *week*, 2 *weeks*. Since the amount of HTF we had was limited, it was decided to test only the gels with a percentage of

nanoclay of 6% and increasing PEO. Results are plotted below.

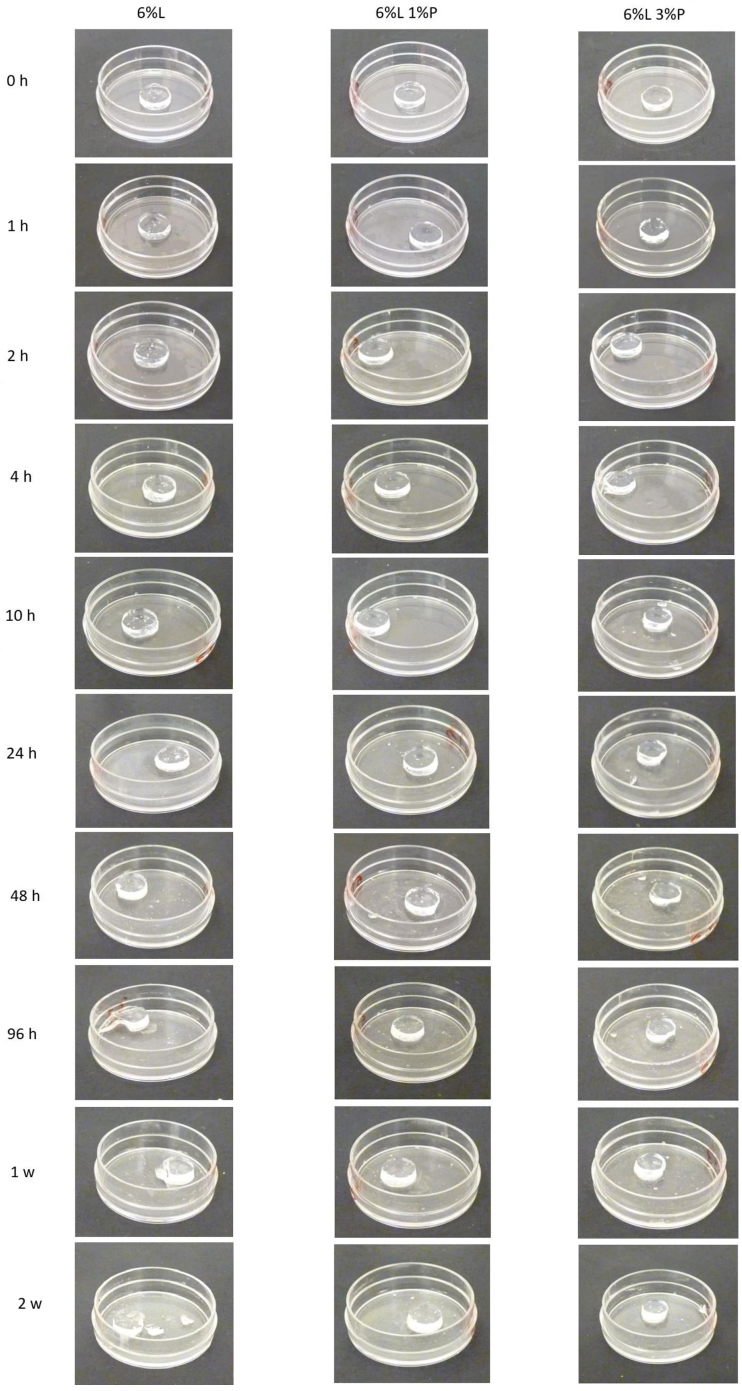


FIGURE 2.21: *Stability in HTF. Changes in weight were observed at the showed timepoints (obviously once the medium is removed) and at each time-point pictures were taken. Four samples for each composition were prepared and between them the most representative was chosen to be plotted. The more stable material in HTF seems to be 6% Laponite 1% PEO.*

### 2.2.5 Swelling and Degradation Tests

One important characteristic of hydrogels is the ability to swell when they are immersed in a solution. The molecules of the solvent can easily penetrate the network of which the gel is made and the material changes in volume. This property can be interesting for a better adhesion inside the Fallopian tube, but, at the same time, a high degree of swelling can encourage the disintegration of the network and the loss of compactness necessary to block external fluids in the organ. The test was performed using 2 ml Eppendorf tubes, deploying almost 1 g of the material inside them and adding 1 ml of medium. Four samples for each material were prepared and stored inside the 37°C incubator in the periods between the timepoints.

This test is different from the stability test because the medium is in contact only with the upper face of the material while, by using Petri dishes, all the surfaces except one are exposed to it.

As a ratio of weights, the percentage of swelling is calculated like:

$$D_{swell} = (W_f - W_i)/W_i \times 100$$

where $D_{swell}$ is the rate of swelling, $W_f$ is the final weight and $W_i$ is the initial weight.

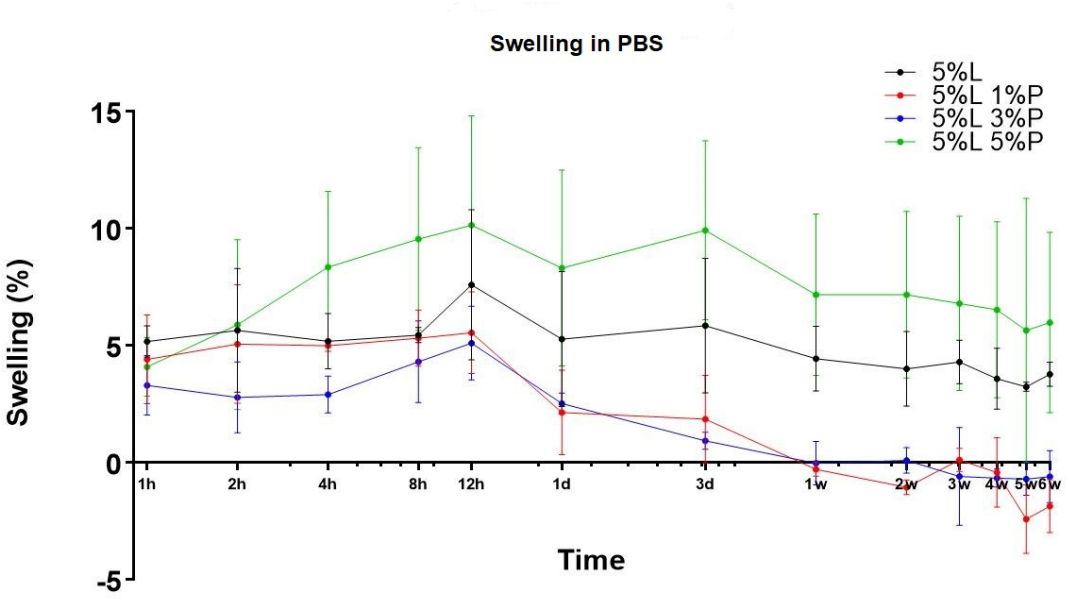


FIGURE 2.22: *Swelling of gels with 5% Laponite in PBS.*

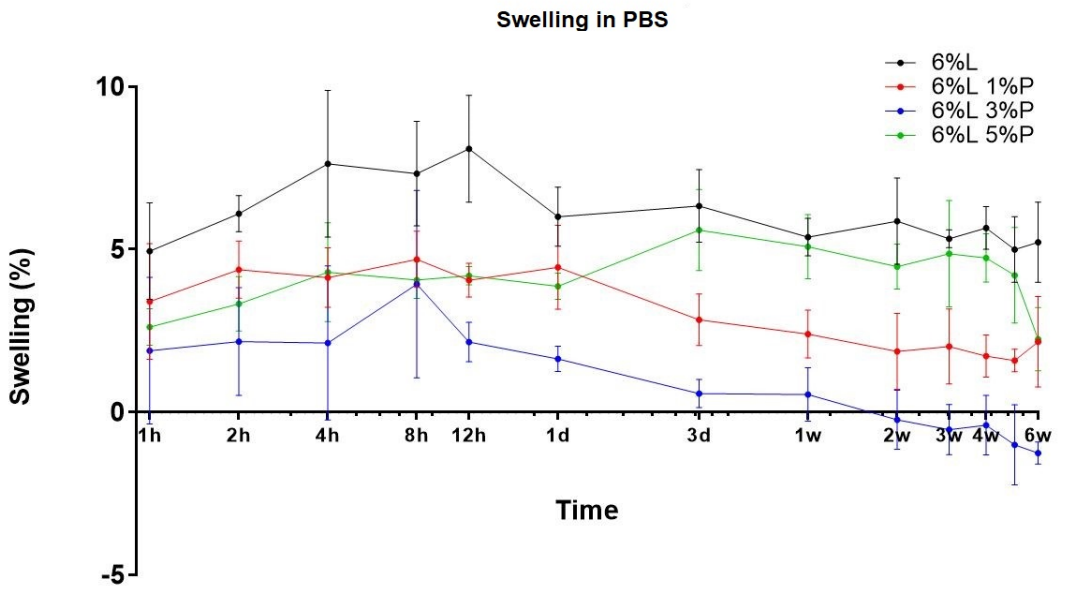


FIGURE 2.23: *Swelling of gels with 6% Laponite in PBS.*

Actually, swelling and degradation are the natural continuation of the stability test: when it has been found that the shear-thinning biomaterial was stable in PBS and HTF, it was decided to study the swelling and the degradation properties of it.

The used time points to observe the changes in weight for the sample in PBS are: 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 72 h, 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks.

Generally, swelling is not a continuous process: after few hours, the osmotic force is balanced and the deformation inhibited. Anyway, in this case no swelling was detected, the material is stable and does not degrade after 6 weeks. Looking at the graphs it is very difficult to find a trend: it seems that 1% PEO and 3% PEO compositions are the less swelled but those with the highest rate of degradation too. This might be in contrast with what it was found in the stability test.

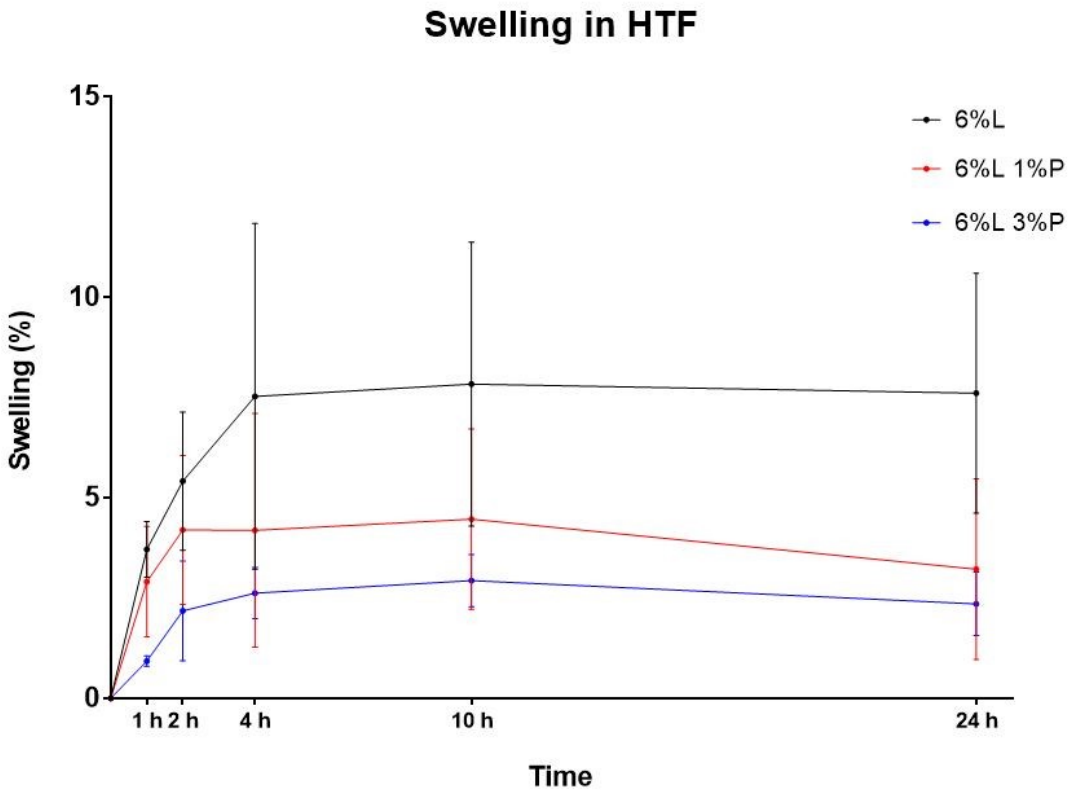


FIGURE 2.24: *Swelling of reduced set of gels in HTF until 24 h. This experiment was done with Eppendorf tubes, putting three materials (6% Laponite, 6% Laponite 1% PEO and 6% Laponite 3% PEO) in contact with HTF for 24 hours at 37°C. The 6% Laponite is the one that swells better compared to the others but, it is a very small swelling, irrelevant.*

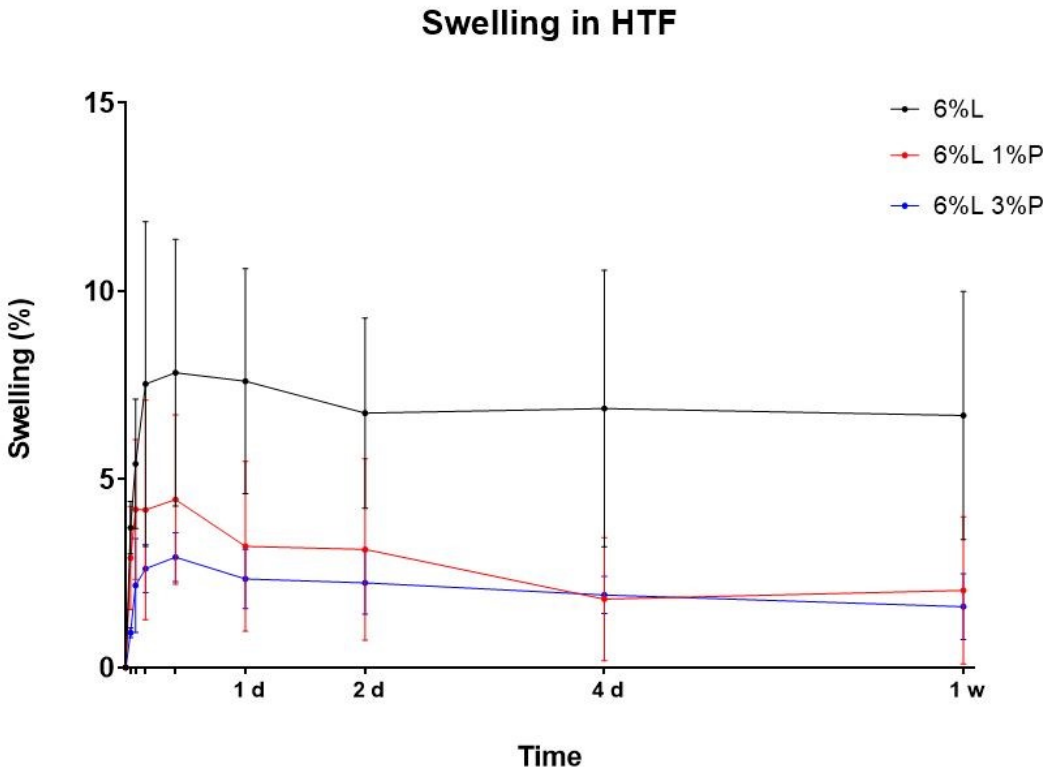


FIGURE 2.25: *Swelling of reduced set of gels in HTF until 1 week. This experiment was done with Eppendorf tubes, putting three materials (6% Laponite, 6% Laponite 1% PEO and 6% Laponite 3% PEO) in contact with HTF for 1 week at 37°C. The 6% Laponite is the one that swells better compared to the others but, it is a very small swelling, irrelevant.*

For the Human Tubal Fluid it is the same: the 6% Laponite is the more swelled while the other two have a very low value of swelling percentage. In any case, all the 6% Laponite gels can be considered long lasting gels.

To perform degradation test, the protocol is different. For this test, the focus was on the changes in the amount of solid particles when the materials are in contact with PBS. The experiment was divided in two parts: long term and short term degradation. The main difference with the swelling procedure is that for the degradation test, to measure the weight of the solid particles, each sample should be freeze-dried to remove all the water content. If the freeze dryer is not immediately available, when the timepoints occur, to stop the degradation process, the sample is immediately stored at -80°C. The critical steps of this procedure are: the removal of the medium, the holes used to freeze-dry, the too low amount of material initially deposed. The

degradation rate is calculated comparing two dried weights but the initial solid content is measured when using a wet mass, when it is a gel. To measure the solid content at 0 h, the sample is directly put inside the freezer at -80°C and then freeze-dried, never being in contact with the medium. 4 specimens for each composition were used and the initial solid content for all of them was measured like following:

$$SolidContent_{solcont0} = W_{0af}/W_{0bf}$$

where $W_{0af}$ is the weight of the sample at time point 0 after freeze drying and $W_{0bf}$ is the weight of the samples at time point 0 before freeze drying. The calculated value is then averaged with other values at time point 0 h. The rate of degradation is calculated for each time point i as :

$$SolidContent_i = W_{af_i}/(W_{bf_i} * SolidContent_{average0h})$$

where $W_{afi}$ is the weight of the sample at time point i after freeze drying, $W_{bfi}$ is the initial weight of the samples at time point i before the medium is inserted and Solid content $_{average0h}$ is the average value of solid content at time point 0 h.

Results are shown in Figure 2.26-2.29.

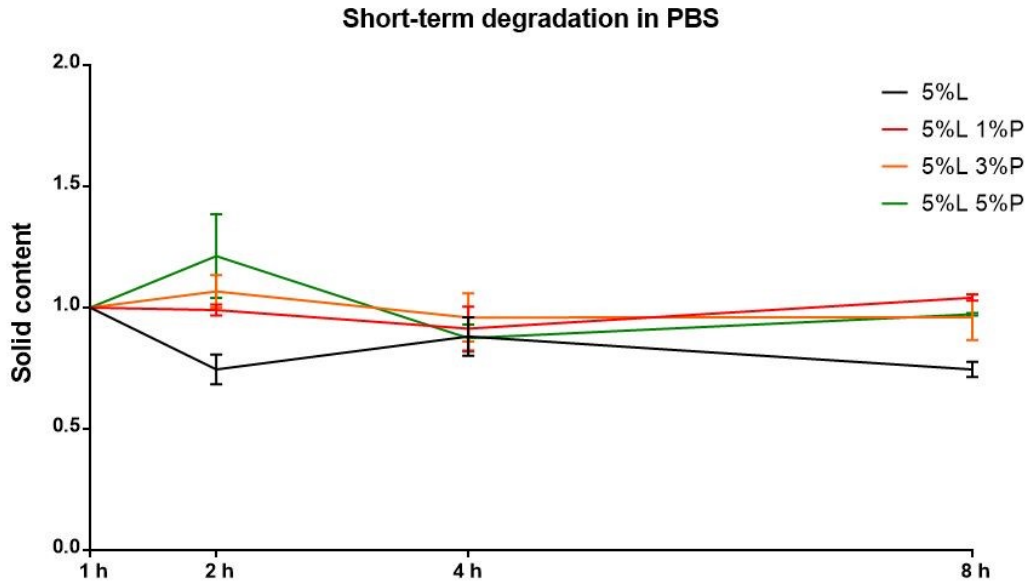


FIGURE 2.26: *Degradation of gels with 5% Laponite in PBS until 8 h.*

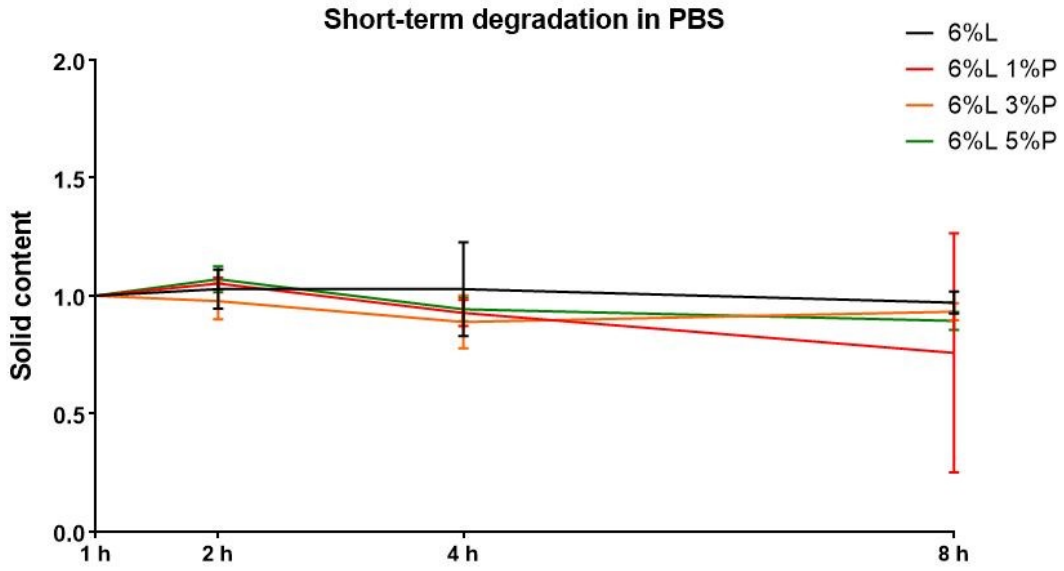


FIGURE 2.27: *Degradation of gels with 6% Laponite in PBS until 8 h.*

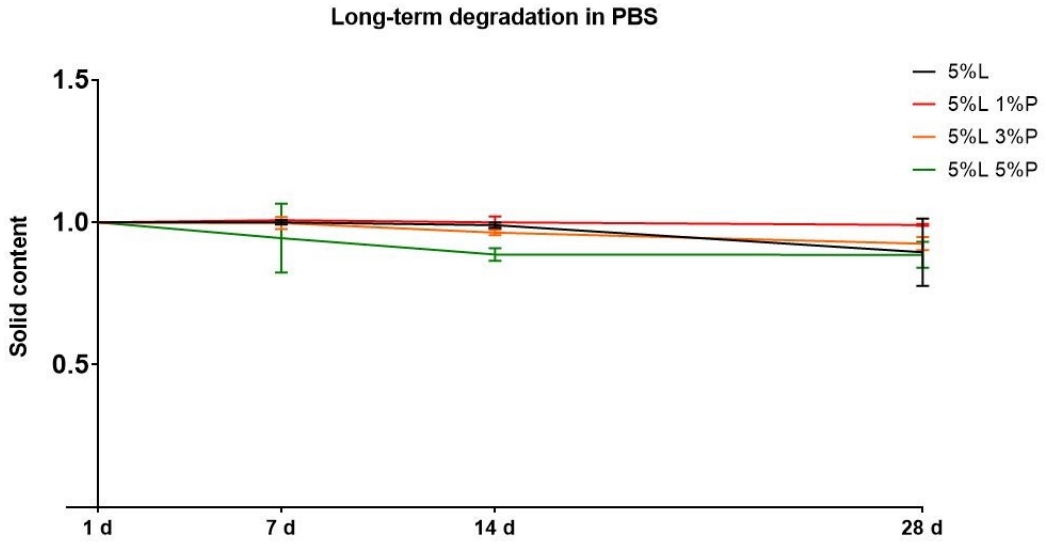


FIGURE 2.28: *Degradation of gels with 5% Laponite in PBS until 28 days.*

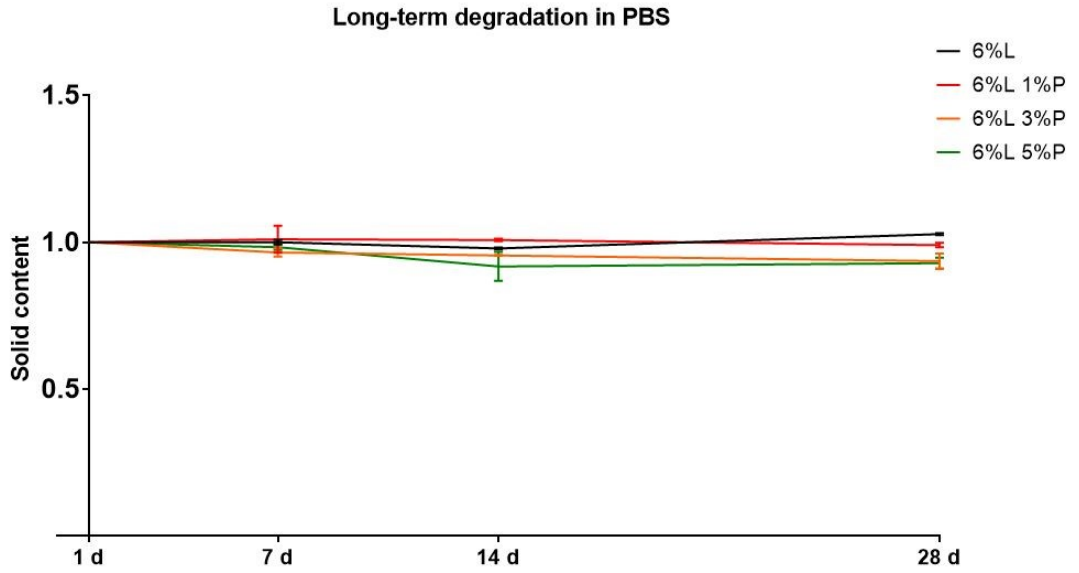


FIGURE 2.29: *Degradation of gels with 6% Laponite in PBS until 28 days.*

Analysing the data, it is noted that all the Laponite-PEO gels are extremely stable in PBS over time: no one of them is characterized by fast degradation properties, independently form the ratio of nanoclay or polymer.

### 2.2.6 Reversibily Test

Permanent sterilizationty is seen from many women like the most efficient methods for birth control. However, is statistically proven that, of all the women who chose sterilization, 2-13% regret it, within the first 6 years [51]. Therefore, reversibility of the occlusion is very important. To prove the reversibility of our material we performed an experiment that is very similar to the stability one but, instead of PBS or HTF, water is the medium. Small Petri dishes and a mold with an inner diameter of 1.5 cm to give cylindrical shape to the gel were used. The experiment was performed at 37°C, considered as physiological temperature. The used timepoints to observe the changes in weight are 1 h, 2 h, 3 h, 4 h. It was not possible to reduce the time between one timepoint and another one, monitoring more frequently the gels because of the big number of samples (four for each mixture, for 8 mixtures). At every timepoint water was carefully removed, without touching or sucking the

materials and the dish was weighted and photographed. Results are in Figure 2.30 and Figure 2.31.

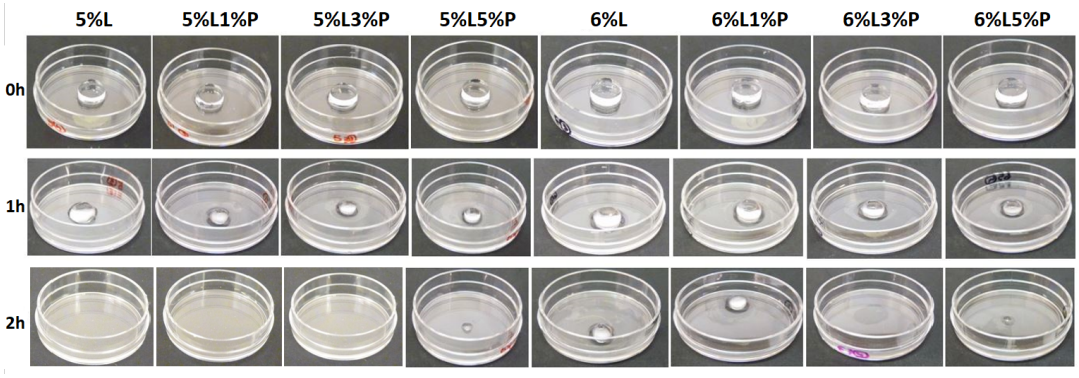


FIGURE 2.30: *Reversibility test. Changes in weight were observed at the showed timepoints (obviously once the medium is removed) and at each time-point pictures were taken. Four samples for each composition were prepared and between them the most representative was chosen to be plotted. There is a visible trend: the higher the PEO concentration, the more reversible is the gel formation over time. Moreover, materials with less Laponite dissolve faster and heavier.*

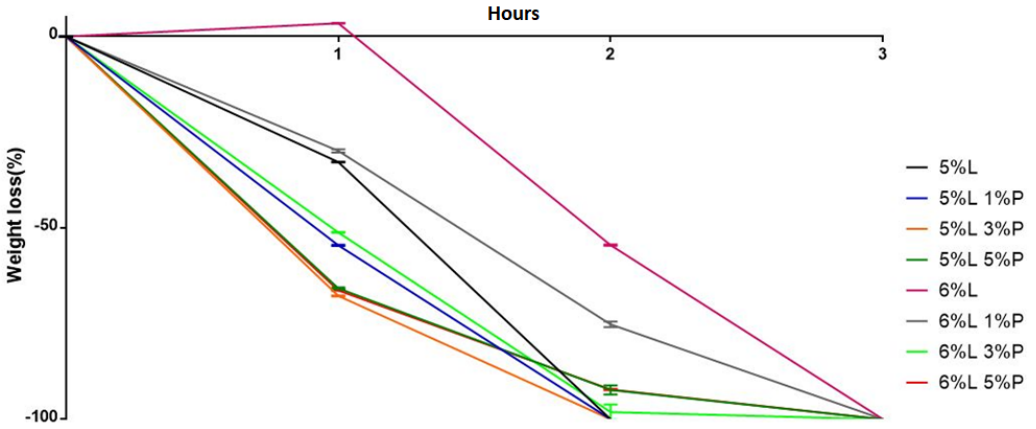


FIGURE 2.31: *Weight loss (%) vs time. The graphs are about changes in weight over time. Results match the pictures. After 3 h, all the samples were completely dissolved in water, starting from those with lower content of Laponite which after 2 h were around -100% of the initial weight.*

There is a visible trend: the higher the PEO concentration, the more reversible is the gel formation over time. Moreover, materials with less Laponite dissolve faster and heavier. In the graph above, weight loss in percentage versus time measured in hours are shown. The numerical results match the pictures. After 3 h, all the

samples were completely dissolved in water, starting from those with lower content of Laponite which after 2 h were around -100% of the initial weight. Physically, the behaviour of the shear thinning biomaterial is due to a simple dilution, since, as mentioned in the begging of this work, for concentration of Laponite lower than the 4% the biomaterial is not a gel. The decisive factor is the percentage of Laponite: mixtures with 5% are easier to dissolve than those with 6% Laponite. It could be interesting to occlude a transparent tube with the hydrogel, and to inject water with a catheter to wash away the material; the experiment could be video-recorded to better understand the dissolution rate and kinetics.

### 2.2.7 Compression Test

To better understand the mechanical response of our set of materials (6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO, 6% Laponite 5% PEO) a compression test was performed.

While the sample experience a uniaxial compressive, load, stress, strain and deformation were recorded and the stress-strain curves were plotted. By analysing them it is possible to obtain the elastic limit, the yield stress point, the Modulus of elasticity or Young's Modulus and other essential parameters to define the behaviour of our mixtures.

The compression test was performed using the mechanical testing machine INSTRON 5943, placing the sample between two plates and with the relevant sensor that is compatible with 100 N force. All the samples were tested three times, at $21°C$ and the compression rate was 0.3 $mm/min$.

The hydrogels were prepared for the test using a PMMA mold in a Petri dish (Figure 2.32). To have the mold, a sheet of PMMA (with a thickness of 3.2 $mm$) was cut with the laser cutter, making two concentric circles so that the final mold has an outer diameter of 3 $cm$ and an inner diameter of 1.5 $cm$.

The data acquisition and processing were performed with BlueHill software and the test was considered started when the upper plate touches the surface of the sample and the machine measures a load of 0.01 $N$ (for more details, see Appendix C). The experimental setup is shown in Figure 2.33.

Below, Figure 2.34 shows the stress-strain curve for each material.

The Young's Modulus of the gel was calculated using Origin, fitting the linear region of the stress-strain curve between 0% and the 10% of the maximum strain measured, and calculating the ratio of stress to strain. The results are shown in Figure 2.35. Among different PEO contents, the materials with lower percentage of PEO have a higher Young's Modulus: this means that, remaining in the elastic domain, the resistance of these samples to changes in length was higher.

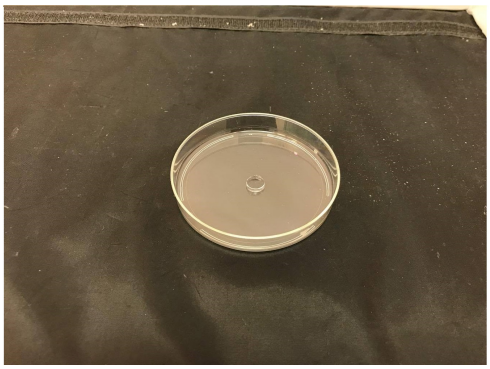

FIGURE 2.32: *Final appearance of one of the cylindrical samples used for the compression test. The diameter is* 1.5 *cm, the height is* 3.2 *mm. The sample was prepared using a PMMA mold, made with the laser cutter.*


FIGURE 2.33: *Experimental setup with the mechanical testing system, Instron 5943, and the Petri dish containing the sample under consideration.*

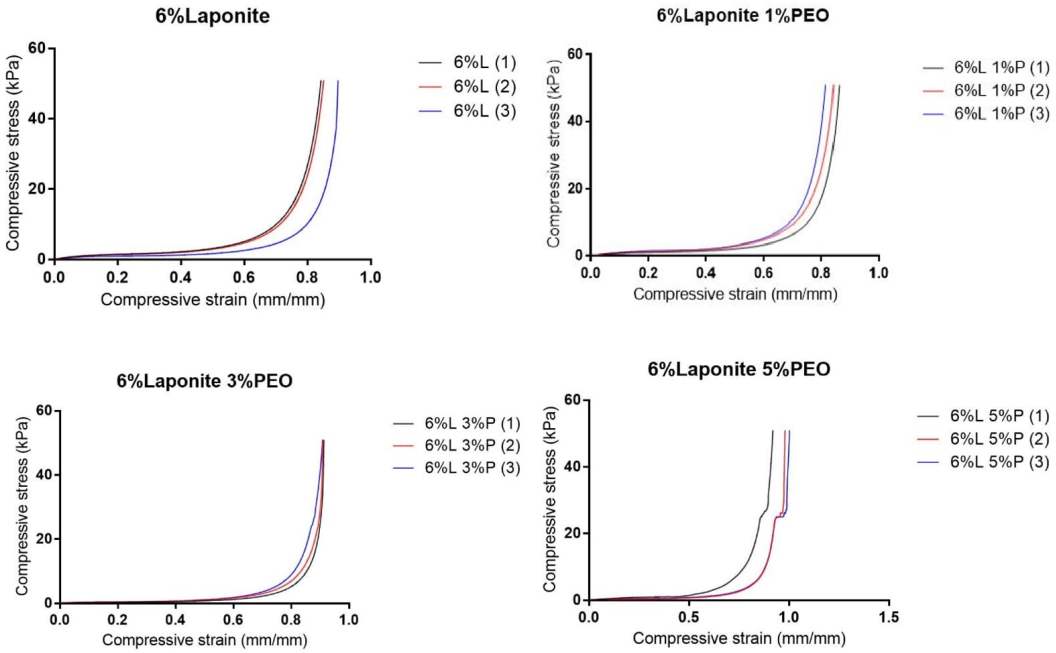


FIGURE 2.34: *Stress versus strain curves of Laponite/PEO gels use to calculate Young's Modulus, fitting the linear region of the stress-strain curve between* 0% *and the* 10% *of the maximum strain measured, and calculating the ratio.*

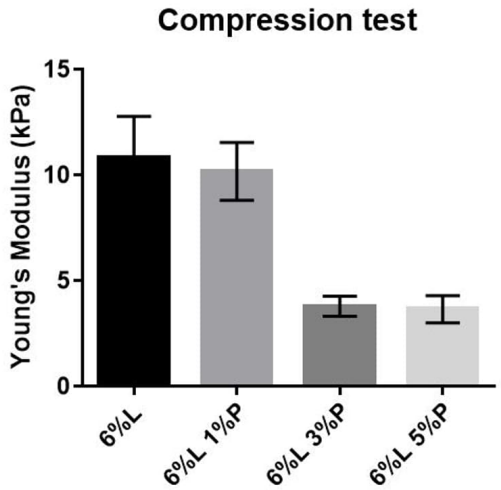


FIGURE 2.35: *Young's compressive Modulus (E) vs composition. It is clear that materials with less PEO have a higher E so higher mechanical properties.*

### 2.2.8 Adhesive Strength Test

One of the most common ways to quantify the adhesive strength is to perform a lap-shear tensile test. In this test, two samples are bonded together using an adhesive material and tested, applying tensile force. For the application of the shear-thinning gel in study, it is important to be sticky, so that it can stay attached to the walls of the tube without moving.

The test uses two partially overlapped specimens, with an adhesive between them, and a mechanical tester equipped with grips. After defreezing the tissue and preparing the samples, the tensile test was performed using the mechanical testing unit INSTRON 5943 that pulled at 20 mm/min one of the samples until failure.

In our experiment, porcine skin and porcine Fallopian tubes were used, after cutting them in 3 cm x 1 cm strips. Then, 0.01 ml of material was injected in an area of 1 cm x 1 cm of one of the two strips and they were pressed together, using a clamp for 3 min, to increase the contact force (Figure 2.36). The load needed to pull the specimen on top (while the other specimen is fixed) versus extension is shown. The experimental setup for the lap-shear test is in (Figure 2.37). The reached peak is the failure load; after that, the samples are detached. The tensile strength and the failure load, using porcine skin and Fallopian tube tissue are given below, in Figure 2.38-2.39.

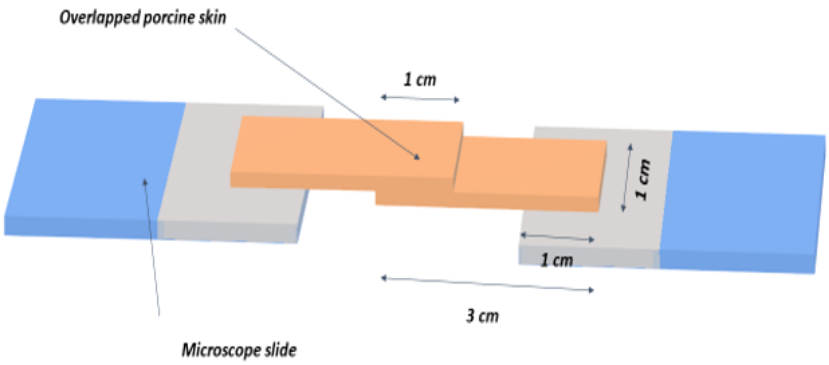


FIGURE 2.36: *Preparation of the sample. After cutting the tissue in 3 cm x 1 cm strips, 0.01 ml of material was injected in an area of 1 cm x 1 cm of one of the two strips and they were pressed together, using a clamp for 3 min, to increase the contact force.*

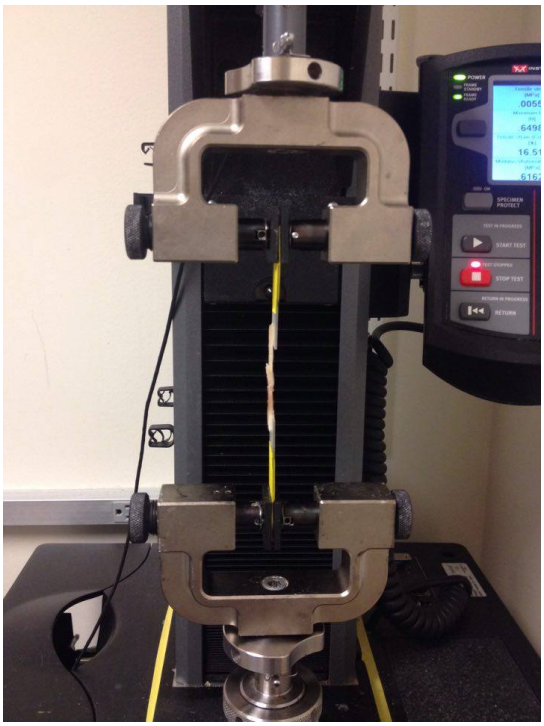

FIGURE 2.37: *Experimental setup lap-shear test. The tensile test was performed using the mechanical testing unit INSTRON 5943 that pulled at 20 mm/min one of the samples until failure (the other one is fixed).*

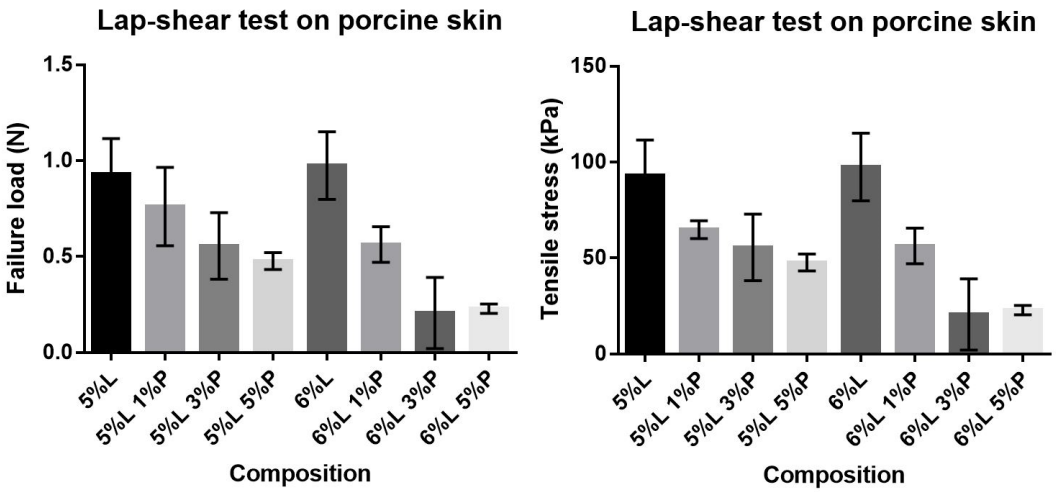


FIGURE 2.38: *Failure load and tensile stress vs composition using porcine skin. Bar chart representing the changes in adhesiveness versus the composition. Increasing the amount PEO is not convenient in term of adhesion: the failure load and the tensile strength are lower.*

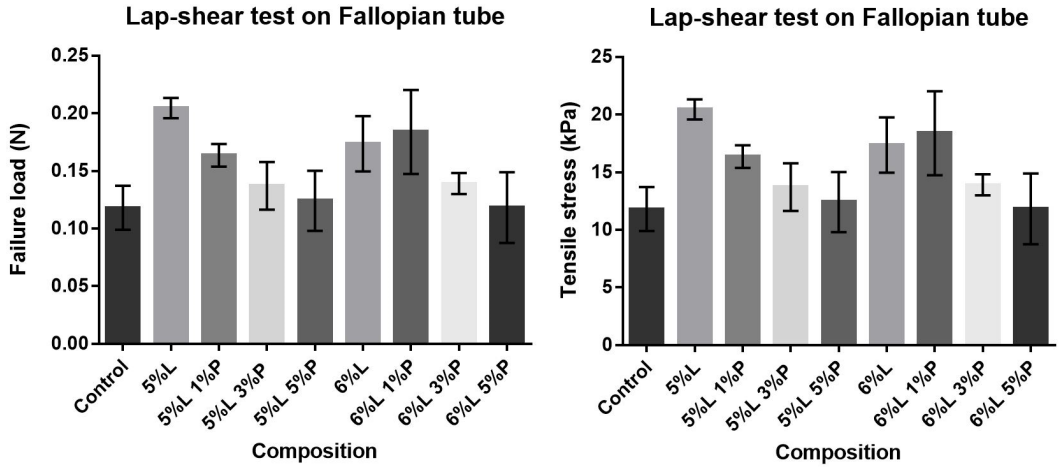


FIGURE 2.39: *Failure load and tensile stress vs composition using porcine Fallopian tube. Bar chart representing the changes in adhesiveness versus the composition. In this graph values are lower and the decline due to the change in material is less evident. This might be since the Fallopian tube are generally more wet and "slippery" so the adhesion strength is weak.*

The bar charts report that increasing the amount of PEO is, in general, not convenient in term of adhesion: the failure load is lower, so the tensile strength. The control bar in the test that use Fallopian tube represents the situation where the overlapped area is created by putting one edge of tissue on top of the other one, without interposition of material.

While in the first graphs there is a dramatic fall when the percentage of used PEO grows, in the second set of results values are lower and the decline due to the change in material is less evident. This might be since the Fallopian tube are generally more wet and "slippery" so the adhesion strength is weak. Observing the results for different concentrations of Laponite, for both uterine tube tissue and skin, a higher amount of nanoclay seems to be useless for an adhesive purpose, considering that the values are very close.

On the other hand, a low concentration of PEO, like in 6% Laponite 1% PEO, seems to improve the failure load value, when the experiment is performed with tissue from Fallopian tube, even if for 5% Laponite this observation is not valid and the standard deviation ranges make the material comparable to the one with only Laponite. Again, 6% Laponite and 6% Laponite 1% PEO are among the best materials, but solutions to increase the adhesiveness have been investigated (such as conjugation of

PEO with DOPA).

### 2.2.9 Shelf Life Experiment

The Laponite gels are known to show aging effects [52]. However, the ability to maintain the same properties over time, from an industrial point of view, is a very important characteristic, that will reduce the cost (due to an indispensable express transport, for example) and optimize the production. Considering that the synthesis of this shear-thinning material is not extremely simple, a preparation in the hospital is excluded. The shelf time is the maximum time for the material to be stored, without losing his intrinsic properties.

Evaluating how the time affect the injectability of the material through a $4F$ catheter is the aim of the shelf-life experiment. So far, two time points were observed: day 3 and day 14, but the experiment is still going on until 30 days. Moreover, since many chemical reactions are accelerated by temperature, half of the gels were stored inside the fridge, at $4°C$. For each material, once prepared a big amount of it, and tested his injection force to be sure about the quality, 6 syringes were prepared and sealed: 3 of them were put in the fridge and the others were kept outside. Before the injection force measurement, the syringe stored at $4°C$ was kept at room temperature for 1 hour at least. Four materials were compared: 6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO and 6% Laponite 5% PEO; the results are shown in Figure 2.40.

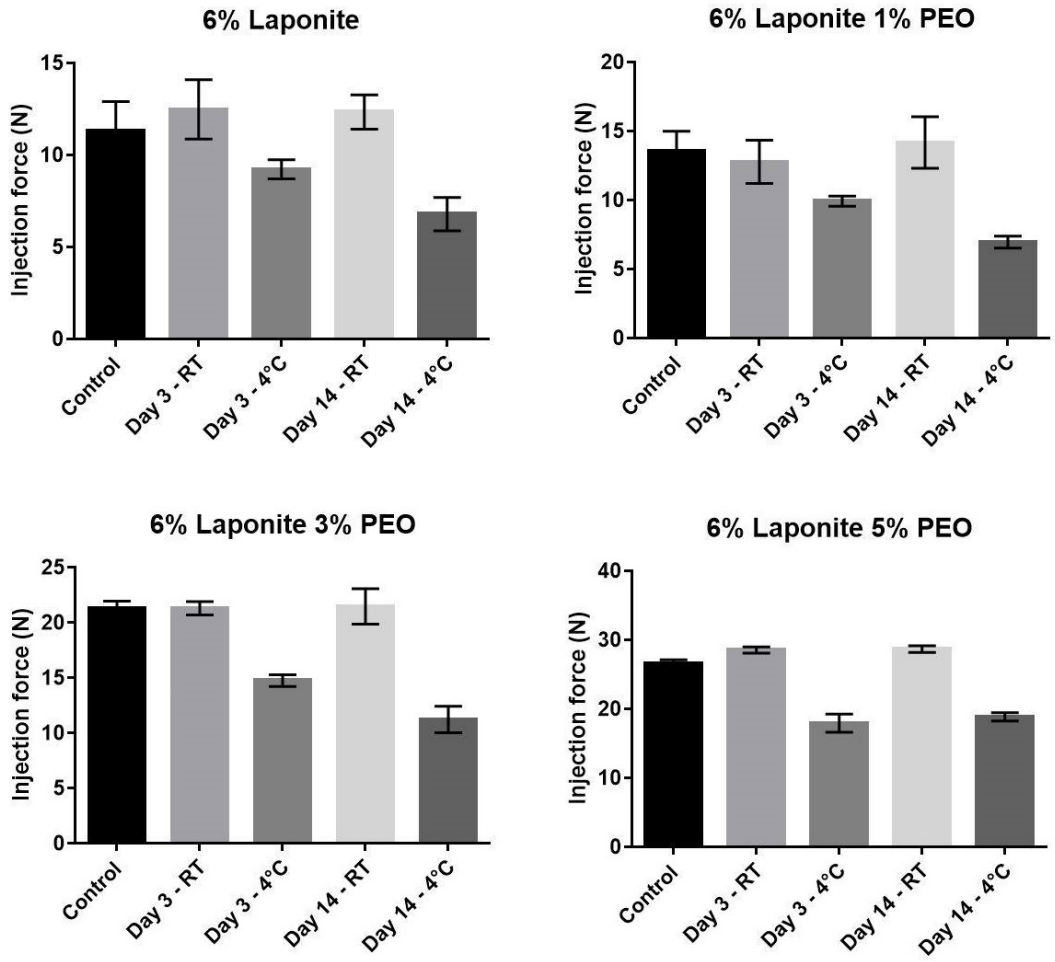


FIGURE 2.40: *Aging effect on shear-thinning biomaterials. The evaluated compositions are, from the top left to the bottom right, with a percentage of laponite of 6% and increasing PEO. The temperatures of storage are 25°C (room temperature, RT) and 4°C, the observed timepoints are 3 days and 14 days. The materials look very stable at RT, even when they are stored for 14 days. However, their injectability decreases drastically when they stay inside the fridge.*

The control is the measured injection force after 6 h from the synthesis. Observing the graph, it is possible to say that independently from the content of PEO, the material looks very stable after 14 days at 25°C while the storage at low temperature is not recommended since the lifespan is evidently reduced when the gel is kept at 4°C. The 6% Laponite 3% PEO and the 6% Laponite 5% PEO are the most critical materials, the most sensitive to changes in temperature. Overall, these results are very promising, in fact the possibility to store the material at 25°C means no costs for refrigeration.

### 2.2.10 Rheological Characterization

Shear-dependent (or non-Newtonian) fluids are fluids in which the viscosity function changes; particularly, if the viscosity increases for increasing applied shear rate the fluid is called shear-thickening fluid, if the viscosity function is decreasing the fluid is a shear-thinning fluid [53]. The shear-thinning behaviour is very common between gels and the applications in engineering for these materials are several. Among the interesting properties of shear thinning fluids there are drip resistance, suspension stability and injectability, the most important for us.

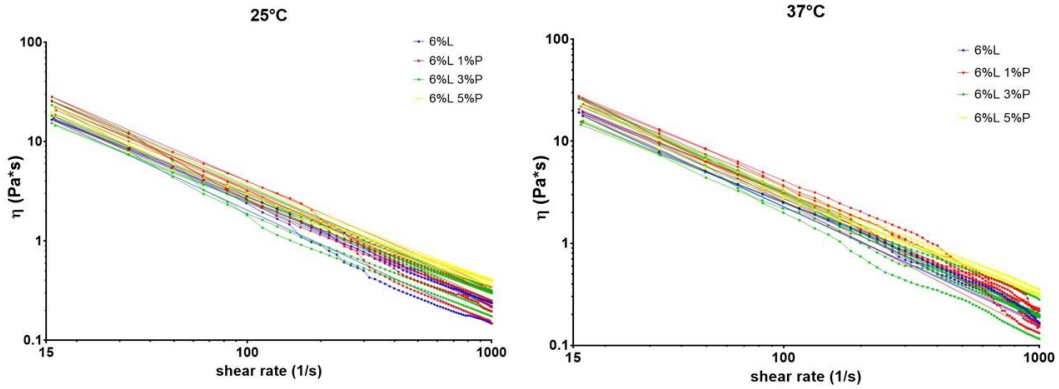


FIGURE 2.41: *Viscosity profile of the evaluated gels at 25°C and at 37°C. These viscosity versus shear rate curves show the shear-thinning behaviour of our materials; this means that they can deform and behave like liquids when a stress is applied and fast recover their original nature.*

In our study, rheological experiments were performed with a AR-G2 Rheometer [TA Instruments]. All the experiments were done using a cone and plate geometry (20 mm diameter, 1° cone angle) with a truncation gap of 56 $\mu$m.
Samples were loaded onto the plate and allowed to equilibrate for 2 minutes. For shear rheology, the viscosity versus shear rate was monitored at 0.1-1000 Hz.

As shown in Figure 2.41, the shear thinning hydrogel we developed can be easily injected by applying, during injection through the catheter, a shear stress and quickly change it back, once the shear is removed.

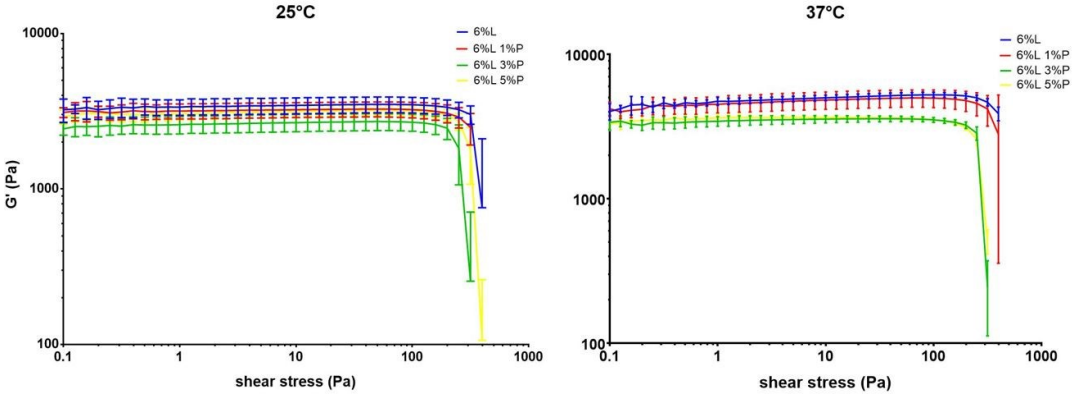


FIGURE 2.42: *Storage modulus vs shear stress at* 25°C *and* 37°C*. Oscillatory stress sweep was conducted at 0.1-1000 Pa and 1 Hz. The curves are plotted on log-log plot. For all our materials G′ is bigger than G″ so the material behaves in theory like an elastic solid. 6% Laponite 3% PEO and 6% Laponite 5% PEO are softer materials but weaker than the other gels.*

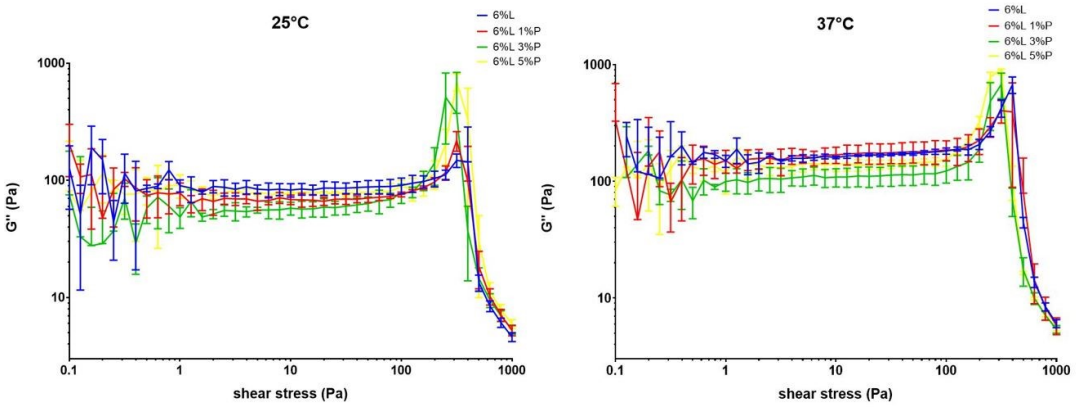


FIGURE 2.43: *Loss modulus vs shear stress at* 25°C *and* 37°C*. Oscillatory stress sweep was conducted at 0.1-1000 Pa and 1 Hz. The curves are plotted on log-log plot. For all our materials G′ is bigger than G″ so the material behaves in theory like an elastic solid. 6% Laponite 3% PEO and 6% Laponite 5% PEO are softer materials but weaker than the other gels.*

The second rheological test we performed was the oscillation test: amplitude sweep and frequency sweep. In the first case the amplitude of the deformation or of the shear stress changes while the frequency is kept constant and in the second one is the amplitude of the shear stress that is kept constant.

Oscillatory stress sweep was conducted at 0.1-1000 Pa and 1 Hz (Figure 2.42-2.43), at 25°C and 37°C. The curves are plotted on log-log plot.

Generally speaking, there is a region where the values of G′ and G″ have a plateau at low value of shear stress: this region is called linear-viscoelastic. The constant value of the storage modulus is the elastic solid-like behavior (G′) and the constant value of the loss modulus is the viscous response (G″). Different informations can be obtained from these values.

For all our materials G′ is bigger than G″ so the material behaves in theory like an elastic solid, not dissipating energy but storing it and being able to return it. If the G″ is bigger than the G′ then the viscoelastic fluid behave will prevail.

So this rheological test is used to understand how strong is the material microstructurally, applying a small amplitude oscillatory shear. When the material undergoes yield and the structure is destroyed, the mechanical energy given to the material is dissipated, thus the material flows. At that time, first G″ becomes greater than G′ and then loss and storage moduli decrease.

In the case of our gels, 6% Laponite 3% PEO and 6% Laponite 5% PEO are softer materials (because the initial storage modulus value is lower) but weaker (because the curve decreases earlier) than gel with less PEO content.

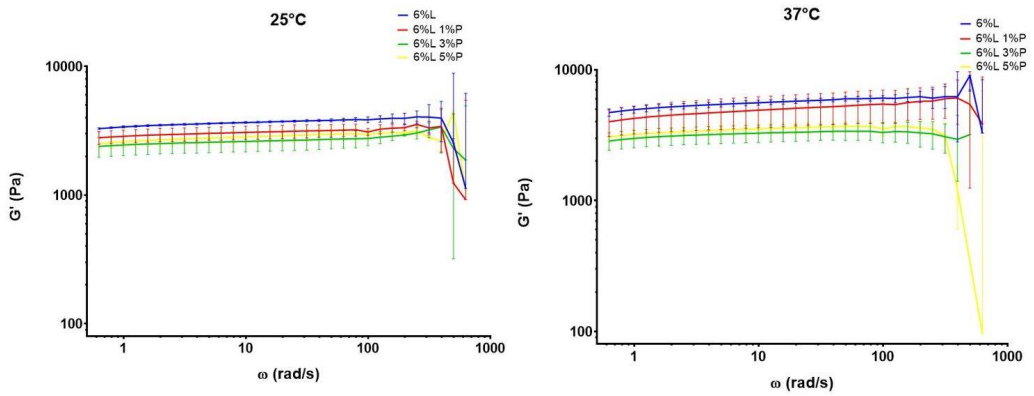


FIGURE 2.44: *Storage modulus vs angular frequency at 25°C and 37°C. Oscillatory frequency sweep was conducted at 0.1-100 Hz and 10 Pa. The curves are plotted on log-log plot. Gel moduli were not so dependent on oscillatory deformation frequency and were a bit more elastic at high shear rates but also more viscous at low shear rates.*

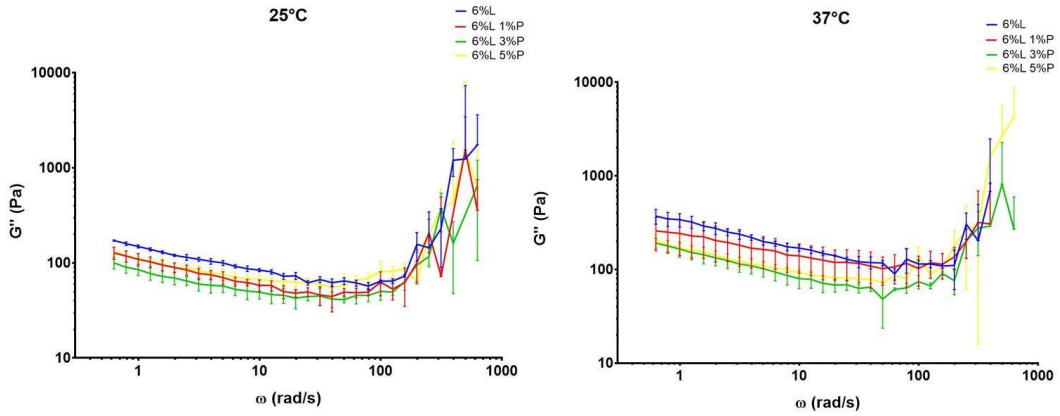


FIGURE 2.45: *Loss modulus vs angular frequency at 25°C and 37°C. Oscillatory frequency sweep was conducted at 0.1-100 Hz and 10 Pa. The curves are plotted on log-log plot. Gel moduli were not so dependent on oscillatory deformation frequency and were a bit more elastic at high shear rates but also more viscous at low shear rates.*

The next rheological test, oscillatory frequency sweep, was performed at 0.1-100 Hz and 10 Pa (Figure 2.44-2.44) for all the samples at 25°C and 37°C, and plotted on log-log plot. For shear rate sweeps, samples were equilibrated at 37°C for 10 min prior to testing, sheared at 10 s-1 for 2 min, and equilibrated for an additional 10 s. Following this, shear rates were ramped from 0.001-10 s-1 at a density of 10 points per decade. This test is done to understand the nature of the structure in the fluid.

Gel moduli were not so dependent on oscillatory deformation frequency and were a bit more elastic at high shear rates but also more viscous at low shear rates. In our case the gel composition has relatively little effect on moduli and shear thinning properties.

### Creep and Recovery Test

In order to simulate conditions closer to the real application in which the gel is inserted inside the tubal lumen and must not permanently deform because of muscle contractions, creep recovery tests and multiple stress creep recovery tests were performed. Performing a creep test, a loading followed by an unloading is executed. Thus, in the first part of the experiment, a constant stress in linear viscoelastic interval is applied for a period of a creep testing time of 30 min and the strain is monitored as a function of the time; while, in the second part, which lasts 30 min too, stress is

removed and the system attempt to regain the initial conditions and dimensions. Fully recovering is not reached so the residual deformation is recorded. This permanent value has important information in the engineering field because can led to loss of stability[54]. The creep was varied between 10 and 30 Pa. Results are shown in Figure 2.46-2.50.

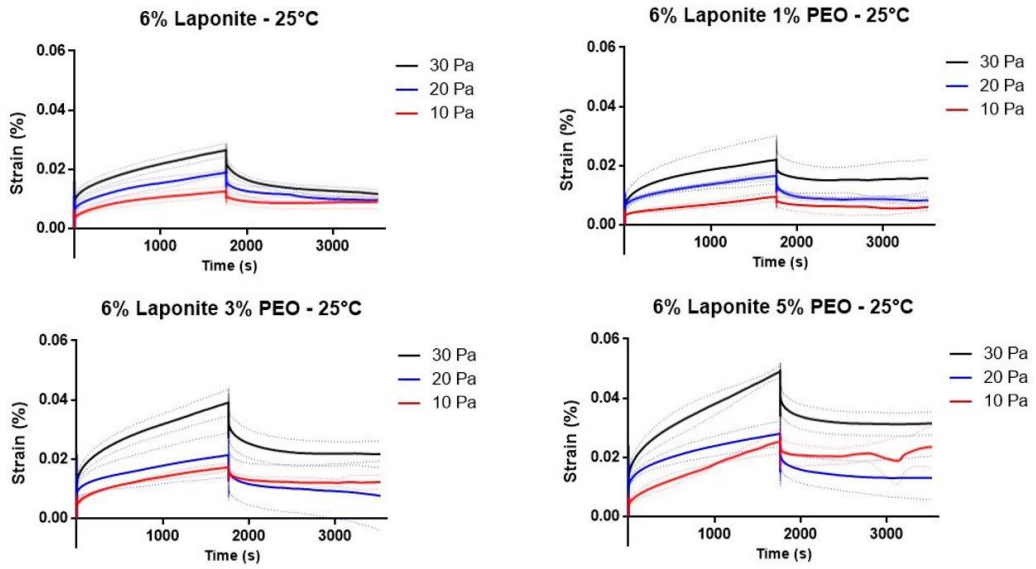


FIGURE 2.46: *Rheological creep and recovery test: strain vs time. Four gels were tested at* 25°C *and three different loads were applied: 10 Pa, 20 Pa, 30 Pa.*

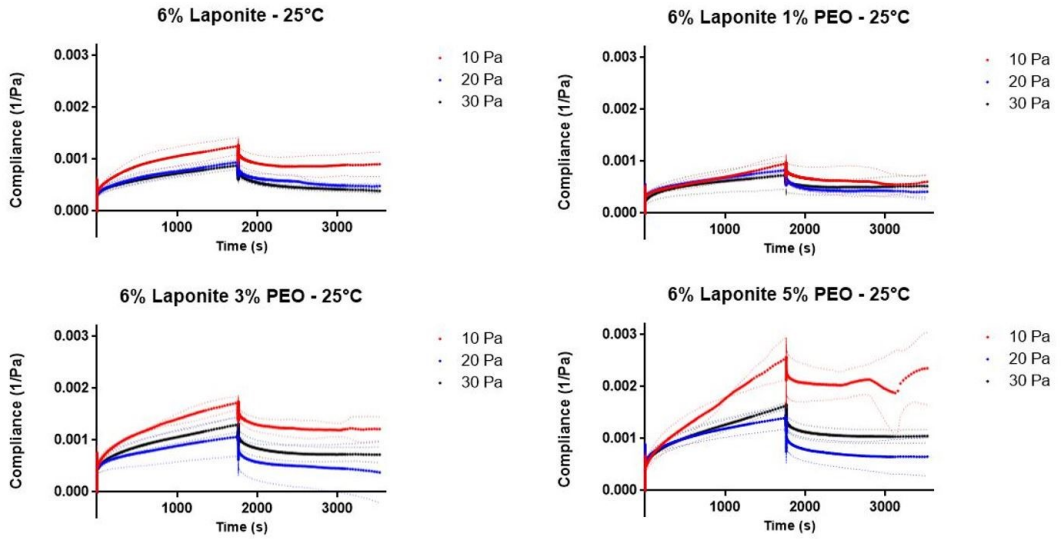


FIGURE 2.47: *Rheological creep and recovery test: compliance vs time. Four gels were tested at* 25°C *and three different loads were applied: 10 Pa, 20 Pa, 30 Pa.*

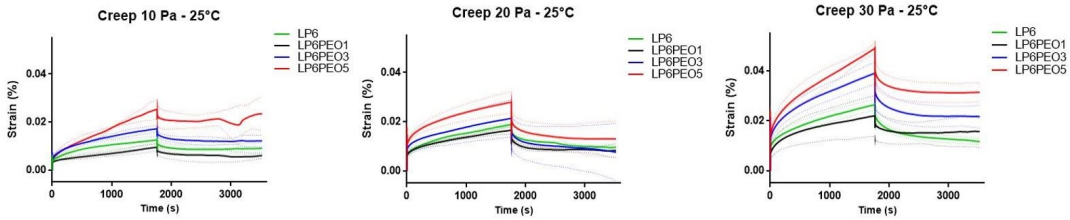


FIGURE 2.48: *Rheological creep and recovery tests for 10 Pa, 20 Pa, 30 Pa. 6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO, 6% Laponite 5% PEO were tested at* 25°C *and three different loads were applied: 10 Pa, 20 Pa, 30 Pa.*

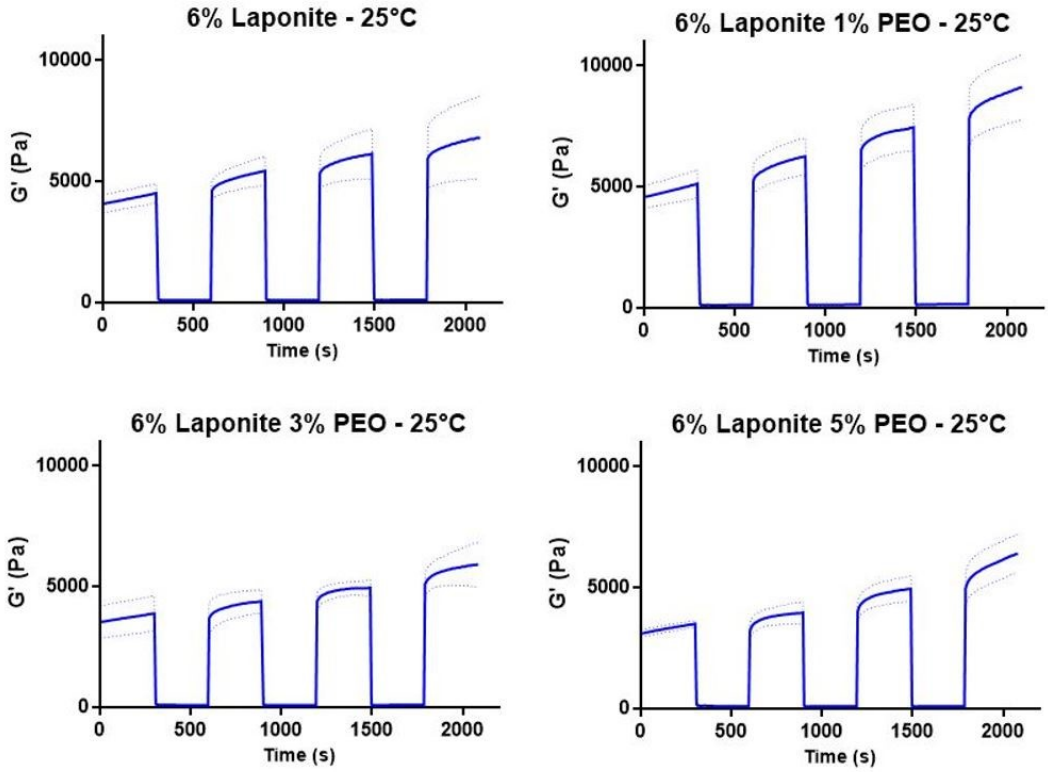


FIGURE 2.49: *Multiple stress creep recovery test: storage modulus vs time. The test was performed at* 25°C*, for four compositions: 6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO, 6% Laponite 5% PEO.*

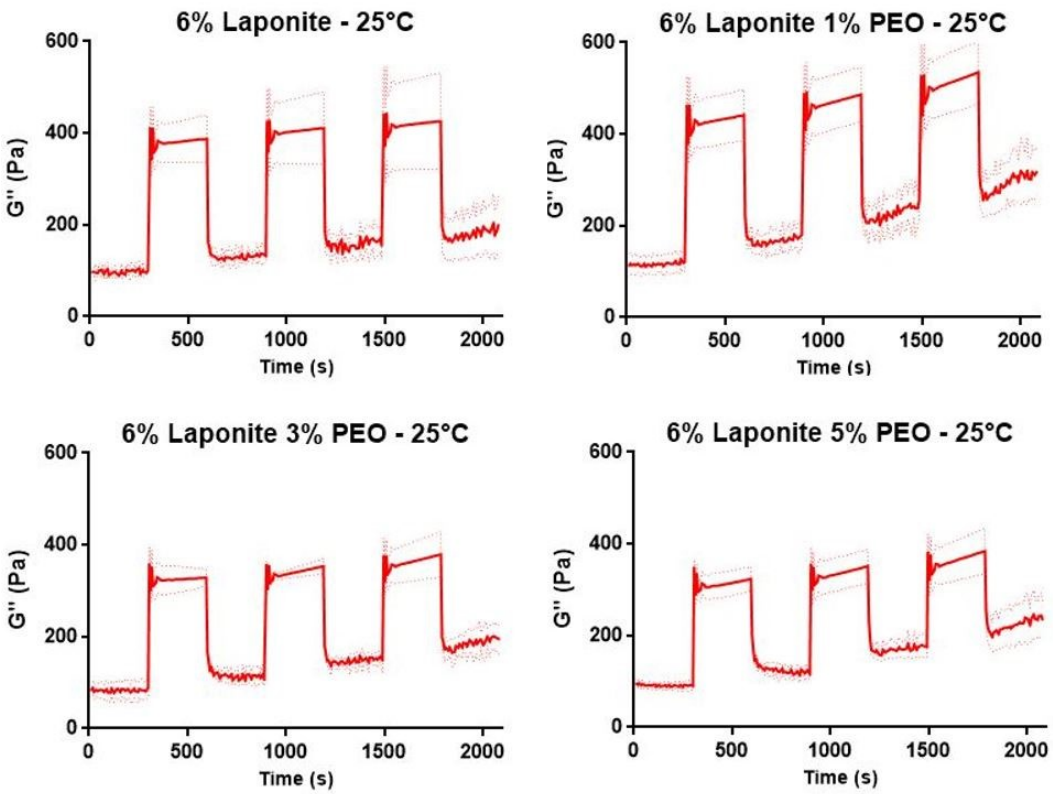


FIGURE 2.50: *Multiple stress creep recovery test: loss modulus vs time. The test was performed at* 25°C*, for four compositions: 6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO, 6% Laponite 5% PEO. For all of them G" is lower than G', so the elastic portion is the main one compared to the viscous.*

For the rheological creep and recovery test, four gels were tested: 6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO, 6% Laponite 5% PEO. The experiment was performed at room temperature.

All the graphs, as expected, show that the strain is not recovered completely: in the curves strain versus time, the unrecovered strain for each material is its final value of strain. The percentage of recovery can be calculated subtracting the unrecovered strain from the strain peak and dividing everything for the strain peak.

Subsequently, the ratio strain versus stress, the compliance, was calculated, and plotted. Of course, the loading phase in the compliance graph has the same trend of the strain in the first phase. The higher concentration of PEO demonstrates the higher creep deformation and so the higher time-dependence for all the loading phases as for 6%Laponite 5%PEO and 6%Laponite 3%PEO and the same behaviour

can be found in the recovery region since the higher concentration of PEO the higher delayed recovery. Furthemore, unstable recovery can be noticed at lower loading phase for the highest PEO concentration at $25°C$ and lower recovery with respect to the higher strain imposed.

In the multiple stress creep recovery test performed at $25°C$, after controlling that the increasing stress creep applied is always under the yield stress, consists in a multiple stress application-relaxation cycles. Unrecovered strain increases at every cycle; for all the materials G'' is lower than G', so the elastic portion is always the main one compared to the viscous.

### 2.2.11 Cytotoxicity

In this study, 3T3 cells are used to evaluate the effect of Laponite, PEO, and both together on cells growth and proliferation. 3T3 are fibroblast adherent cells, from mouse embryo tissue. They grow relatively easily in the $37°C$ incubator; the ones that were used for our experiments have a low passage number.

In order to achieve our goal, a PrestoBlue analysis was performed in combination with a Live/Dead assay so that the overall results of one method can act as a confirmation for the other one.

The required materials are $1\times$ Trypsin, sterile D-PBS Buffer solution, DMEM cell culture medium [DMEM Dulbecco's Modified Eagle Medium, Gibco], FBS, Pen/Strep, Laponite, PEO and PrestoBlue stock solution [PrestoBlue Cell Viability Reagent, Invitrogen].

Before starting the experiment, it is important to understand that all the cell culture rooms must be free from pathogenic microorganisms, and the work area must be absolutely aseptic so hands and equipment must be always sprayed with ethanol before entering the hood.

The first procedure to learn, to perform a test with cells, is cell passaging. 3T3 cells grow fast in a favourable environment until they reach confluence and occupy all the substrate. Once this happens, cell passaging is needed: cells are put in a new flask with fresh growth media; this allows them to proliferate more and to live longer.

In our case, T75 flasks are used. When the confluence is approximately from 80% to 100%, the media is removed and the flask is washed with DPBS. After removing the

DPBS, we proceed adding 2 $mL$ of trypsin inside the flask and leaving the flask at 37°$C$ for 2 $min$. Trypsine is an enzyme able to detach strongly adherent cells from the surface of the substrate.

The trypsine was neutralized by adding the double volume of media to the flask and with it subsequently the bottom of the flask was gently washed. The solution was then transfer in a 15 $mL$ Falcon tube and centrifuged at 1200 RPM for 5 $min$ to separate cells from the rest. This step is useful because then the media is removed and 1 $mL$ of fresh medium is added to the cell pellet, gently pipetting to create a suspended solution. The last step consists in using the cell counter to determine approximately the total number of cells in the solution and the volume of the cell suspension needed for a certain seeding density (for a detailed cell passaging protocol see Appendix C).

To test the cytotoxicity of Laponite and PEO, it was decided to add them to the culture medium, considering different concentrations. For the Laponite we used the following concentrations: 1000 $\mu g$ in 1 $mL$ of media, 500 $\mu g$ in 1 $mL$ of media, 100 $\mu g$ in 1 $mL$ of media, 10 $\mu g$ in 1 $mL$ of media, 1 $\mu g$ in 1 $mL$ of media, 0.1 $\mu g$ in 1 $mL$ of media. The cell behaviour due to the PEO effect was evaluated for the same concentrations while the effect of both of them together (Laponite and PEO) was evaluated for 100 $\mu g/mL$ of Laponite and 100 $\mu g/mL$ of PEO, 500 $\mu g/mL$ of Laponite and 500 $\mu g/mL$ of PEO, 100 $\mu g/mL$ of Laponite and 100 $\mu g/mL$ of PEO, 10 $\mu g/mL$ of Laponite and 10 $\mu g/mL$ of PEO, 1 $\mu g/mL$ of Laponite and 1 $\mu g/mL$ of PEO, 0.1 $\mu g/mL$ of Laponite and 0.1 $\mu g/mL$ of PEO (see Appendix C for the stock solution preparation protocol).

**PrestoBlue Assay**

For the PrestoBlue, 4 repetitions per condition were performed. Once that the cells are in solutions with Laponite and PEO, they are kept in incubator for 24 $h$. The PrestoBlue is conducted 3 times: 24 $h$ after the passaging before the changing of the media, 24 $h$ after using the new media with the chemicals inside, 3 days after using the new media with the chemicals inside. PrestoBlue is a reagent used to evaluate cell viability and cytotoxicity *in vitro* [55]. It is a resazurin-based solution able to

penetrate the living cells; once entered the mitochondrial enzymes convert it to his reduced form, and the solution changes colour and becomes fluorescent.

To perform the experiment, first, the hood lamp was turned off because the solution is sensitive to light, so the reagent was diluted ($10\times$) in fresh media; the tube containing the mixture was then wrapped in aluminium foil. Once the old medium is removed from the wells, 200 $\mu L$ of PrestoBlue medium were put in each well, so the plate was incubated for 90 $min$. After 90 $min$, in 50 $\mu L$ steps, the supernatant was transferred to three clean 96 wells plates, so in the old well plate the cells are still covered in 50 $\mu L$ of solution and each new well plate has three PrestoBlue replicates. Finally, a plate reader was used to read the data (see Appendix C to know more about PrestoBlue Assay protocol).

The results are shown in Figure 2.51.

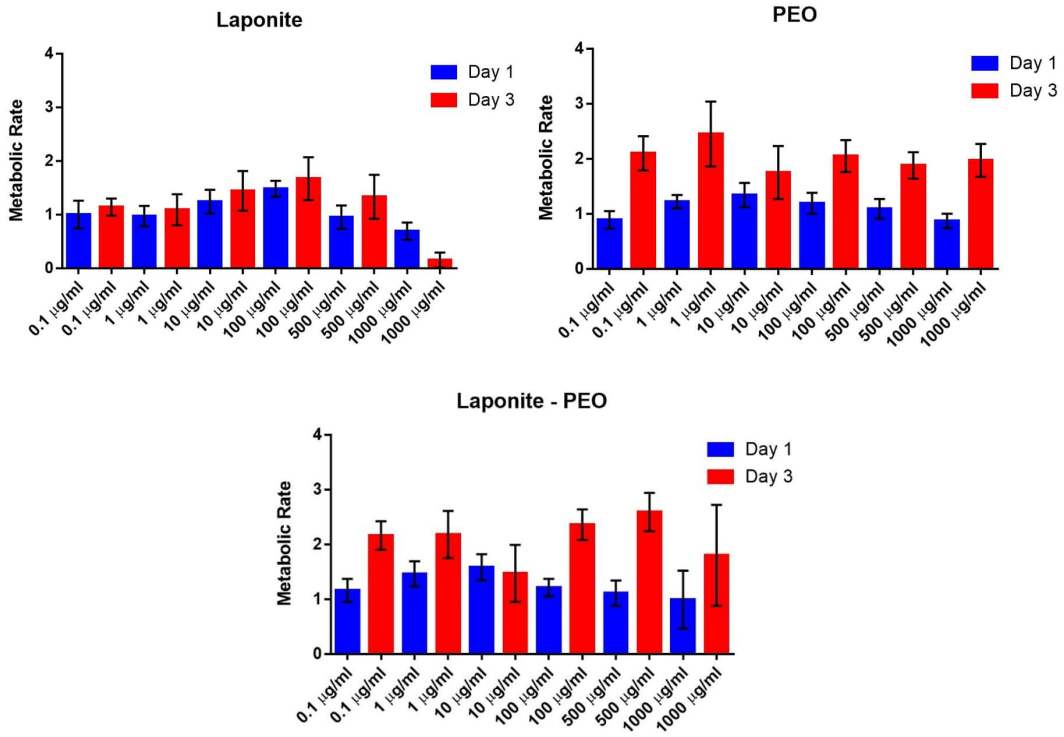


FIGURE 2.51: *Metabolic rate obtained with PrestoBlue Assay. All the values represented are relatively low and not dramatically different: the experiment needs to be continued until day 7 to have a complete information of cells behaviour. Only Laponite at high concentration looks toxic.*

For this experiment a concentration of 5000 $cells/mL$ was used. The bar chart illustrates the metabolic rate versus the concentration of chemical inside the medium, varying between 0.1 $/mug/mL$ to 1000 $\mu g/mL$.
All the values represented are relatively low and not dramatically different: certainly, the experiment needs to be continued until day 7 to have a complete information of cells behaviour. The stability of the obtained numbers is evident especially for the wells containing only Laponite, even if in each graph there is a slight trend: for the graph regarding Laponite the optimum concentration after 24 $h$ seems to be 100 $\mu g/mL$ but the biggest rise after 3 days in metabolic activity is for 500 $\mu g/mL$; Laponite at higher concentrations looks toxic.
In the second bar chart, a peak of metabolic rate is reached using 1 $\mu g/mL$ of PEO at day 3 but the standard deviation for this measurement is quite high. Differently from the previous graph, using PEO every bar is increased over time, there is an increase in metabolic activity of cells.
Combining powders of Laponite and PEO together, the most interesting gain is due to a concentration of 500 $\mu g/mL$ for both the materials. In spite of this, the concentrations that were used are lower than the real concentrations of Laponite and PEO inside the studied shear-thinning biomaterials, where for example the maximum concentration of Laponite is 66 $mg/mL$. Unfortunately, a so high concentration cannot be tested because the dispersion of the nanoclay inside the medium might be complicated.

**Live/Dead Assay**

The Live/Dead staining is a two-colour fluorescence assay used to discriminate live cells, in green, (thanks to the fluorescence of Calcein-AM) from dead cells (identified by the red fluorescence of the Ethidium Homodimer-1). This assay was performed with 2 repetitions. The first step was preparing a stock solution containing 99.75% DPBS, 0.2% of 2$mM$ EthD-1 stock solution and 0.05% of 4$mM$ Calcein-AM stock solution. Once removed the previous medium from the wells that have to be analysed, 50 $\mu L$ of Live/Dead solution were added to each well; then the well-plate was wrapped and incubated for 15 $min$. After that, the solution was removed and the

cells were left in 50 $\mu L$ of DPBS (for the complete protocol, check Appendix C). The outputs are read with a fluorescent microscope.

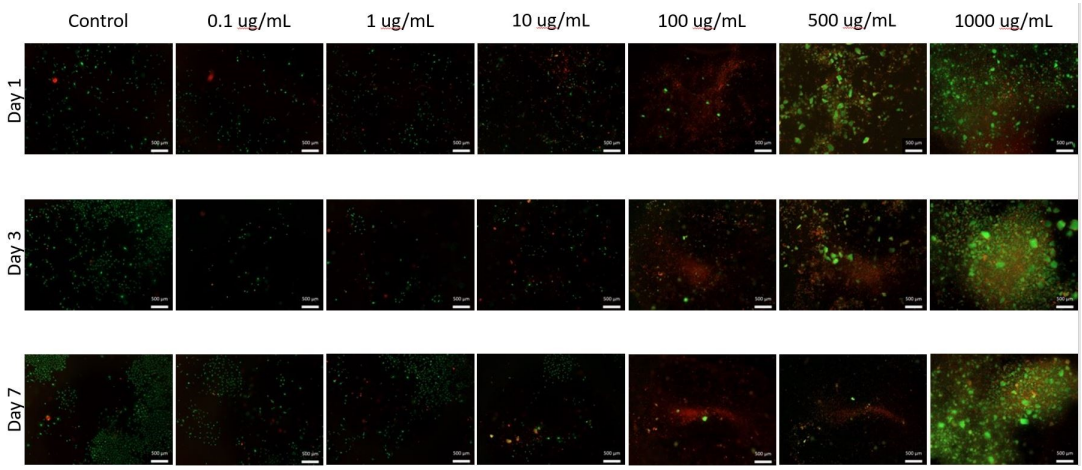


FIGURE 2.52: *Live/Dead Assay on 3T3 cells to evaluate the cytotoxicity of laponite particles. These are the most representative pictures of all the wells. Live/Dead staining stains live cells in green and dead cells in red. However, the Laponite particles reflect the fluorescent light, in particular the red light, and make impossible to identify properly the dead cells.*

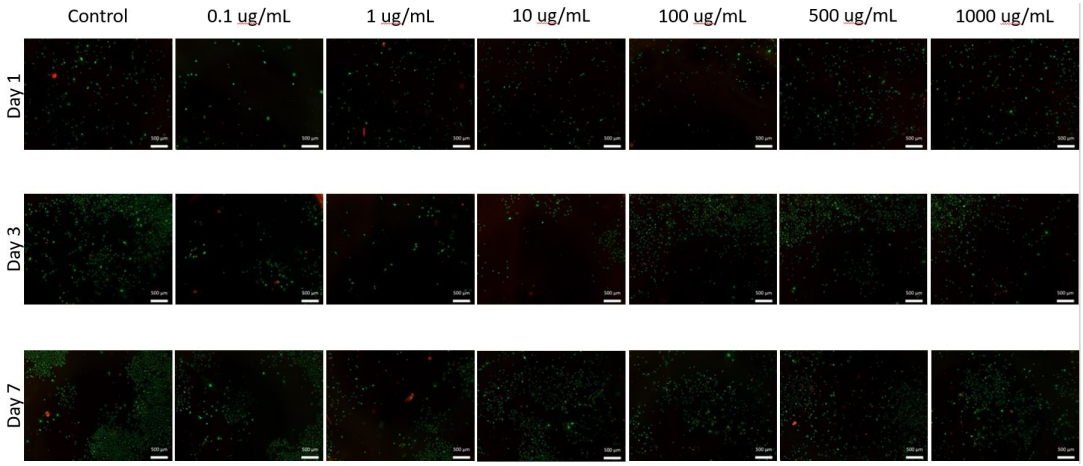


FIGURE 2.53: *Live/Dead Assay on 3T3 cells to evaluate the cytotoxicity of PEO putting directly the powder of the polymer inside the medium. These are the most representative pictures of all the wells. Unlike Laponite, the PEO gave us no problems of reflections and the live cells, in green, are easy to recognize from the red ones, which are dead cells.*

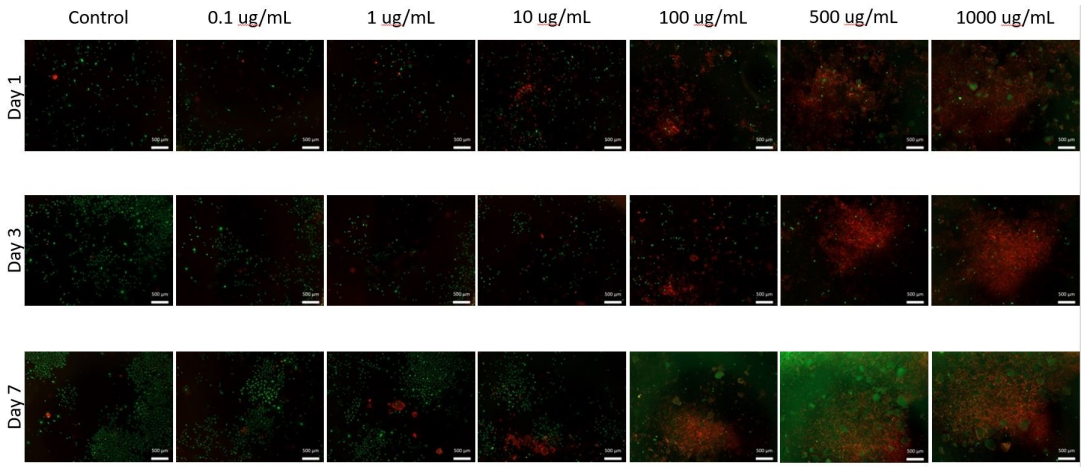


FIGURE 2.54: *Live/Dead Assay on 3T3 cells performed in order to assess the effect of Laponite and PEO together on cells viability. These are the most representative pictures of all the wells. Live/Dead staining stains live cells in green and dead cells in red. Laponite, used alone, gave us imaging troubles, and when it is mixed with PEO, the difficulty to distinguish dead from live cells remains.*

The Live/Dead Assay, commonly, is very sensitive as technique to discriminate live cells from dead cells. However, in our case, the Laponite particles reflect the fluorescent light, and this made impossible to identify in particular the dead cells. PEO gave us no trouble but, when it was mixed with Laponite, the higher the concentration, the harder it was to see cells.

### 2.2.12 Cells-Material Interaction

In our project, for the future utilization of the shear-thinning material in study it is important to understand how this gel can affect the behaviour of cells. To have a not permanent occlusion, the gel should not act like a fibrotic tissue promoting agent but at the same time it should not be systemically toxic. The aim of this experiment is to determine the effects of 6% Laponite, 6% Laponite 1% PEO and 6% Laponite 3% PEO on cells adhesion and spreading, to be sure that the material is not supporting tissue formation. To obtain this information bright-field imaging, Live/Dead staining and PrestoBlue analysis are used. These methods should present consistent information amongst each other to confirm the effects of the gels. It should be noted that the set of materials is further reduced: the reason for this is that the 6% Laponite 5% PEO was the less stable in PBS and mediocre in embolizing, so it cannot be considerate

a good candidate for tube occlusion. The first step includes the gel preparation on well-plates and the cell-seeding (check Appendix C for the full protocols). 0.1 $mL$ of each gel was added to its respective well in a 48-well plate and spread at the bottom. Cells were seeded on top of this layer at a seeding density of 10000 cells per well with a cell culture media volume of 300 $\mu L$ and their adhesion, spreading and metabolic rate were evaluated using the different techniques mentioned before. There is a difference in the protocol for cell seeding (or cell passaging) for this test because in this case there are a total of 60 wells that will have cells so it is better to calculate the cell suspension volume for 70 or 80 wells and this time the seeding density changes. So, before placing the samples inside the incubator, 300 $\mu L$ of cell suspension should be added to the wells that will have cells (there are gel controls without cells, to them we added 300 $\mu L$ without cells). The PrestoBlue protocol is different too: the volume of cell solution added to each well is 300 $\mu L$, and the same amount of pure media is also added at the end, before placing back the samples in the incubator. About the Live/Dead staining, this time, 100 $\mu L$ of Live/Dead stock solution were added to each well and, once removed the Live/Dead solution, 70 $\mu L$ of PBS should be added to each well (for other information, go to the Appendix C).

**Bright-field imaging**

For the bright-field imaging, 3 replicates per gel were done and 3 control replicates were added; imaging is done at 4, 8, 12, 24, 48 and 72 hours after incubation, for a total of 12 wells. Results are shown in Figure 2.55-2.56.

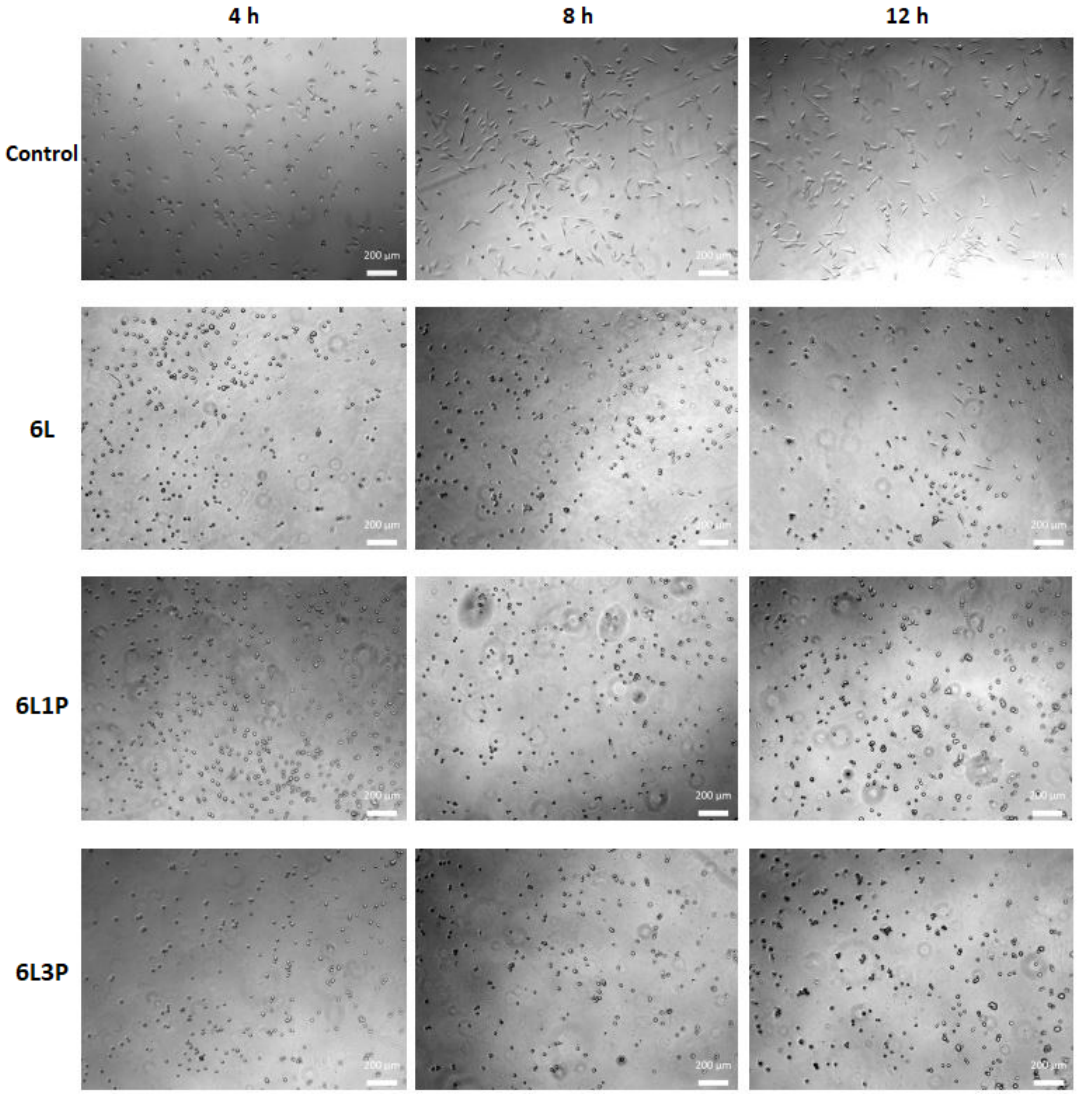


FIGURE 2.55: *Bright-field images showing cells-material interaction. The biological effects of three different gels (6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO) were tested. The control is only normal media. The time points are 4 h, 8 h, 12 h. A trend is visible: the higher the PEO content, the less cells adhere to the substrate.*

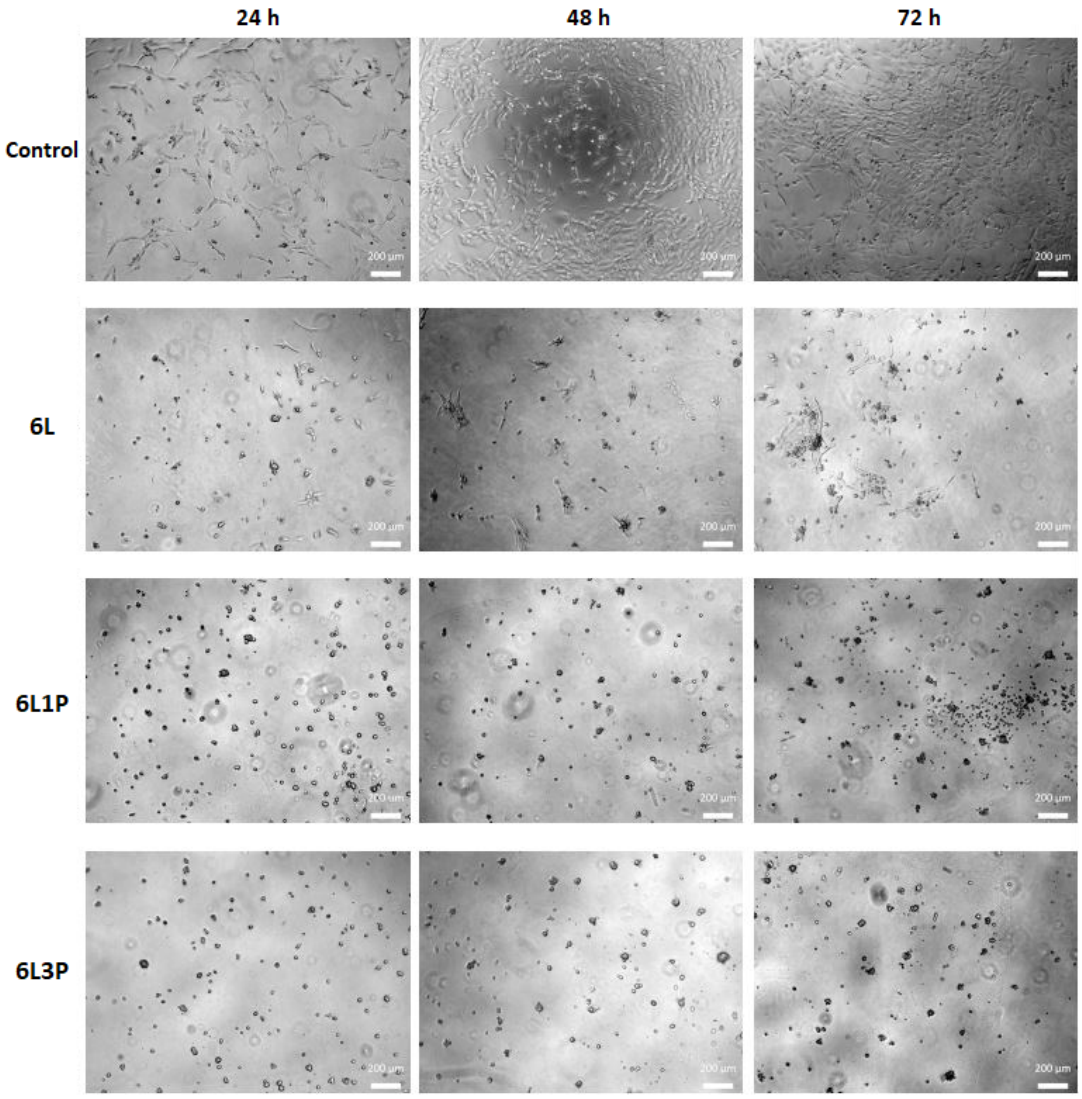


FIGURE 2.56: *Bright-field images showing cells-material interaction. The biological effects of three different gels (6% Laponite, 6% Laponite 1% PEO, 6% Laponite 3% PEO) were tested. The control is only normal media. The time points are 24 h, 48 h, 72 h. A trend is visible: the higher the PEO content, the less cells adhere to the substrate.*

Bright-field images showed that, after the control made by only media without gel, 6% Laponite is the material in which cells spread more easily. Cells seeded on gels composed of 6% Laponite and 1% or 3% PEO do not spread at all but they maintain a round shape and after 3 days, they are shrunk. Therefore, it can be said that a trend is visible: the higher the PEO content, the less cells adhere to the substrate, exactly as it was expected. The limit of this experiment is that, unfortunately, the not perfectly flat surface of the gel made the focus difficult.

**PrestoBlue Assay**

For the PrestoBlue, 4 replicates with cells per gel, 4 replicates without cells per gel and 4 control replicates on days 1 and 3 were done; we use the same wells plate for both days (28 wells in total). The obtained data are plotted in the following figure as metabolic rate versus composition and normalized rate (normalized with the control) versus composition, to better compare the materials.

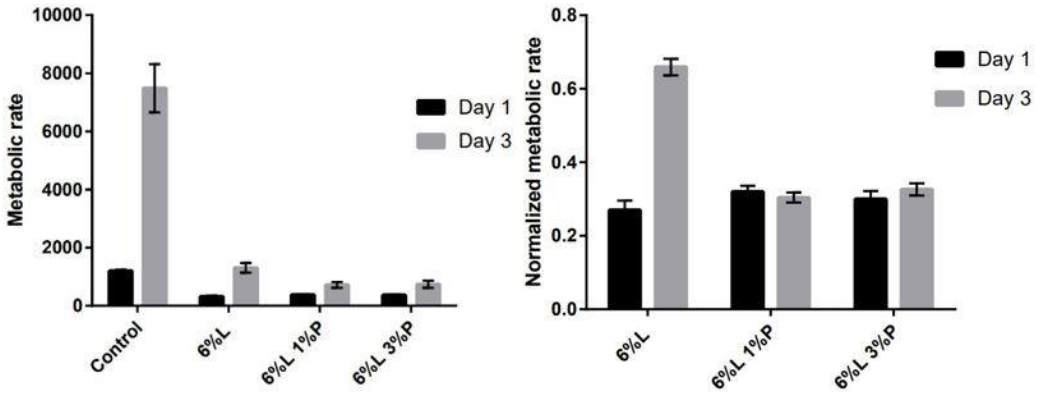


FIGURE 2.57: *Metabolic rate versus composition and normalized rate (normalized with the control) versus composition. The PrestoBlue analysis showed that cells on 6% Laponite are alive and have a higher metabolic rate than on the other 2 gels.*

The PrestoBlue data confirm that cells on 6% Laponite are alive and have a higher metabolic rate than on the other 2 gels. Even if the cells on 6% Laponite 1% PEO and 6% Laponite 3% PEO might be still alive they are not growing properly, they have a low metabolic rate.

**Live/Dead Assay**

In this case, 2 replicates per gel and 2 control replicates on days 1 and 3 were done; two different well plates per day were used.

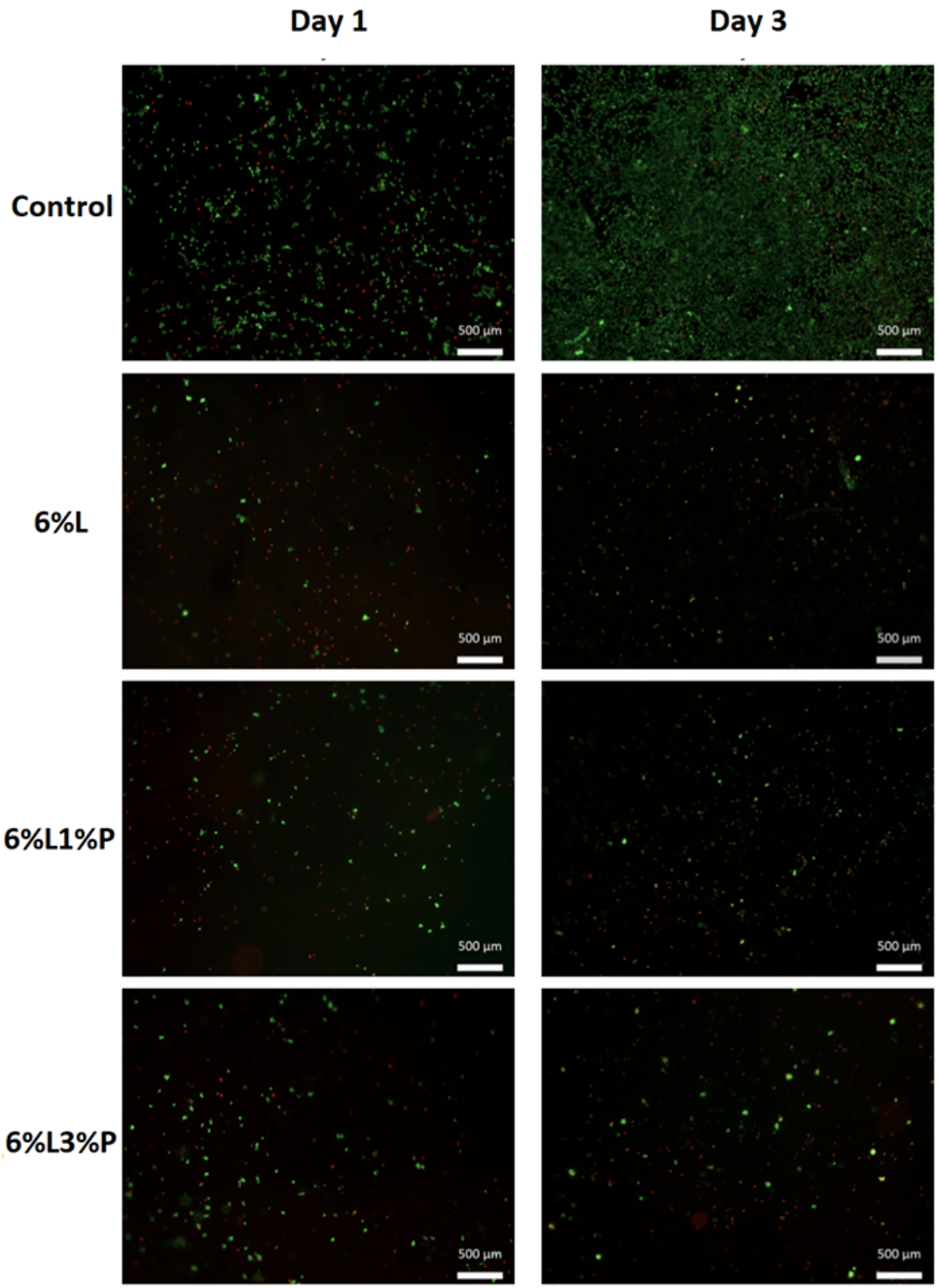


FIGURE 2.58: *Live/Dead Assay on 3T3 cells put on top of 3 gels: 6% Laponite, 6% Laponite 1% PEO and 6% Laponite 3% PEO. The layer of gel on the bottom creates a green shadow but it is possible to see that 6% Laponite has less living cells while 6% Laponite 1% PEO and 3% PEO gave almost the same results.*

With the Live/Dead Assay it is possible to see that even if the cells are not attached on the gels they are still alive. In these images, it is difficult to recognize the number of dead cells since the layer on the bottom creates a green shadow: anyway, 6% Laponite seems to have less living cells while 6% Laponite 1% PEO and 3% PEO gave almost the same results.

# Chapter 3

# Conclusions and Future Plans

In this work, different nanocomposites made of Laponite and Polyetilene oxide were synthetized for the application of Fallopian tube occlusion. Laponite was chosen because of its good mechanical properties, while the PEO was used for its cells antiadhesive properties. The result is a gel with shear-thinning properties thanks to which it behaves like a fluid when a shear stress is applied on it; as a matter of fact, when it is injected through a microcatheter, its viscosity decreases proportionally to the amplitude of the applied stress. This property makes the biomaterial extremely injectable and, at the same time, very resistant when the injection force ceases.

In the initial design of the experiment, to determine the optimal formulation of Laponite-PEO mixtures, material with 4% to 8% of nanoclay concentration and with 0% to 10% of polymer were prepared. Previous studies demonstrated that Laponite for concentrations lower than 3% doesn't form a gel; with the tube inversion test, we demonstrated that also 4%, Laponite with 0%, 5%, 10% of polymer is not immediately a gel. A trend is visible: the probability to have a gel increases with an increase of the Laponite concentration and it decreases with an increase of PEO concentration. Another preliminary experiment, injection by hand, showed that due to the high content of Laponite, starting from 6.5% Laponite 5% PEO toward higher nanoclay concentrations, the gel is hardly injectable. Thus, the range of interesting materials goes from 5% to 6% Laponite and from 0% to 3% of PEO.

According to our experimental plan, several tests were performed to have a complete characterization of the eight main materials in study. As described in the chapter 2, the accurate measurement of the injection force using Instron was also used to

evaluate the reproducibility of all the gels, since same materials have similar injection forces. The Instron step was considered a fundamental one after the material preparation, for the quality control. Regarding the injection force, with both catheter and needle, it can be noticed that the higher Laponite content, the less easy to inject, and same from low to higher PEO concentration, although the material is softer.

Our aim was to block sperm entering the oviduct so the main property in choosing our best material is occlusivity: *in vitro*, using different Tygon tubes to model the Fallopian ones, it was demonstrated that the higher the concentration of Laponite the higher the occlusion pressure that the material can resist, so the less the occlusion fails, whereas, among the same concentration of Laponite, by increasing the concentration of PEO, the occlusion pressure decreases. For all the tube's diameters and injected volumes, the same trend can be found, even when the medium changes.

Among the most promising materials, besides the good injectability of the 5% Laponite combinations, the 6% Laponite and 6% Laponite 1% PEO gave us a good performance beside a higher occlusivity. Subsequently, to have a more realistic *in vitro* model, the body temperature effect on the strength of the occlusion was evaluated on only one of our best materials: 6% Laponite 1% PEO. What we found out is that occlusion pressure doesn't change because of the temperature and is stable over time.

From the previous experiment was born the idea to see until which day the occlusion ability of 6% Laponite 1% PEO was the same. The experiment of aging for the occlusion pressure was performed until day 7 and the pressure remained constant.

Later, an *ex vivo* test using porcine Fallopian tubes was done but due to the huge size of the organs, (20-10 times bigger than the human ones) this experiment is considered an extreme case: we were forced to change some parameters so the results are not comparable with the *in vitro* ones and the obtained pressures were very small. Another future *ex vivo* step could be measuring the occlusivity on rabbit oviducts because they are smaller and less challenging.

To simulate the wet environment were the material should stay first PBS, because of its popularity in biology and its pH, and then HTF, a synthetic medium that mimics the composition of the oviduct fluid in human were used. Results showed a trend: the higher the PEO concentration, the less stable is the material over time.

The more stable material in HTF seems to be 6% Laponite 1% PEO.

As natural consequence of stability, swelling and degradation standard tests were performed. The results confirmed what was observed during the stability: the materials don't swell and don't degrade up to 6 weeks, so there is no possibility that an high degree of swelling encourages loss of compactness or disintegration in PBS or HTF, at physiological temperature.

However, in water, after 3 h, all the tested samples were completely dissolved, starting from those with lower content of Laponite. This is a very promising result because water could be used to reverse the gelification process so the temporary sterility, since it is very far as medium to simulate the fluid in the uterine tubes, unlike HTF and PBS. For the future, it could be interesting to occlude a transparent tube with the hydrogel, and to inject water with a catheter to wash away the material; the experiment could be video-recorded to better understand the dissolution rate and kinetics.

To understand the mechanical response a uniaxial compression to our set of materials was applied and then the Young's Modulus was calculated considering the linear region of the stress-strain curve between 0% and the 10% of the maximum strain measured. Among different PEO contents, the materials with lower percentage of PEO have a higher Young's Modulus: this means that, remaining in the elastic domain, the resistance of these samples to changes in length was higher.

For the application of the shear-thinning gel in study, it is important to be sticky, so that it can stay attached to the walls of the tube without moving. To quantify the adhesive strength, lap-shear tensile test was executed. Increasing the amount PEO is not convenient in term of adhesion: the failure load and the tensile strength are lower but, number says that a higher amount of Laponite is useless.
Very important from an industrial point of view, shelf-life experiment is crucial and our results are very interesting: materials can be kept at room temperature, outside the fridge for 14 days, without losing their properties. This means no costs for refrigeration and no need to prepare the material in the hospital were the equipment can be not proper. Moreover, the rheological tests showed the shear thinning behaviour independently from the formulation of the gel, and oscillation stress sweeps showed that 6% Laponite 3% PEO and 6% Laponite 5% PEO are softer materials but weaker

than the other gels. In order to simulate conditions closer to the real application in which the gel is inserted inside the tubal lumen and must not permanently deform because of muscle contractions, creep recovery tests and multiple stress creep recovery tests were then performed. At every cycle, there is an unrecovered strain but our suggestion is to repeat them at $37°C$ to mimic the real situation.

The last part of the thesis described results concerning cytotoxicity. 3T3 cells are used to evaluate the effect of Laponite, PEO, and both together on cells growth and proliferation, adding them in the culture media, analysing them with a PrestoBlue assay in combination with a Live/Dead assay. The experiment needs to be continued until day 7 to have a complete information of cells behaviour. After 3 days Laponite at higher concentration looks toxic; for PEO there is an increase of metabolic activity over time. In spite of this, the concentrations that were used are lower than the real concentrations of Laponite and PEO inside the studied shear-thinning biomaterials, where for example the maximum concentration of Laponite is 66 mg/mL. Unfortunately, a so high concentration cannot be tested because the dispersion of the nanoclay inside the medium might be complicated.

The last experiment with cells evaluated how the gels can affect the behaviour of cells. To have a not permanent occlusion, the gel should not act like a fibrotic tissue promoting agent but at the same time it should not be systemically toxic. A trend is visible: the higher the PEO content, the less cells adhere to the substrate, exactly as it was expected. The 6% Laponite 1% PEO is our best material.

Since the optimization can be considered done, it is crucial for our project proceeding with in vivo studies, so an evaluation of the quality of the material prepared using ULTRAVIST, an x-ray contrast, was done during the last week. This test will allow us to understand if there is an ingrowth of tissue or not and perform histological experiments on animal tissues.

In perfect accordance with the aim of the project that includes creating a biomaterial able to adhere at the wet walls of the oviduct, a conjugation with DOPA was evaluated. Some of the promising preliminary results are shown in Figure 3.1; anyway, they can't be completely trusted because of some issues in the preparation. The experiment should be repeated.

A possible problem can be if the material is sperm-permeable: this will lead to

have ectopic pregnancies since the fertilization could happen but the implantation no. This is a reason why it is important to study the interaction sperm-material.

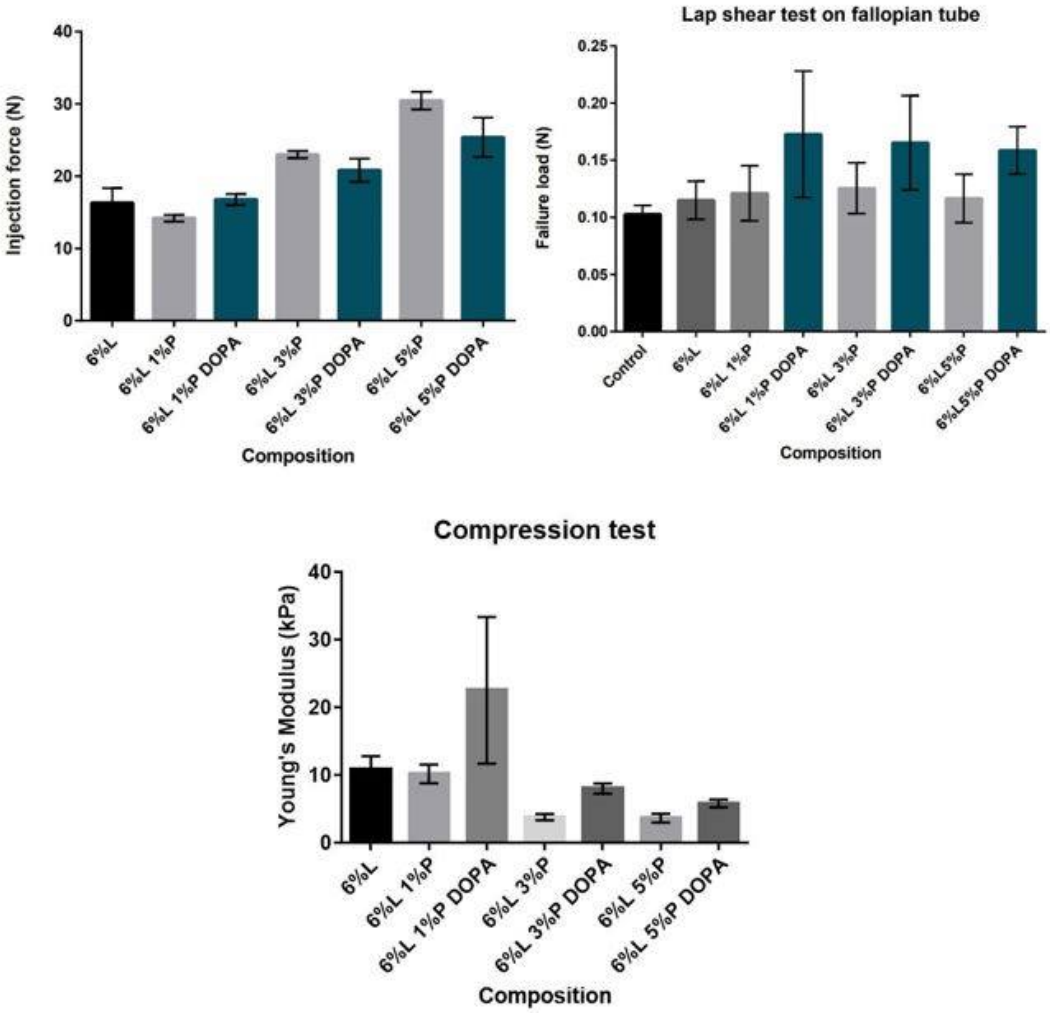


FIGURE 3.1: *DOPA conjugation, preliminary data. Four of the gels were conjugated with DOPA to enhance the biological adhesiveness. Then, injection force measurements, lap shear tests and compression tests were performed: the adhesive strength test showed, as expected, a better adhesiveness, with an improvement of the 30%, the Young's Modulus calculation showed an improvement also in stiffness while the material became more easily injectable. Anyway, the data can't be completely trusted because the of some issues in the preparation.*

# Appendix A

# Materials and Compound

## A.1 Laponite XLG-XR

Laponite XLG-XR), BYK Additives Inc, Gonzales TX. More details on www.byk.com, info@addcompnorthamerica.com reorder from Eckart America 440-954-7600.

## A.2 PEO

Poly(ethylene glycol), Mw: 20,000, Sigma Aldrich. More details on www.sigmaaldrich.com, Product ref. no. 24887748.

## A.3 HTF

HTF (Human Tubal Fluid) no BSA/ PVA, Cytospring, In vitro Fertilization Media HTF More details on www.cytospring.com, Product ref. no. H0004.

## A.4 Milli-Q water

Ultrapure Milli-Q water.

## A.5 FBS

FBS (Fetal Bovine Serum) gibco by life technologies, Thermo Fisher Scientific More details on www.thermofisher.com, Product ref. no. 10437028.

## A.6 DPBS

DPBS (Dulbecco's Phosphate Buffered Saline) gibco by life technologies, Thermo Fisher Scientific More details on www.thermofisher.com, Product ref. no. 14190250.

## A.7 DMEM

DMEM (Dulbecco's Modified Eagle Medium) gibco by life technologies, Thermo Fisher Scientific More details on www.thermofisher.com, Product ref. no. 11965092.

## A.8 Pen-Strep

Penicillin-Streptomycin-Glutamine (100X) More details on www.thermofisher.com, Product ref. no. 10378016.

## A.9 Trypsin

Trypsin (2.5%, 10X), no phenol red. More details on www.thermofisher.com, Product ref. no. 15090046.

## A.10 PrestoBlue®

PrestoBlue®Cell Viability Reagent, Thermo Fisher Scientific More details on www.thermofisher.com, Product ref. no. A13261.

## A.11 LIVE/DEAD™

LIVE/DEAD™Viability/Cytotoxicity Kit for mammalian cells, Thermo Fisher Scientific More details on www.thermofisher.com, Product ref. no. L3224.

# Appendix B

# Instrumentation

## B.1 Centrifuge Tubes

50mL Polypropylene Conical Centrifuge Tubes (sterile). Length 115mm, Cap Diameter 33mm, FALCON™15mL Polypropylene Conical Centrifuge Tubes (sterile). Length 120mm, Cap Diameter 20mm, FALCON™More details on https://www.fishersci.com

## B.2 Catheter

AngioDynamics, Soft-Vu™Angiographic Catheters, 5F X 65 cm More details on http://www.angiodynamics.com, catalog no. 10709001.

## B.3 Well Plates

Multiwell Tissue Polystirene Culture Plate,12 Well Polystyrene, FALCON™More details on https://www.fishersci.com

## B.4 Needles

BD PrecisionGlide Needle™: Single-use Needles: Regular Bevel - Regular Wall.

| Color | Needle Gauge | Material | Dimensions | Catalogue N. |
|---|---|---|---|---|
| Pink | 16G x 1 | Stainless Steel | $1.6mmx25mm$ | 305197 |
| Pink | 18G x 1 1/2 | Stainless Steel | $1.2mmx40mm$ | 305196 |
| Turquoise | 23G x 1 IM TW | Stainless Steel | $0.6mmx25mm$ | 305145 |
| Turquoise | 23G x 1 1/2 | Stainless Steel | $0.6mmx40mm$ | 305120 |
| Blue | 25G x 1 1/2 | Stainless Steel | $0.5mmx40mm$ | 305197 |

More details on www.fishersci.ca.

## B.5 Tubes

| Inner Diameter | Inner Diameter | Outer Diameter | Product name | Material | Company | Cat. no. |
|---|---|---|---|---|---|---|
| 1 mm | 0.040" ID | 0.070"OD | Tygon Microbore tubing,100 ft/roll | Tygon | Cole-Parmer | EW-06419-04 |
| 1.8 mm | 1/16″ ID | 3/16" OD | 1/16" Wall Tygon® 2375 Ultra Chemical Resistant Tubing | Tygon 2375 | United States Plastic Corps | 57545 |
| 3 mm | 1/8" ID | 1/4" OD | 1/16" Wall Tygon® 2375 Ultra Chemical Resistant Tubing | Tygon 2375 | United States Plastic Corps | 57547 |
| 4 mm | 1/8" ID | 1/4" OD | 1/16" Wall Tygon® 2375 Ultra Chemical Resistant Tubing | Tygon 2375 | United States Plastic Corps | 57549 |
| 10 mm | 3/8" ID | 1/2" OD | 1/16" Wall Tygon® 2375 Ultra Chemical Resistant Tubing | Tygon 2375 | United States Plastic Corps | 57552 |

## B.6 Syringes

BD disposable sterile syringes. With BD Luer-LokTM tip. Total volume of 1mL, 3mL, 5mL, 10mL and 60mL. More details on www.bd.com, model ref. no. 309628,

309657, 309646, 309604 and 309653 respectively.

## B.7 Vials

Low Background Glass Scintillation Vials, 20ml Capacity, 22mm Cap Size, Polypropylene Unattached Caps, Metal Foil Liners, RPI RESEARCH PRODUCTS INTERNATIONAl. More details on www.rpicorp.com, model ref. no. 121000.

## B.8 Petri Dishes

Plastic Petri Dishes 35 x 10mm, 60 x 15mm, 100 x 15mm Style Polystyrene, FALCON™. More details on www.fishersci.com, catalog no. 351007.

## B.9 Glass Slides

VWR®Micro Slides 2in x 3in, 1.2 mm thick. More details on www.us.vwr.com.

## B.10 Parafilm®

BRAND®PARAFILM®sealing film. More details on www.sigmaaldrich.com, prod ref. no. 701605.

## B.11 Well Plate Reader

BioTek Synergy MX™. More details on www.biotek.com.

## B.12 Heating Plate

Heating plate, CORNING PC-420D.

## B.13 Freeze Dryer

Freeze dryer, Labconco Freeze dry system freezone 4.5.

## B.14 Vortex

Fisher Vortex Genie 2, Thermo Fisher Scientific.

## B.15 Rheometer

Rheometer AR-G2, TA Instruments, New Castle, DE, USA.

## B.16 Instron®

Instron®5943 (Instron, Norwood, MA, USA).

## B.17 Laser Cutter

Versalazer, Universal Laser Systems.

## B.18 Caliper

CD-4 CSX ABSOLUTE Digimatic Caliper Series 500, Mitutoyo Corp.

## B.19 Microscope

Zeiss Axio observer D1, Zeiss, Thornwood, NY, USA.

## B.20 Super Glue

Scotch Super Glue Gel.

## B.21 Incubator

Incubator 37 °C, Thermo Scientific Precision 6LM Incubator Mechanical Convection. More information on www.fishersci.com.

## B.22 Syringe Pump

Harvard PHD 22/70-2000 Advance Syringe Pump.

| | |
|---|---|
| # of syringes | 2 |
| Accuracy | $\pm 0.35\%$ |
| Average Linear Force | $23kg$ |
| Depth | $27.9cm$ |
| Width | $22.8cm$ |
| Height | $15.9cm$ |
| Maximum Flow Rate | $220mm/min$ |
| Minimum Flow Rate | $0.00001\mu L/h$ |
| Syringe Minimum Size | $140mL$ |
| Syringe Maximum Size | $0.5\mu Lcm$ |

More details on `www.harvardapparatus.com`, model ref. no. 70-2000.

## B.23 Pressure Sensor

PASCO Dual Pressure Sensor. More informations on www.pasco.com.

## B.24 USB link

USB link, PASCO.

## B.25 3-way Connector

Discofix 3-way Stopcock blue, B. Braun. More informations on www.bbraun.com.

## B.26 Oven 80°C

VWR/ ShelLab Model 1370 GM Gravity Convection Oven.

## B.27 Eppendorf tubes ®

Eppendorf®Safe-Lock microcentrifuge tubes, natural, volume 1.5 and 2.0 mL. More details on www.sigmaaldrich.com.

## B.28 Mini Centrifuge

Fisher Scientific 05-090-100 Microcentrifuge. Details on www.fishersci.com.

# Appendix C

# Solution Preparations and Protocols

## C.1 Preparation of 15% Laponite Gel

For 10 $mL$ of solution:

1. put 10 $mL$ of Milli-Q water on ice for at least 10 $min$ until it is cold;
2. weigh 1.76 $g$ of Laponite and add it to the cold Milli-Q water;
3. vortex the solution immediately for at least 10 $min$. It is important to use a vortex speed of 3200 $RPM$.

## C.2 Preparation of 25% PEO Solution

For 7.5 $mL$ of solution:

1. weigh 2.5 $g$ of PEO and add it into 7.5 $g$ of Milli-Q water;
2. vortex the solution for at least 5 $min$, until it is dissolved. The solution will be white, but no bulks will be visible;
3. put the solution into the 80°$C$ oven for 10 $min$ until it becomes transparent.

## C.3 Preparation of Laponite-PEO Mixtures

1. Weigh the desired amount of PEO, Laponite and Milli-Q water in this order, according to the wanted concentration (the table below shows two examples),

into a 50 $mL$ Falcon tube and vortex the mixture for 15 $min$. Sometimes the Laponite gel sticks to the wall of the tube; if it happens, shake vigorously the Falcon tube to take this material in the bottom.

2. Put the resulting material for 20 $min$ into the 80°$C$ oven.

3. Centrifuge the material for 5 $min$ at 3200 $RPM$, then use a spatula to mix the material inside the tube. Repeat this procedure for three times to have a completely bubble-free material.

4. Keep the material at room temperature for at least 4 $h$ until further usage; if the material is used too early, the material properties might deviate from the reported ones.

TABLE C.1: **Two examples of specific materials composition.**

| | **PEO stock solution (g)** | **Laponite stock solution (g)** | **Water (mL)** |
|---|---|---|---|
| **6% Laponite 1% PEO** | 0.48 | 4.8 | 6.72 |
| **6% Laponite 3% PEO** | 1.44 | 4.8 | 5.76 |

## C.4 Injection Force Measurement

1. Transfer the material into a 3 $mL$ syringe (BD 3 $mL$ Syringe Luer-LokTM Tip) using a spatula.

2. Insert a cap to seal the tip of the syringe and place the syringe inside a 50 $mL$ Falcon tube. Centrifuge at 3000 $RPM$ for 5 $min$ to clear bubbles. Air bubbles may generate a noisy curve.

3. Remove the cap and squeeze some of the material until the 3 $mL$ syringe is filled up to the 2.5 $mL$ mark. Then connect the syringe to the 4$F$ catheter

(0.035" inner diameter) [Angio Dynamics Inc.] or to the 18*G* needle, [BD PrecisionGlide Needle] (pink needle).

4. Use the mechanical testing machine, INSTRON® 5943, with a relevant sensor that is compatible with 100 *N* force.

5. Make 3 or more measurements with the same material to ensure the results are reproducible. Between each measurement, wash and clean thoroughly the catheter.

## C.5 Occlusion Pressure Measurement

1. Cut the required tube to 10 cm length.

2. Fill the tube until the desired length (15 mm or 30 mm) by using the 3 ml syringe with a needle and if the measurement is in water or PBS, fill the rest of the tube with water or PBS.

   - To fill a 1 mm tube was used a 25G needle.
   - To fill a 1.8 mm tube was used a 23G needle (0.6 mm x 40 mm).
   - To fill a 3 mm tube was used a 18G needle.
   - To fill a 4 mm tube was used a 18G needle.
   - For the tube with a diameter of 10 mm was used a 18G needle.

3. Link the tube to a needle connected to the 3-way connector. Put Parafilm all around.

   - To connect the 1 mm tube a 23G needle was used (0.6 mm x 25 mm).
   - To connect the 1.8 mm tube a 16G needle was used.
   - The 3 mm tube was directly connected to the 4-way connector.
   - The 4 mm tube was directly connected to the 4-way connector.
   - The 10 mm tube was connected with an adaptor created by cutting an Eppendorf tube of 1.5 ml and gluing it to the tube with a Scotch Super Glue Gel.

4. Connect the 3-way connector to the 60 ml syringe, filled with air, water or PBS.
5. Connect the third entrance to the PASCO sensor and this one to the computer with the USB link.
6. Open the software Pasco Capstone.
7. Set up 1 ml/min as flow rate and 31 mm as diameter in the syringe pump.
8. Start the syringe pump and observe the measured pressure; whenever the pressure shows a peak and then a decrease in slope is because the material is not occluding anymore.

### C.5.1 Occlusion Pressure Measurement in Fallopian Tube

**NOTE**: try to keep the Fallopian tube hydrate and cold using ice, to prevent the tissue to go waste.

1. Check visually the entirety of the organ.
2. Put the catheter [5F, Angio Dynamics Inc] inside the whole organ, so passing through cervix, reach the closest part of one Fallopian tube, the intramural section.
3. Connect the catheter to a syringe [10 $mL$, BD Luer-Lok Tip, Becton, Dickinson and Company] and insufflate air with it inside the organ.
4. Go deeper inside the uterine tube and repeat step 3 until the end of the organ. This procedure is done to be sure that there are no holes or anatomical problems in the tissue. If yes, it is suggested to use another tube.
5. Repeat from step 2 for the other Fallopian tube (the salpinges are paired organs).
6. Cut the initial part of the Fallopian tube containing the intramural section in a piece of 10 $cm$ like in Figure C.1.
7. Measure the inner diameter using different tubes with a known outer diameter, so as to force the animal tissue to maintain a cylindrical aspect, during the measurement.

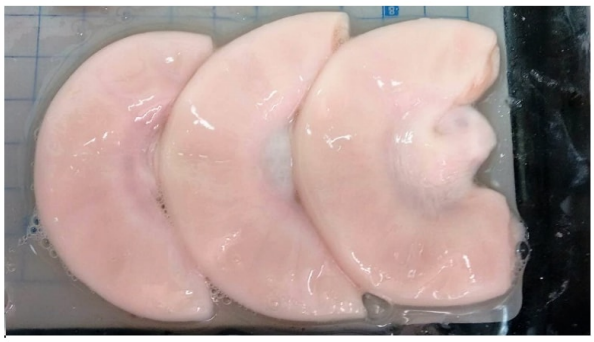

FIGURE C.1: *10 cm pieces of Fallopian tube cut from the whole organ.*

8. Fill the uterine tube with the desired volume of material by using the 3 $mL$ syringe and if the measurement is in water or PBS, fill the rest of the tube with water or PBS.

9. Create a connector taking off the plunger from a 10 $mL$ syringe [BD Luer-Lok Tip, Becton, Dickinson and Company] and cutting the part closer to it, reducing the length of the syringe of 1 $cm$. Then connect it to a soft and flexible Tygon tube [1/16" ID $\times$ 3/16" OD $\times$ 1/16" Wall Tygon® E-1000 Tubing]. Use Parafilm to seal them together.

10. Connect the tube end of the created connector with the 3-way connector [Discofix 3-way Stopcock blue, B. Braun]. Put Parafilm all around.

11. Connect the 3-way connector to the 60 $mL$ [BD Luer-Lok Tip, Becton, Dickinson and Company] syringe, filled with air, water or PBS.

12. Connect the third entrance to the PASCO sensor [Wireless Pressure Sensor, PASCO] and this one to the computer with the USB link [USB Pasco linker].

13. Open the software Pasco Capstone.

14. Set up 20 $mL/min$ as flow rate and 31 $mm$ as diameter in the syringe pump [Syringe pump, Harvard apparatus PHD 2000 Infuse/Withdraw].

15. If water or PBS are used, fill the homemade connector too with them, turning on the syringe pump, lifting the free larger end of the connector and waiting until the it is completely full.

16. Connect the Fallopian tube with the larger end of the created connector and keep it close as much as possible to not let the medium go back outside.

17. Start the syringe pump and observe the measured pressure; whenever the pressure shows a peak and then a decrease in slope is because the material is not occluding anymore.

## C.6 Stability

1. Transfer the material into a 5 ml syringe [BD Syringe Luer-LokTM Tip] using a spatula.

2. Weigh an empty Petri dish (35 x 10 mm).

3. To have the PMMA mold, cut with the laser cutter a sheet of PMMA (with thickness of 3.2 mm), making two concentric circles so that the final mold has an outer diameter of 3 cm and an inner diameter of 1.5 cm.

4. Place the mold in the small Petri dish.

5. Inject the material in the center of the mold and use the spatula to flatten the material surface. Then, with a blade, take off the material in excess.

6. To get the mold out of the dish, turn upside down for few seconds the dish containing the material and the PMMA mold. The mold is heavier than the gel, so it falls down, disparting from the material.

7. Weigh the dish with the material inside.

8. Put 4 ml of medium into the dish, around the cylinder of material.

9. To prevent drying and evaporation, seal the dish with Parafilm and store it at $37°C$ in between the time points.

10. At every time point:

    - the medium should be carefully removed, without touching or sucking the material;
    - weigh the dish;

- put new 4 ml of medium inside the dish, seal it and place it back at 37°C.

The used timepoints to observe the changes in weight are different depending on the medium. Repeat the procedure at least three times for each material.

## C.7 Swelling test

1. Transfer the material into a 5 ml syringe [BD Syringe Luer-LokTM Tip] using a spatula.
2. Weigh a 2 ml Eppendorf tube.
3. Deploy 1 g of material into the tube.
4. Weigh the Eppendorf tube with the material inside.
5. Centrifuge the Eppendorf in a mini-centrifuge for 10 s.
6. Put gently 1 ml on top of the gel using a 1 ml pipette, and close the tube.
7. Store the Eppendorf at 37°C in between the timepoints.
8. At every time point
    - the PBS or the HTF should be carefully removed, without touching or sucking the material
    - weigh the Eppendorf
    - put 1 ml of PBS or HTF on the material, close it and place it back at 37°C.

Repeat the procedure at least three times for each material.

## C.8 Degradation test in PBS

### C.8.1 Short-Term Degradation in PBS

1. Transfer the material into a 5 ml syringe [BD Syringe Luer-LokTM Tip] using a spatula.
2. Weigh a 1.5 ml Eppendorf tube.

3. Deploy 0.3 g of material into the tube.
4. Weigh the Eppendorf tube with the material inside.
5. Centrifuge the Eppendorf in a mini-centrifuge for 10 s.
6. Put gently 0.5 ml on top of the gel using a 1 ml pipette, and close the tube, except for the sample used for the 0 h time point that must be frozen at -80°C directly, without going in contact with PBS.
7. Store the Eppendorf at 37°C in between the timepoint.
8. At every time point
    - the PBS should be carefully removed, without touching or sucking the material
    - put the sample in -80°Cfridge -
    - freeze dry the sample -
    - weigh the Eppendorf.

The used timepoints to observe the changes in weight are: 0 h, 1 h, 2 h, 4 h, 8 h. Repeat the procedure at least three times for each material.

### C.8.2 Long-Term Degradation in PBS

Transfer the material into a 5 ml syringe [BD Syringe Luer-LokTM Tip] using a spatula.

Weigh a 2 ml Eppendorf tube.

Deploy 0.5 g of material into the tube.

Weigh the Eppendorf tube with the material inside.

Centrifuge the Eppendorf in a mini-centrifuge for 10 s.

Put gently 1 ml of PBS on top of the gel using a 1 ml pipette, and close the tube, except for the samples used for the 0 h time point that must be frozen at -80°C directly, without going in contact with PBS.

Store the Eppendorf in incubator at 37°C until the timepoint.

At every time point

- the PBS should be carefully removed, without touching or sucking the material
- put the sample in $-80°C$ fridge
- freeze dry the sample
- weigh the Eppendorf.

The used timepoints to observe the changes in weight are: 0 h, 1 day, 7 days, 14 days, 28 days. For each material at each timepoint will be prepared at least 3 samples.

## C.9 Compression Test

1. Transfer the material into a 5 $mL$ syringe (BD 5 $mL$ Syringe Luer-LokTM Tip) using a spatula.
2. To have the PMMA mold, cut with the laser cutter a sheet of PMMA (with thickness of 3.2 $mm$), making two concentric circles so that the final mold has an outer diameter of 3 $cm$ and an inner diameter of 1.5 $cm$.
3. Place the mold in an empty Petri dish ($100 \times 15$ $mm$).
4. Inject the material in the center of the mold and use the spatula to flatten the material surface. Then, with a blade, take off the material in excess.
5. To get the mold out of the dish, turn upside down for few seconds the dish containing the material and the PMMA mold. The mold is heavier than the gel, so it falls down, disparting from the material.
6. Use the mechanical testing machine INSTRON 5943 placing the sample between two plates and with the relevant sensor that is compatible with 100 $N$ force.
7. The test starts when the upper plate touches the surface of the sample and the machine measures a load of 0.01 $N$.

8. Make 3 or more measurements with the same material to ensure the results are reproducible. The raw data should resemble the measurements shown in Figure C.2.

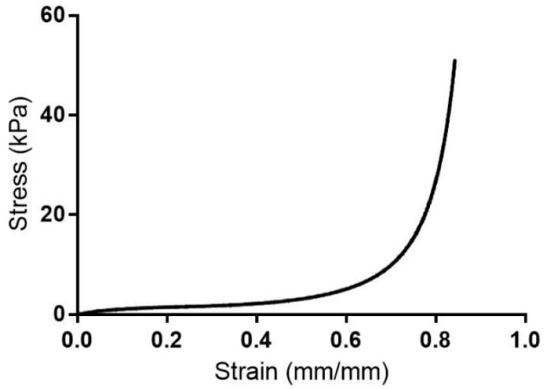


FIGURE C.2: *Example of stress vs strain curve.*

## C.10 Lap-Shear Adhesive Strength Test On Porcine Skin/Fallopian Tube

1. Transfer the material into a 3 $mL$ syringe [BD Syringe Luer-LokTM Tip] using a spatula.
2. Inject the material into a 1 $mL$ syringe [BD Syringe Luer-LokTM Tip].
3. Cut with a single edge blade two 3 $cm \times 1$ $cm$ strips of porcine skin or Fallopian tube.
4. Cut a microscope slide [VWR Micro Slides, 25 $mm \times 75$ $mm$] in the middle with a glass cutter.
5. Attach each strip in the rough part of the microscope slide.
6. Inject 0.01 $mL$ of material in an area of 1 $cm \times 1$ $cm$ of one of the two strips and press them together.
7. Use a clamp to increase the contact force for 3 $min$.

8. Put tape in the free edge of each slide's piece.

9. Proceed with the mechanical tester INSTRON 5943. The chosen speed is 20 $mm/min$.

10. Make 3 or more measurements with the same material.

11. Clean the overlapped surfaces to prepare the tissue for a new measurement, paying attention to keep the biological sample humid.

## C.11 Cell Passaging

**NOTE**: make sure to have sterile manipulation, all the times, during this procedure. Always spray hands and equipment with 70% ethanol before using them inside the hood.

1. Take $1\times$ trypsin and culture media (89% DMEM, 10% FBS, 1% Pen/Strep) out of the freezer and place both solutions in the water bath at $37^{\circ}C$ because cold solutions can have negative effects on cells.

2. Take T75 flask out of the incubator and check under the microscope whether the cells are fine.

3. Remove media from the flask using a serological pipette. Never touch the tip of the pipette or the flask, opening it, as it can lead to contamination.

4. Wash the flask with 6 $mL$ of DPBS by slowly making infinity signs.

5. Remove the DPBS by using a serological pipette.

6. Add 2 $mL$ of trypsin inside the flask.

7. Put the flask inside the incubator for 2 $min$.

8. Neutralize the trypsin by adding double the amount of trypsin used in media to the flask (4 $mL$).

9. Wash the bottom of the flask by gently pipetting part of the solution and rise it over the bottom of the flask.

10. Transfer the solution in the flask to a 15 $mL$ Falcon tube using a 10 $mL$ pipette.
11. Centrifuge the solution at 1200 $RPM$ for 5 $min$ to create a cell pellet.
12. Remove the media from the 15 $mL$ Falcon tube to retrieve the cell pellet.
13. Add at least 1 $mL$ of fresh medium to the cell pellet. If the flask was extremely confluent more medium might be required in order to be able to count the cells. Mix well by pipetting the pellet gently up and down until a well suspended solution is made.
14. Count the cells (cell counter).
15. To determine the volume of the cell suspension needed for a certain seeding density, the following equation is used:

$$(CellCountingAverage \times 10^4)/1\ mL = SeedingDensity/X$$

where $X$ is the seeding volume for 1 well. A cell suspension for all the wells has to be made. The total volume added to each well is 200 $\mu L$. For a 96 well-plate, cell suspension for 110 wells should be prepared to avoid eventual depletion of media due to small pipetting errors.

16. Incubate cells for 24 $h$.

*Protocol transcription, thanks to Melvin Gurian and Mariana Garcia-Corral.*

## C.12 Stock Solution Preparation for PrestoBlue

1. Put 10 $mg$ of Laponite, PEO and 10 $mg$ of both Laponite and PEO in 3 separate 15 $mL$ Falcon tubes.
2. Sterilize the 3 tubes under UV-light for 10 $min$ at 600 $mW$, 10 $cm$ away from the UV light source.
3. Add 10 $mL$ of cell culture medium to each tube (1 $mg/mL$).
4. Vortex the solutions for 30 $s$.

5. Use the stock solution to create a serial dilution by using 10% $v/v$ ratio of the previous higher concentration for the next lower one. Starting from the stock solution, this will give the concentration ranging from 1000, 100, 10, 1, 0.1 $\mu g/mL$ of particles. For the 500 $\mu g/mL$ use equal parts of medium and the particle stock solution. **NOTE**: before using any particle solution for any step in the experiment, resuspend the solutions thoroughly and work fast with the solutions, as the particles tend to precipitate extremely fast. This is especially important for the high concentration solutions.

*Protocol transcription, thanks to Melvin Gurian and Mariana Garcia-Corral.*

## C.13 PrestoBlue Assay

1. Place culture media in the water bath.
2. Prepare a PrestoBlue solution (10% PrestoBlue and 90% media) in a 15 $mL$ or 50 $mL$ Falcon tube, based on the needed amount. Each well will be filled with 200 $\mu L$ PrestoBlue medium per well. Prepare again a stock solution for at least 10 wells more. **NOTE**: the Falcon tube has to be wrapped in aluminium foil to minimize the exposure to light.
3. Remove medium from the wells.
4. Put 200 $\mu L$ of PrestoBlue medium in each well and wrap the wells plate in aluminium foil afterwards. Work in a dark environment to do all these steps.
5. Incubate the well plate for 90 minutes.
6. Transfer the supernatant, in 50 $\mu L$ steps, to three clean 96 well-plates, so the cells are still covered in 50 $\mu L$ of solution and each well has three PrestoBlue replicates.
7. Wrap new well-plates in aluminium foil.
8. Remove the remaining medium and add 200 $\mu L$ of the media with particles to the respective wells (6 wells per condition).
9. Read out the data with a plate reader.

*Protocol transcription, thanks to Melvin Gurian and Mariana Garcia-Corral.*

## C.14 Live/Dead Assay

1. Take the Live/Dead reagents from the freezer and let them to warm up to room temperature.
2. Prepare a stock solution containing 99.75% DPBS, 0.2% of 2 $mM$ EthD-1 stock solution and 0.05% of 4 $mM$ Calcein-AM stock solution.
3. Remove medium from the wells that should be analysed.
4. Add 50 $\mu L$ of the Life/Dead solution to each well.
5. Wrap the well-plate in aluminium foil and incubate for 15 $min$.
6. Following incubation, remove the stain and leave the cells in 50 $\mu L$ of DPBS.
7. View labelled cells under a fluorescence microscope.

*Protocol transcription, thanks to Melvin Gurian and Mariana Garcia-Corral.*

## C.15 Gel Spreading on Well Plate

1. Take the materials (6% Laponite, 6% Laponite 1% PEO and 6% Laponite 3% PEO) and use a spatula to transfer each of them into a 5 mL syringe [BD Luer-Lok Tip, Becton, Dickinson and Company].
2. With the 5 mL syringe fill two 1 mL syringes [BD Luer-Lok Tip, Becton, Dickinson and Company] per material.
3. Add 0.1 mL of each gel to its respective well in a 48-well plate.
4. Centrifuge well plates for 20 min at 3500 RPM.
5. Place the open well plates inside the cell culture hood and use the hoods UV for 10 min to sterilize the gels.

NOTE: it is important to use sterile manipulation all the times during this procedure. Always spray hands and equipment with ethanol before entering the hood.

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