UNIVERSITA' DI PADOVA  FACOLTA' DI INGEGNERIA

TESI DI LAUREA

A NEW DEVICE FOR VISCOSUPPLEMENTATION TREATMENT: DEVELOPMENT OF MANUFACTURING AND CONTROL PROCESSES

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ITALIAN ABSTRACT

Il presente lavoro di tesi è frutto dell'attività di stage svolta presso il dipartimento R&D di Lima Corporate s.p.a. di Villanova di San Daniele del Friuli (UD). Il programma di tesi si inserisce all'interno di un progetto di ricerca e sviluppo finalizzato alla realizzazione di un dispositivo per la viscosupplementazione basato su di un nuovo materiale a matrice polisaccaridica. Il prodotto in esame è un composto di polimero e gel di CMCA (carbossimetil cellulosa ammidata) da utilizzare come dispositivo per la viscosupplementazione nell'artrosi del ginocchio per ripristinare la funzione di lubrificazione e di *shock absorber* che il fluido sinoviale patologico non è più in grado di fornire.

La parte iniziale del lavoro è stata incentrata sulla caratterizzazione dei processi produttivi; dato che il processo di sintesi del prodotto era già stato sviluppato ed ottimizzato, sono state messe a punto ed ottimizzate le lavorazioni successive ovvero il riempimento del composito all'interno delle siringhe, il confezionamento e la sterilizzazione.

L'analisi delle proprietà chimiche, meccaniche e reologiche ha costituito una parte del lavoro svolto; grazie allo sviluppo di test mirati, come l'analisi attraverso lo spettrofotometro IR, il pH-metro, il densimetro ed il reometro, è stato possibile valutare le caratteristiche del prodotto fornendo sia un sistema di controllo in produzione che sul prodotto finito. E' stato anche sviluppato uno studio sulla stabilità del prodotto grazie ai dati forniti da esame di tipo reologico su campioni di prodotto sottoposto ad invecchiamento a temperatura ambiente e ad alta temperatura, simulando un invecchiamento accelerato.

Al termine di questa fase di sviluppo di metodi di controllo dei processi, sul prodotto è stata svolta un'analisi di performance tramite test di usura su protesi, in diversi accoppiamenti articolari eseguiti in simulatori di passo di anca e ginocchio. Al termine del test è stato possibile valutare il livello di protezione fornito dal dispositivo in esame, tramite l'analisi dell'usura dei componenti in polietilene (UHMWPE) confrontandola con quella subita da liners identici ma che avevano lavorato in presenza di siero bovino.

Con lo scopo di valutare un possibile ulteriore utilizzo del composto di polimero e gel di CMCA è stato sviluppato un ulteriore test utilizzando un simulatore di passo con protesi d'anca caratterizzate dall'accoppiamento metallo-metallo. Si è andati così a valutare se l'iniezione del prodotto, nel caso di pazienti con protesi in Co-Cr-Mo, sia in grado di ridurre il rilascio di ioni metallici, la cui presenza è stata analizzata all'interno del fluido lubrificante usato (composto polimero-gel di CMCA o siero bovino).

CHAPTER 0: INTRODUCTION

The present thesis work is the result of four months of internship in the R&D department of Lima Corporate s.p.a, located in Villanova di San Daniele del Friuli (UD), Italy, which is an Italian multinational company specialized in orthopedics and traumatology. The core business is focused on the production and distribution of implantable prosthesis for knee, hip and shoulder and on research of new materials.

This project is part of a R&D program that is targeted to the realization of a device for viscosupplementation based on a new technology.

The product herewith described is a CMCA (amidated carboxymethyl cellulose) polymer-gel dispersion, used to reduce pain in the treatment of osteoarthritis by improving mechanical, and rheological behaviour of the synovial fluid; at present, this device is concluding the validation phase and it is going to be evaluated in a clinical trial on humans.

Because the production process was already defined and validated, this thesis had been focused on optimization and characterization of the processes, i.e. filling, packaging and sterilization that have been outsourced.

The thesis is oriented to the development of the manufacturing and control processes including the analysis of mechanical, rheological, chemical and stability properties of the CMCA polymer-gel solution for viscosupplementation in relation to the production process and the intended use. Almost all the tests described have been performed internally, with the exception of the rheological characterization that has been performed in collaboration with Department of Industrial Engineering and Information Technology of Trieste.

Figure 0.1 presents the production flowchart of CMC powder amidation to the final product storage.

Moreover, an analysis of the contest in which the material has to be used has been performed: the viscosupplementation technique in the osteoarthritic knee. According to this overview, the mechanism of action of viscosupplements has been investigated; then the characteristic properties, that are critical for such devices to be effective, have been evaluated on the new product.

The technique has been described with related advantages and disadvantages, reporting data from different studies aimed to investigate its efficacy and safety in treating osteoarthritic disease.

The CMCA composite material has been compared to other commercial products, giving evidence of the structural differences in the origin, in the biocompatibility and in the rheological response as the rationale for the development of this product.

Particular attention is given to the manufacturing process in all its phases, from raw material control, packaging, sterilization and storage of the final product (because of their great importance in determining product properties) and its aging.

As to shelf life evaluation, a study was performed in order to understand product properties trend by measuring two parameters: Elastic and Viscous Moduli, in a frequency range between 0,02Hz and 10Hz. This study allowed to predict how this characteristics change in time and how fast they do it, comparing real time aging and accelerated aging, obtained by high temperature exposition. Another important information that can be obtained from this study, once defined the acceptable variation range of the analyzed properties, is the product's shelf life.

Finally, three experiments that exhaust the wear of prosthetic implants in presence of CMCA polymer-gel dispersions are presented. The first simulates the lubricating action in the knee by the use of a machine reproducing the movement of this joint. The second analyzes the effects of the presented product, in presence of Metal on Metal (MoM) total hip prosthesis: this test also allowed verifying the ion release, connected to the wear of the Cr-Co-Mo prosthesis in the presence of the CMCA polymer-gel mixture.

The three mechanical tests, due to their complexity, have been carried out with the project management approach in order to have the possibility to control, handle and, if necessary, to re-organize activities in the best way.

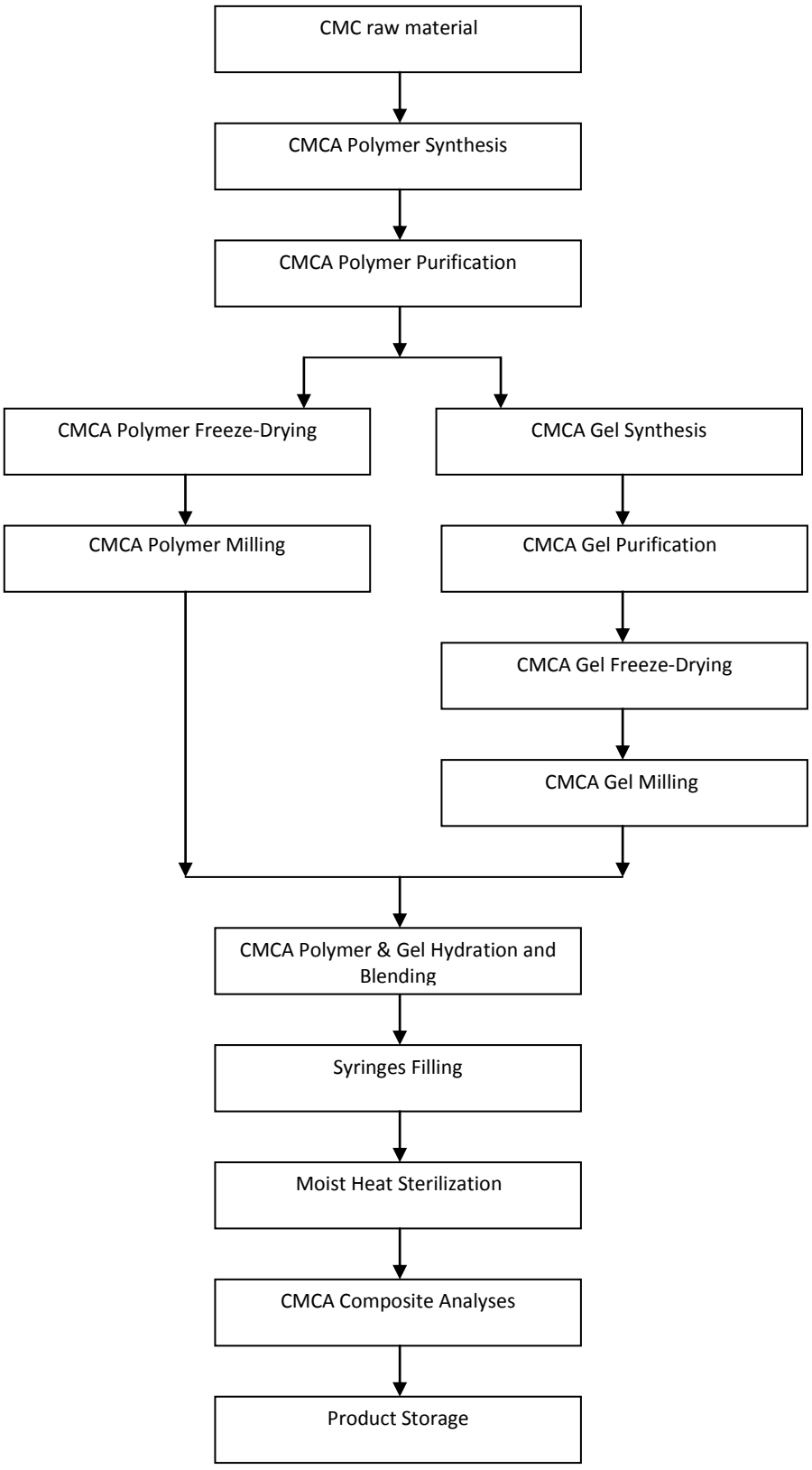
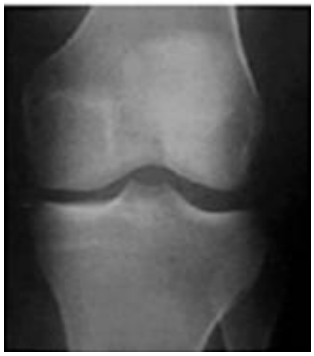
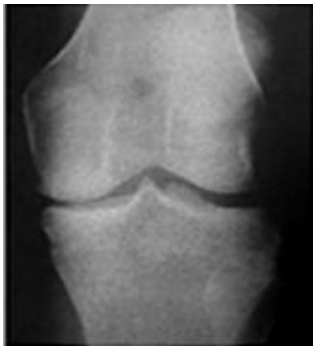


Figure 0.1
Production Process

CHAPTER 1: VISCOSUPPLEMENTATION

1.1 OSTEOARTHRITIS AND VISCOSUPPLEMENTATION

Osteoarthritis (OA) is a chronic degenerative multifactorial joint disease characterized by a progressive degradation of the articular cartilage, a drop in the articular joint space, subchondral sclerosis, cysts, synovial inflammation and osteophyte formation **[1]**. It is the most widespread form of joint disease and it has been worldwide estimated that 9,6% of men and 18,0% of women older than 60 years suffer of OA **[2]**.

a.  b. 

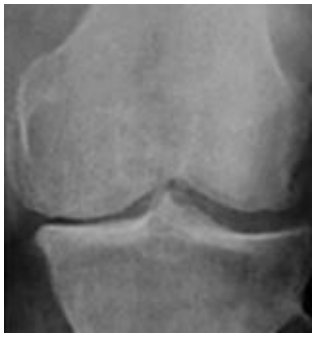
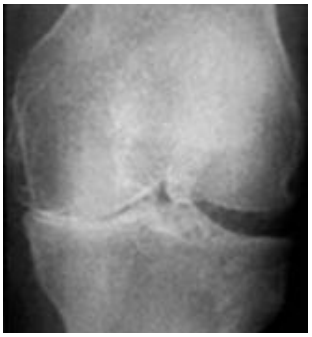
c.  d. 

Figure 1.1
OA progression from a normal joint (a.) to a severe OA with drop in articular joint space and osteophytes formation (d.) ***[3]****.*

The knee is a joint where OA is quite common because of aging. *Figure 1.1* shows the OA progression in the knee from a normal joint (a) to a severe OA (d) in which the articular joint space is reduced and the formation of osteophytes is visible. Diarthrodial joints, also known as synovial joints, such as the knee, are those which can move more freely and contain synovial fluid. Normal synovial joints essentially provide frictionless motion between limb segment and they are characterized by a very low friction coefficient between articular cartilage surfaces, in healthy conditions, due to the presence of the synovial fluid. Diarthroid joints operate well under a wide range of conditions from low loadings and high shear rates to high loadings and low shear rates.

Synovial fluid (SF), or synovial, is a clear viscous liquid fluid, secreted by synovial lining cells, that occupies joint's cavity with its lubricating and nutritive action. It supplies nutrients, necessary for chondrocyte metabolism, and removes catabolic products since normal articular cartilage has no blood supply. The primary role of SF is protective, by limiting axial forces on the articular surfaces and decreasing friction between joint surfaces **[1]**.

The most abundant macromolecules in synovial fluid are sodium salt of hyaluronic acid (NaHA in a concentration of 3,5 mg/ml and a high average molecular weight) and blood plasma proteins (10-30mg/ml) as albumin (8-13mg/ml) **[4].**

Hyaluronic Acid (HA) is a very long chained (between 3 and 30µm) unbranched non-sulfated GAG made by the repetition of a disaccharide unit of N-acetyl-glucosamine and residues of glucuronic acid linked with a glicosidic bond (see *Figure 1.2*).

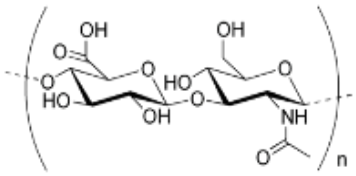


Figure 1.2
The disaccharidic unit (glucuronic acid and N-acetilglucosamine) repeated in the main structure of HA.

HA, which is the most abundant glycosanminoglican (GAG) in mammalian tissue and in diarthroid, in healthy joints reaches the concentration of about 0,5-4mg/ml essential for normal joints functions.

The presence of Hyaluronic acid (hyaluronan) governs synovial fluid's viscous and lubricant behaviour. It also bonds the opposing surfaces of the joints to each other creating a tensile strength enabling opposing surfaces to slide freely across each other and limiting their separation. Synovial fluid's lubricating and chondroprotective ability is not entirely dependent upon viscosity but also to its ability to change physicochemical nature of a surface, repelling one to another in a process called "boundary lubrication". This process is mediated by the lubricin which is a synovial fluid glycoprotein **[5]**. Hyaluronic acid, instead, is responsible for synovial fluid's rheological properties, enabling it to act as a lubricant or shock-absorber **[6]**.

Considering the knee joint, the volume of synovial fluid contained is well documented in literature as shown by the following chart.

SOURCE	JOINT	VOLUME
[7]	All synovial joints even the largest	< 2 ml
[8]	Generic joint cavity considering type and inter-individual variability	[0.1-4] ml
[9]	Not specified	< 4 ml
[10]	Not specified	≤ 3.5ml
[11]	Knee	[0.13-3.5] cm^3 average 1.1 cm^3
[12]	Knee	≤ 3.5 ml average 1ml

[13]	Knee	≤ [3-4] ml
[14]	Knee	≤ 5 ml
[15]	Knee	[0.5-1.5] ml
[16]	Knee	[0.5-4] ml
[17]	Knee	[0.3-1] ml
[18]	All synovial joints even knee	< 3 ml
[19]	Synovial joints	≈ 3 ml
[20]	All synovial joints even the largest	[0.2-0.5] ml
[21]	Knee	≤ 4 ml
[22]	Knee	≤ 4 ml
[23]	Knee	≤4 ml
[24]	Knee	[0.5-4] ml

Table 1.1
Synovial Fluid Volume and References

In osteoarthritis, the synovial fluid is more abundant and less viscous in fact hyaluronan becomes depolymerised and its concentration and molecular weight (MW) are reduced causing a drop in elastoviscous properties **[1,6]**. These changes increase cartilage susceptibility to injuries and damage.

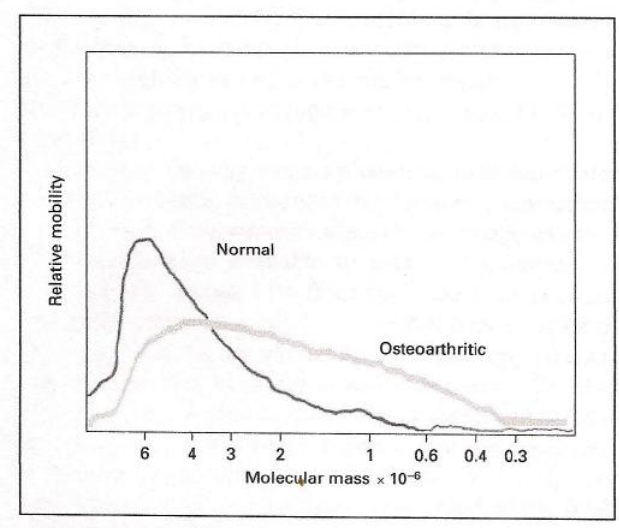


Figure 1.3
*Agarose gel electrophoresis of Normal and OA Synovial Fluid **[25]***

The polydispersity profile of hyaluronan in osteoarthritic fluid is more variable than in healthy patients as it could be seen in *Figure 1.3*; however it always contains a small variable amount of high molecular mass fraction (>2 million Da) **[25]**.

Viscosupplementation by repeated intra-articular injections of viscoelastic solutions is used in the treatment of symptomatic knee osteoarthritis. Viscosupplement usually injected are exogenous HA or its derivatives: they decrease pain and improve knee functions through the restoration of the synovial fluid's rheological properties.

There are different injection techniques that differ by knee position (bent, straight or partially bent) and injection site (infra-pattellar, medial or lateral) and the choice is made by the physician depending on his experience and on the patient. The technique does not influence so much the onset of adverse effects although knee partially bent reduces them **[2,6]**.

The first evidence that joint injections of hyaluronan could reduce pain was given by Balazs et al. in 1960s. They used hyaluronan solutions made of highly purified Na-hyaluronan of relatively high MW (>2million Da) in concentrated (1%) solutions (dissolved in phosphate-buffered physiological NaCl). The hyaluronan was taken from human umbilical cord or rooster comb and was sterile, pyrogen-free and caused no inflammation while injected into various tissues and tissue spaces of animals such as vitreous, joints and peritoneal cavity. This highly purified fraction with only traces of protein was non-immunogenic and was called the "non-inflammatory fraction of Na-hyaluronan" (NIF-NaHA). The first preparation made for medical use was called with the trade name Healon® (Biotrics, Arlington, Mass., USA, 1972) **[25]**.

In early 1970s viscosupplementation with hyaluronan was used to reduce pain of racing horses **[26]**. In 1987 hyaluronan solutions were approved for the treatment of arthritic pain in Japan and Italy and in 1997 they were approved by Food and Drug Administration (FDA) **[1,27]**.

Hylans are cross-linked forms of purified hyaluronan with extremely high molecular weights (about 6million Da) developed to obtain solutions with greater viscoelastic behaviour without affecting the two specific groups of the molecule **[26,28]**. The aim to produce high molecular weight and high concentrated products is due to the greater

analgesic effect they obtain after injection. Because of their greater viscosity, crosslinked hyaluronans prevent their removal from the synovial cavity increasing their residence time **[5]**.

The therapy's analgesic effect is thought to be achieved by multiple mechanisms as for example the reduction of pain provided by the creation of a viscoelastic protective barrier around the nociceptive afferent fibers in the intercellular matrix **[6,27,29]**. It has been demonstrated that a mechanical stress can induce stretch-activated ion channels opening in cellular membrane and that their activity can be reduced if cell membrane are bathed in elastoviscous hylan solutions **[25]**. There are some sensory endings in direct contact with the hyaluronan-rich extracellular matrix so a change in elastoviscous properties (caused by concentration or MW variations) can influence the sensitivity of these terminals to mechanical and chemical stimuli **[25]**.

Studies on cats demonstrated that injection of elastoviscous hylan in the inflamed knee joint significantly reduces the inflammation-evoked ongoing and movement-evoked nerve discharges. Non-elastoviscous hylan does not produce this effect.

The attenuating effect of hylan on nociceptive afferent discharges cannot be explained by the volume increase caused by the injected fluid, because this effect could never be obtained with the same volume of nonelastoviscous hylan. Most importantly, the nonelastoviscous hylan solutions contain the same concentration of chemically identical but smaller molecules of hylan than the elastoviscous hylan solution. The difference in mean molecular weight results in differences in physical (rheological) properties. This strongly indicates that different effects are related to the viscoelasticity of the solution. The lack of effect of nonelastoviscous hylan cannot be explained by differences in diffusion in the joint, because, at the time of measurement (1–2 h after injection), these molecules are still present in the joint. The reduction of nociceptive discharges in articular afferent fibers by elastoviscous hylan solutions described in the present study provides experimental support to the clinical observation that viscosupplementation with elastoviscous hyaluronan and hylan solution decreases pain and improves the mobility of joints in race horses with traumatic arthritis and in osteoarthritic human knee **[30]**.

Another advantage of viscosupplementation is that the injection of hyaluronan or hylan stimulate synovial cells to synthesize endogenous, high quality, hyaluronic acid giving a very long efficacy to the treatment, also exceeding resident time of the injected product in the joint **[6,29,31]**.

OA causes motility problems such as slow speed, limited peak knee flexion and extension degrees, decreased weight bearing that could be improved with intra-articular injection of hylan inducing an alteration on the natural history of the disease by decreasing excessive loading in the knee **[31]**.

The American College of Rheumatology suggests an initial non-operative, non-invasive treatment of OA consisting in physical therapy and weight loss followed by pharmacological intervention including topical and oral analgesics, non steroidal anti-inflammatory drugs (NSAIDs), opioids and steroids. The long-term use of these pharmacologic agents can induce deleterious effects and some patients may have gastrointestinal or renal intolerance of NSAIDs. Considering this, viscosupplementation provides a pharmacologic alternative for who is functionally limited, due to osteoarthritic knee pain, especially for those who wish to postpone surgical intervention or for whom this solution is inappropriate **[32]**.

The viscosupplementation technique is not free of local adverse events (AEs) which are the same observed with other intra-articular injections including corticosteroids as well as saline **[33,34]**. The etiology and pathophysiology of these local AEs is not clear but it has been hypothesized a connection with the procedure, the sensitivity of synovium, the disease stage of process, the inflammatory reaction to the product, patient activity after the treatment, age, time since diagnosis, infection or allergy to avian products **[2,33]**. However most adverse events reported in literature are minor and transient at the injection site. Painful post-injection reaction occurres in 1-2% patients and it do not last more than 72 hours. Systemic allergic reactions due to individual hypersensitivity were rarely recorded **[28]**.

Improper intra-articular needle placement is thought to cause local tissue damage, pain and swelling for example in the case of injection into the perisynovial fat pad but, if

correctly executed, the injection technique does not influence the possibility to experience AEs instead **[2,33]**.

Concluding, viscosupplementation has been demonstrated to be effective in reducing pain connected to OA and also in improving motion ability and to stimulate new production of healthy HA. These effects are much more greater in underweight patients, in males and in patients more recently diagnosed of OA

1.2 COMMERCIAL PRODUCTS

There are many commercial HA preparations available for viscosupplementation; they have animal or synthetic origin and they can be based on hyaluronic acid (or its derivatives) or not. Most are hydrogel formulations of HA in water for injection and may contain addition excipients such as sodium chloride for isotonization and sodium hydrogenphosphate for pH adjustment. Most contain low molecular weight HA (0,5-1,5 million Da) while one formulation (Synvisc®) contains cross-linked HA (hylan) with 6-7 million Da molecular weight **[4]**.

The animal origin, usually by isolation from rooster comb or by fermentation (e.g. *Streptococcus equii*), can induce allergic reaction, due to the presence of protein traces, if injected in sensitive people. Moreover, cellulose derivatives are characterized by great similarity in mechanical, chemical and rheological properties to human hyaluronic acid.

This similarity is the final goal of all commercial products because it is the key to properly act after injection, reducing the pain and affording mechanical stress.

Synovial fluid is characterized by both elastic and viscous properties depending on size, conformation, interaction and number of hyaluronic acid molecules in the fluid. The elastoviscous characteristic can be detected through the dynamic shear modulus (or dynamic rigidity G*) measured at various frequencies. It can be splitted into its two components: the elastic modulus G’ and the viscous modulus G’’ (*Equation 1.1*).

G* is the ratio between the peak stress to the peak strain considering a sinusoidal solicitation (*Equation 1.2*).

$$|G^*| = \sqrt{(G')^2 + (G'')^2}$$

Equation 1.1
Dynamic Rigidity components

$$G^* = \frac{\sigma_{peak}}{\gamma_{peak}};$$

Equation 1.2
Dynamic Rigidity Definition

G'' is also called dynamic loss modulus since it is connected with the energy dissipated as heat when a strain is applied; G' is also known as storage modulus and represents the energy stored during strain.

While increasing strain frequency the synovial fluid rapidly transits from a viscous to an elastic body. This transformation is reversible and has no deteriorating effect on the molecules.

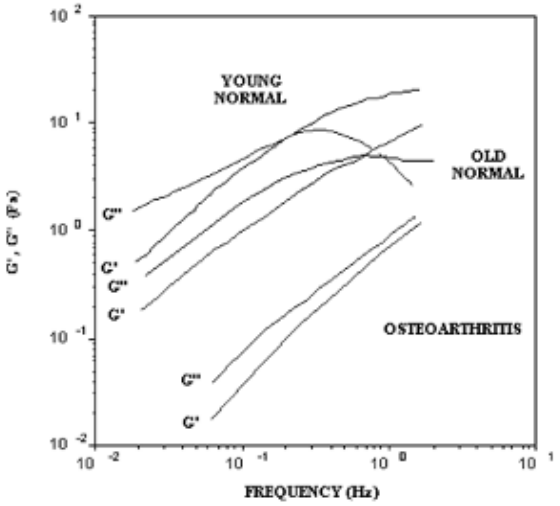


Figure 1.4
Elastic modulus (G') and viscous modulus (G'') of synovial fluid.
(Balzas et al.) ***[35]***

As can be seen in *Figure 1.4* OA is a pathologic condition in which both moduli have a lower value and G'' is always greater than G'. The viscosupplementation technique aims at restoring a situation much more similar to the healthy one.

In 1997 in the USA Hylan G-F 20 (commercial name Synvisc®) was approved as a VS product. It is a high molecular weight HA derivate composed of two hylan polymers within a buffered physiological NaCl solution. The first polymer called hylan A is native hyaluronan which is soluble and with high MW; the second polymer is hylan B that is an insoluble gel. The crosslinking between the two molecules produces a product with very high MW (6 million Da) and creates a continuous molecular network. Hylan G-F 20 is non-immunogenic, non-inflammatory and does not cause foreign-body reactions **[1,27]**. Other products available are for example Orthovisc® (1,2 million Da **[25]**) and Ostenil® both medium molecular weight hyaluronan but it has been demonstrated that lower MW HA present lower values for G' and G" in fact Hylan G-F 20 presents greater elasticity and viscosity at all frequencies **[26]**.

	G′ at Hz (Pa)			G′′ at Hz (Pa)				
	0.01	0.1	2.5	0.01	0.1	2.5	η^*	ω
Hylan G-F 20	16	46	91	17	24	20	502	0.01
LMW HA	0.1	0.3	9	0.2	1	16	3	7.5

Table 1.2
*Comparative elastoviscosity of hylan G-F 20 and that of lower-molecular-weight (LMW) hyaluronan **[26]**.*

The fact, highlighted in *Table 1.*2, is that high molecular weight hyaluronans present higher values of both moduli at the considered frequencies, showing, in this range, superior viscoelastic properties. This consideration is sustained by the fact that patients treated with higher MW hyaluronan feel a greater improvement in weight-bearing pain from the initial condition before treatment **[26]**. Hyalgan® (0,5 million Da) is another product for VS but due to its low molecular weight does not produce any effect. Orthovisc® with its medium MW induces some improvements but they last for a lower period than Synvisc® **[25]**.

Table 1.3 presents some hyaluronic acid commercial products for viscosupplementation with their MW, and origin.

Trade name	Molecular weight	Source	Mg/ml
Hyalgan	500–730 KDa	HA extracted from rooster combs	20 mg/2 ml
Hyalubrix ≷	1500 KDa	Fermentative HA	30 mg/2 ml
Artz (Supartz)	800–1170 KDa	HA extracted from rooster combs	25 mg/2.5 ml
Synovial (Jointex)	800–1200 KDa HA	Fermentative HA	16 mg/ml
Synvisc	6000 KDa	HA extracted from rooster combs	16 mg/2 ml
Durolane	90 millions Da	Synthetic HA	20 mg/ 3 ml
Euflexxa	2.400–3.600 kDa	Fermentative HA	20 mg/2 ml

Table 1.3
MW of some hyaluronic acid products ***[28]****.*

An alternative to hyaluronan and its derivatives is an innovative product derived from cellulose. In particular it is an amide carboxymethyl cellulose amide (CMCA) in which the amidation has been introduce to improve the biocompatibility creating a cellular structure more similar to hyaluronic acid that encourages cell proliferation. CMC is the most common cellulose derivative and it is made of a long polymeric chain of repeated units of D-glucose. The CMCA constitutes the polymer part (up in *Figure 1.5*) while the crosslinked CMCA forms the gel (down in the *Figure 1.5*).

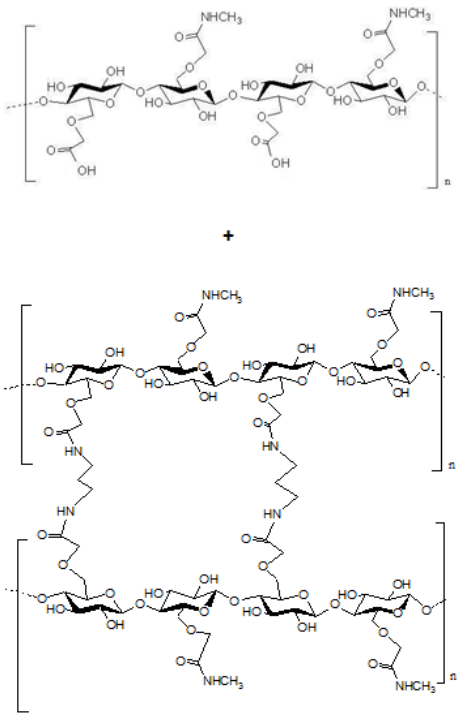


Figure 1.5
CMCA composite: Polymeric part (at the top) and gel part (at the bottom).

The result is a non-Newtonian viscoelastic system composed by two polymeric components: CMCA crosslinked gel dispersed in a polymeric solution of linear CMCA in a volume of saline sufficient to reach the desiderated concentration.

Another important characteristic that have to be considered while designing a viscosupplement is its degradation behaviour. In fact it has to be introduced in an ambient rich of enzymes able to cleave its chemical bonds affecting its chemical and mechanical properties. Two enzymes that are present in synovial fluid are for example lysozyme and hyaluronidase. Lysozime (LZM) is a single chain protein with a MW of 14,3 KDa which catalyzes the hydrolysis of β(1-4) link between N-acetilmuramic acid and N-acetylglucosamine. The synovial fluid concentration of LZM has been determined by Klockars M. et al. who found that the mean concentration in osteoarthritic patients is similar to the one of healthy patients **[36]**. Hyaluronidase is an hydrolitic enzyme that separates hyaluronic acid in its fundamental components (D-glucuronic acid and N-acetyl-D-glucosamine). Comparing hyaluronic acid and the CMCA polymer-gel solution their weight loss was monitored after exposing their freeze-dried form to the degrading solution (hyaluronidase at the concentration of 0,1U/ml or lysozyme at the concentration of 37,73 UI/ l in accord with physiological conditions). After 7 days in hyaluronidase solution the CMCA preparation and hyaluronic acid lost 15,8% and 40% respectively; at the 28^{th} day CMCA losts only 38% while hyaluronic acid losts even 81%. In lysozyme solution CMCA and hyaluronic acid lost 20% and 15,3% respectively, in 7 days; in 28 days they lost 40% and 43% respectively **[37]**. The resistance to enzymatic degradation is an important characteristic due to the fact that it allows the product to withstand into the biological environment.

The number of injections for a complete treatment, is strictly related to the particular product, the volume contained in the syringe, its molecular weight and its concentration. Most formulations propose a dosage of three injections of 2ml, one at week. It has been demonstrated that several injections can increase the intensity of adverse effects to the treatment **[34]**. The incidence of AEs was found to present an approximate two-fold increase in incidence compared in patients previously exposed to hyaluronan or hylans. The mechanism for an increased incidence of local adverse events

in those with previous treatment is not totally known but it is supposed to have an immunologic basis **[2]**.

CHAPTER 2: MANUFACTURING PROCESSES

2.1 PRODUCT PROCESSING

The product manufacturing consists in a series of consequent phases strictly connected one to each other in a temporary order. The flow chart, hereinafter reported, presents all these activities for the processing of the CMCA composite material.

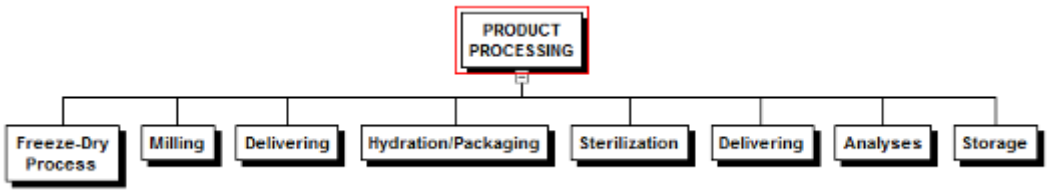


Figure 2.1
Manufacturing Process flow chart.

Each phase will be described in the following Paragraphs with the tests that allowed to consider them a good practice to obtain an effective and safe product. The process starts with the freeze-drying of the material that, after this, is milled and delivered to a

subcontractor to be filled in the syringes and sterilized; after sterilization the material is sent back to Lima Corporate S.p.a, analysed and then stored.

2.2 HYDRATION

The CMCA polymer and the CMCA gel, once produced, are freeze-dried, milled separately, and stored. For the production of the composite material the two freeze-dried powders are mixed, hydrated and filled in syringes of 4ml. The filling procedure is a process carried on by a subcontractor that has a semi-automatic filling process for the filling of high viscous suspensions and gels.

Two critical points connected with the hydration process are the final solution concentration and the non pyrogenicity. To assure this two characteristics, the corresponding tests were performed. In Paragraphs 2.2.1 and 2.2.2 they are described with the procedure adopted and the results obtained.

2.2.1 Density Test

A density test has been designed and performed in order to control the correct hydration concentration; in fact density is a parameter that changes as the concentration does and it is measured by a density meter. The densimeter is composed of a U-shaped borosilicate glass tube in which the sample is introduced (only 1mL is needed) with a syringe.

Figure2.2
The density meter analyzing the content of the syringe.

When in use, the tube containing the product is excited to vibrate due to a magnetic domain; the characteristic frequency changes depending on the sample's density. If the

tube is filled only with air, the oscillatory frequency is checked to correspond to the harmonic oscillation at the fundamental frequency of 440 Hz.

Due to the temperature dependency of the density value, a precise temperature control of the sample is required and measures have to be done at the same temperature (20°C). Density values measurable with the instrument range are from 0 to 3 g/cm^3 and results are affected by a very little error (about 0,001 g/cm^3).

In order to find the relationship between concentration and density, a series of tests have been done. The first test consisted in measuring the density of different samples of the CMC raw material dissolved in water solution at different concentrations (0,1%, 0,5%, 1,0%, 1,5% and 1,7% W/W). The second test was the same but the samples analysed were of CMCA polymer diluted in water at 0,1%, 0,5%, 1,0%, 1,5%, 1,6%, 1,7%, 1,8% and 2,0% W/W. For each concentration, several measures were taken in order to calculate an average value (SD are reported in brackets). *Table 2.1* shows the average density values measured, both for CMC and CMCA polymers.

CONCENTRATION %	CMC Density [g/cm^3]	CMCA Density [g/cm^3]
0,1	0,9986 (0,0002)	0,9982 (0,0004)
0,5	1,0003 (0,0002)	0,9993 (0,0007)
1,0	1,0027 (0,0002)	1,0015 (0,0001)
1,5	1,0049 (0,0007)	1,0032 (0,0001)
1,6	-	1,0036 (0,0001)
1,7	1,0058 (1,0003)	1,0037 (0,0001)
1,8	-	1,0041 (0,0000)
2,0	-	1,0050 (0,0001)

Table 2.1
CMC and CMCA densities at different concentrations.

From these values, two linear relationships were obtained and their expressions are reported below:

$$Y = 0{,}0045X + 0{,}9981$$

Equation 2.1
CMC densities tendency line.

$$Y = 0{,}0036X + 0{,}9977$$

Equation 2.2
CMCA densities tendency line.

The following graphs present the linear relationships described; density is plotted vs concentration. The sample's values are represented in blue while the violet lines in *Figure 2.2* and *Figure 2.3* are *Equation 2.*1 and *Equation 2.2* respectively.

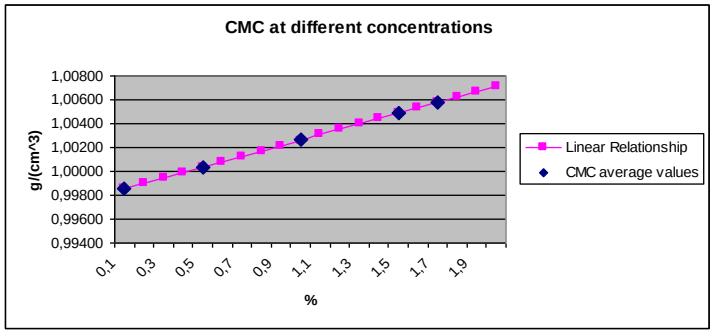


Figure 2.3
CMC polymer densities at different concentrations and tendency line.

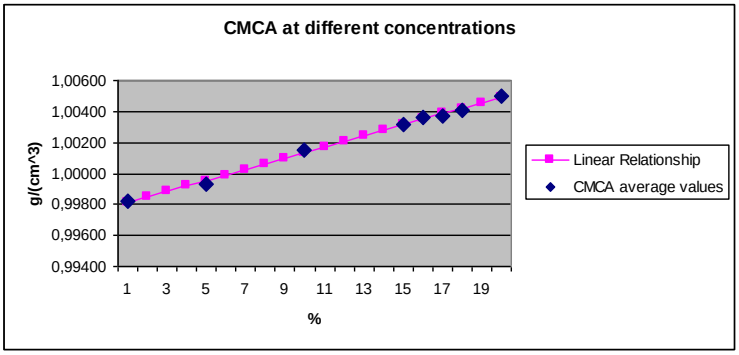


Figure 2.4
CMCA polymer densities at different concentrations and tendency line.

The same test has been performed on seven samples of CMCA composite material, internally prepared, not with the automatic technique performed by the subcontractor, and filled at 1,00%, 2,00%, 3,5%, 3,61%, 3,71%, 3,80% and 4,00% W/W in saline solution. For each concentration a minimum of three measures were done and the average values, presented in *Table 2.2*, were used to find out the tendency line (see *Equation 2.3*). The values in brackets in *Table 2.2* are the standard deviations obtained.

CONCENTRATION %	CMCA Mixture Density [g/cm^3]
1,0	1,0073 (0,0004)
2,0	1,0105 (0,0001)
3,5	1,0150 (0,0006)
3,61	1,0157 (0,0001)
3,71	1,0158 (0,0003)
3,8	1,0164 (0,0001)
4,0	1,0169 (0,0003)

Table 2.2
CMCA Mixture densities at different concentrations.

$$Y = 0{,}0032X + 1{,}004$$

Equation 2.3
CMCA Mixture densities tendency line.

Figure 2.4,shows *Equation 2.3* in violet and the average values reported in *Table 2.2* in blue. The value plotted in yellow is the average value obtained from a sample of CMCA composite material filled with the automatic filling technique, using nitrogen as propellant gas, in a 3,71% saline solution (in the next Paragraph the reason connected to the choice of nitrogen for the standard procedure is discussed).

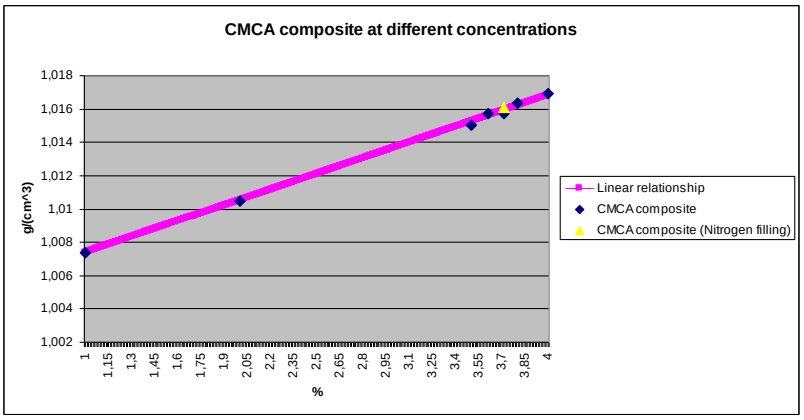


Figure 2.5
CMCA composite densities at different concentrations.

As it can be seen, the tendency line fits well also for the value obtained from a sample prepared using nitrogen, giving evidence to the fact that *Equation 2.3* describes the relationship between concentration and density for a CMCA sample without caring on

the filling technique in fact the automatic filling (yellow sample in *Figure 2.5*) and the internal procedure (blue samples in *Figure 2.5*) present the same trend. The correlation between the values reported and the line is 0,999 showing the great agreement found.
Comparing the three lines obtained (for CMC and CMCA polymers and for CMCA composite material) it is clear that they are very similar and the fact is highlighted by the correlation coefficients:

- CMC - CMCA polymers: 1,000
- CMCpolymer - CMCAcomposite: 0,999
- CMCApolymer - CMCAcomposite: 0,999

These density tests allowed to obtain the linear relationship between density and sample's concentration giving the possibility to perform two controls:

1. Process Control to verify the correct concentration of the CMCA polymer before starting its freeze-dry process and the synthesis of the CMCA gel.
2. Final Product Control to verify that hydration and blending were performed correctly.

The control that is performed with the density measure is very accurate in fact it is possible to distinguish a difference of 0,1% in concentration.

2.2.2 Pyrogen Test

At the beginning of Paragraph 2.2 the problem of pyrogenicity have been presented. To avoid the presence of pyrogens in the final product, the hydration of polymer and gel powders, obtained after freeze-dry process, is performed in non pyrogen and sterile saline solution. The pyrogen test is required for intravenous-injectable products and the CMCA mixture, even if it requires an intra-articular and not intravenous injection, has been tested and resulted pyrogen free. This test was performed in a worse case condition than the condition of intended use.

Pyrogens are substances inducing fever response which can be produced by some gram positive bacteria, mycobacteria, fungi and also viruses. Pyrogens produced by gram negative bacteria, i.e., endotoxins, are important for the pharmaceutical industry **[39]**. There can be several sources of pyrogens in parenteral and medical products and usually they are: water used as solvent or for the processing, packaging components, chemicals, raw materials or the equipment **[39]**.

Non pyrogenicity is a more severe restriction than sterility due to the fact that sterility does not mean non pyrogenicity. A sterile product may contain non vital microorganisms, or parts thereof (cell wall components as lipopolysaccharides) which cannot induce infection but which could induce inflammatory response and fever. This is the reason why non pyrogenicity is required for intravenous-injected products. Pirogenicity is not only induced by microorganisms, but it could be also caused by polysaccharides and other components of medical devices; this consideration allows to distinguish between pyrogenicity of the material itself and pyrogenicity caused by contamination. As a consequence a cellulose derivative product such as the CMCA composite material, has to be tested.

The Material Mediated Pyrogen test evaluates the potential of a material to cause a pyrogenic response, or fever, when introduced into the blood. Lot release testing for pyrogenicity is done in vitro using the bacterial endotoxin (LAL) test and must be validated for each device or material. However for biocompatibility assessment, the rabbit pyrogen test, is preferred. The rabbit test, in addition to bacterial endotoxins detection, is sensitive to material-mediated pyrogens that may be found in test materials or extracts. The European Pharmacopeia **[40]** describes the pyrogen test as a measure of the increase of temperature in rabbits due to an intravenous injection of the sterile solution of the substance under analysis. The difference between the initial temperature and the maximum registered is considered the rabbit's reaction to the injection.

Numero di conigli	Il prodotto soddisfa al saggio se la somma delle risposte non supera	Il prodotto non soddisfa al saggio se la somma delle risposte supera
3	1,15 °C	2,65 °C
6	2,80 °C	4,30 °C
9	4,45 °C	5,95 °C
12	6,60 °C	6,60 °C

Table 2.3
*Evaluation of pyrogen test **[40]**.*

The test has to be performed in one group of three animals at least. The test is passed if the sum of the reactions, for a specific number of animals (first column in *Table 2.3*), is lower than a specific value (second column) for that number of animals; on the other side the test is considered not passed if the sum of the reactions is greater than the values in the third column. If the summed reactions is between the values of the first and the second column, another group of animals is required and the test is repeated.
The pyrogen test on the CMCA composite material has been performed by an external laboratory which injected 1ml/kg of the product, diluted 1:10 in sodium chloride, into the ear vein of three male albino rabbits (white New Zealand rabbits). The product was warmed at room temperature before injection and the temperatures of the rabbits were recorded at intervals of 30 minutes in the three hours after injection, to individuate any temperature raising. The temperature increase was 0,15°C, 0,10°C and 0,55°C and the sum of the responses was 0,80°C that is less than 1,15°C (upper treshold to consider the test passed in the case of one group of rabbits). This test established that the CMCA mixture meets the requirement for the absence of pyrogens.

2.3 PACKAGING AND FILLING TECHNIQUE

Once hydrated, the product has to be packed and stored. According to the *International Standard UNI EN ISO 11607-1* **[41]** the packaging system provides physical protection, maintain sterility up to the point of use and allows identification thanks to appropriate labels. The package must be sterilized without degradation and it has to ensure the stability of the material till the expiration date guaranteeing environmental protection

(barrier from moisture, bacteria, oxygen, light). The package has also to provide dynamic protection resisting to impacts, abrasions and to ensure structural support **[42]**.

The primary packaging of the CMCA composite material is made of a glass syringe and the secondary packaging is made of a plastic blister. Due to the fact that in the past it was used a plastic syringe and a plastic pouch it was necessary to demonstrate that the new package system does not influence the product's characteristics, both physical and chemical, making a new validation. The reason why the glass syringes are preferable to the plastic ones is the better storage characteristics.

Figure 2.6
The glass syringe in the plastic blister (left) and the plastic syringe in the plastic pouch.

To evaluate if the new syringes influenced the material characteristics an infrared (IR) spectroscopy and a pH analysis were done. A viscosity measure was added to find out the effects of the use of air or nitrogen, as propellant gas, in the automatic filling technique.

2.3.1 Techniques Description

Infrared (IR) spectroscopy is an established technique used for structural investigation and compound identification. Basically, it is the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample, absorbing at characteristic IR frequencies. The spectrum is usually given as a transmittance (*T*) measure that is the fraction of incident light, at a specific wavelength,

that passes thought the sample. The absorbance (*A*) is the logarithm of the transmittance's inverse.

$$T = \frac{I}{I_0}$$

Equation 2.4
Transmittance Definition

$$A = log\left(\frac{I_0}{I}\right) = log(T^{-1})$$

Equation 2.5
Absorbance and Transmittance Relationship

where I_0 is the intensity of incident radiation and *I* the intensity of the radiation coming out of the sample **[40]**.

The resolution for the wave length is 4 cm^{-1} and the range analysed in the experiments presented is between 4000 and 450 cm^{-1}.

The pH measure is an electric potential measure reported in pH units. The instrument's probe measures the electric potential with the glass probe containing a silver chloride electrode. The measure is expressed in relation to the known pH of a solution. The pH has to be measured at the same temperature (chosen between 20°C and 25°, in our case 20°C) **[40]**.

Figure 2.7
Vibro Viscometer SV-10 used in the test.

Figure 2.7 shows the vibro viscometer used to measure the viscosity in this test. The instrument relats viscosity to the measure of the opposition to the movement induced by the fluid to the vibration of the sensor plates. This constant vibration is driven by an electric current which induces a constant sine-wave vibration at 30Hz. The viscometer is equipped with a temperature sensor allowing measures between 10°C and 40°C.

The packaging study has been performed over two samples: the first was the product stored in the plastic syringe and the second in the glass one.

2.3.2 Plastic and Glass Syringes Comparison

First of all a spectrometric valuation of the samples has been performed so two IR analyses were done (one for each sample). A little part of the content of each syringe has been analysed. For each sample, 8 measures (resolution 4,00 cm^{-1}) were collected and the results were averaged. This choice results in measures with a good accuracy.

The strong similarity in the spectra of the samples analysed confirms that the differences in materials composition have no influence in the chemical structure. Comparing the two spectra was clear that the only one difference was in the intensity of the peaks giving evidence that glass does not induce chemical changes in the product.

Figure 2.8 shows the great agreement existing: it can be confirmed also by the correlation factor between the two spectra (0,9809).

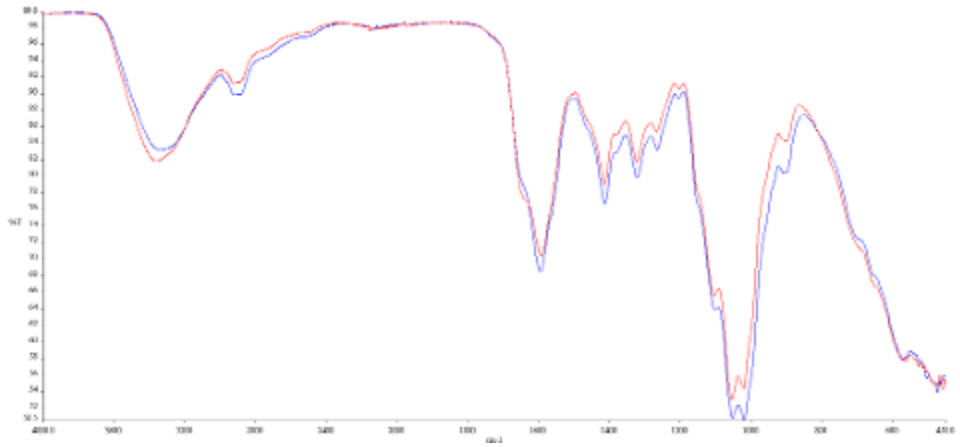


Figure 2.8

Spectrum of the product contained in the plastic syringe (in blue) and in the glass one (in red).

The pH measurements have been done for each sample and the values obtained are reported in *Table 2.4*, showing a great similarity. These results corroborated the conclusions obtained from the IR analysis giving a new proof of them.

SAMPLE	pH
Plastic Syringe	4,65
Glass Syringe	4,68

Table 2.4
Values of pH for plastic and glass syringes

From these results it was clear that glass syringes do not cause chemical or physical changes in the product ensuring a safe use as plastics did.

2.3.3 Propellant Gasses Comparison

In order to prevent possible oxidation caused by oxygen, used as the propellant in the filling technique, it was hypothesized to use an inert gas (nitrogen). The comparative analysis was done to check if the gasses used were safe, efficient or changed chemical and physical properties of the material. IR analysis, pH and viscosity measurements were done with the aim to evaluate any possible changes.

All the tests have been performed on three syringes over two sets: Set One, referring to syringes filled in oxygen, and Set Two, filled with nitrogen. The samples were autoclaved, before performing tests, using three different sterilization cycles (named 1, 2 and 3, respectively) which specimens are reported in the Paragraph 2.4.

Figure 2.9 shows all the spectra obtained, one for each sterilization cycle and gas used. It is clear that they do not influence the chemical structure of the material. Comparing only couples of spectra of the same sterilization cycle, it is evident that the filling technique does not induce any chemical change on the product.

Considering pH and viscosity measures the results obtained are presented in *Table 2.5* where it is shown that pH values are all similar giving strength to the conclusions gained with the IR analysis. The viscosity analysis allowed to understand that the oxygen filling technique induces a greater drop in the viscosity.

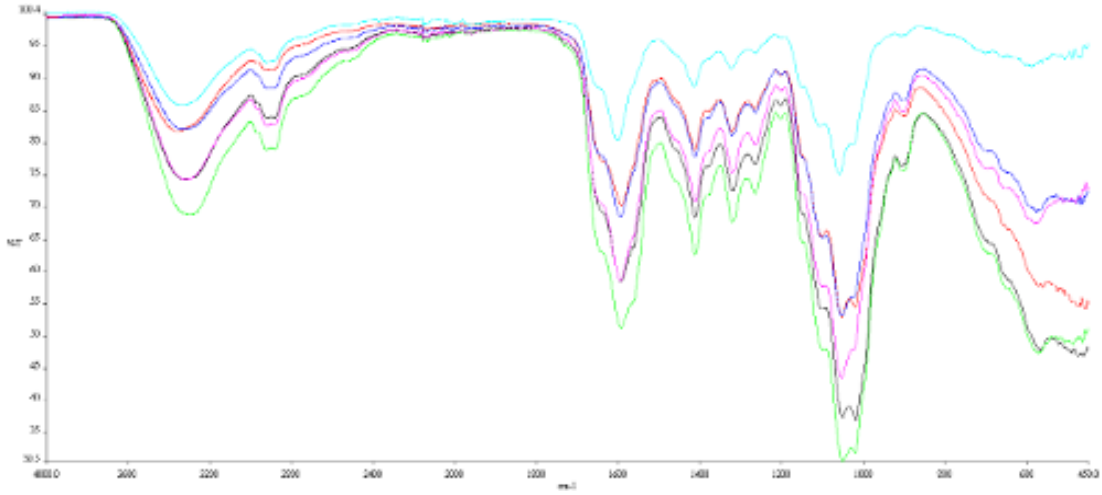


Figure 2.9

Spectra from six different syringes filled in oxygen (red, light-blue, black) or nitrogen (green, blue, violet)and sterilized with Cycle 1 (red, green), Cycle 2 (light-blue, blue), Cycle 3 (black, violet).

This consideration gives evidence that nitrogen filling is better: preserving viscosity is very important in relation to the intended use of the product as a viscosupplement for osteoarthritis.

SAMPLE	pH	VISCOSITY [mPa*s]
1-Oxygen	4,68	918
2-Oxygen	4,61	947
3-Oxygen	4,70	1020
1-Nitrogen	4,65	1825
2-Nitrogen	4,78	1835
3-Nitrogen	4,69	1841

Table 2.5

Values of pH and viscosity of the six samples.

In conclusion, this experiment allowed to affirm that from the chemical point of view the switching from air filling to nitrogen does not induce any chemical modification of CMCA polymer and gel. The only modification that can be observed is in terms of mechanical stability increase, as viscosity values demonstrate.

Considering that nitrogen is an inert, nontoxic gas, widely used in the pharmaceutical industry, it can be assumed that filling with N_2 does not influence the safety of the CMCA composite material, indeed it represents an ameliorative method to preserve mechanical properties.

2.4 STERILIZATION PROCESS

The product in its packaging must be sterilized to guarantee sterility which is a fundamental characteristic for medical devices. In this Paragraph the method used to sterilize the product is described with all the parameters considered to find out the most effective cycle, to assure sterility, and not damage mechanical properties. In order to develop the most adapt sterilization procedure three different sterilization cycles were developed and tested in order to find out their effects on mechanical and rheological properties. To verify these effects the rheological tests were performed in order to compare the values of the elastic and viscous moduli between the samples treated with the different cycles.

Sterile means that the product is free of viable microorganism **[43-45]** because the term "sterile" is applied to pharmaceutical products that have been treated (sterilized) in the way that, at the end of the process, individual items have a probability of being non sterile or have a sterility assurance level (SAL) equal to $1x10^{-6}$ **[43,46,47,48]**. The term bioburden is used to mean the population of vital microorganisms present in or on a product or barrier system. It comes from a sum of different sources including, in the most general case, raw materials, manufacturing, assembling processes and environments, cleaning and packaging and latter aids like compressed gasses, water and lubricants. The purpose of sterilization is to inactivate the microbiological contaminants and thereby transform a non-sterile products into sterile. The kinetics of inactivation of a culture of microorganisms by physical and/or chemical agents used to sterilize medical devices can generally be described by an exponential relationship between the numbers of microorganisms surviving and the extent of treatment with the sterilizing agent (see *Equation 2.6*); therefore, there is always a finite probability that a microorganism may survive regardless to the entity of the treatment.

$$N_t = N_0 * e^{-k*t}$$

Equation 2.6
Kinetics of inactivation of a culture of microorganisms by sterilizing agents.

N_t is the number of surviving organisms after time t, N_0 is the number of microorganisms at time zero (bioburden), t is the exposure time and k is a microbial inactivation rate constant. This expression represents a firs-order kinetic process (see *Figure 2.10*).

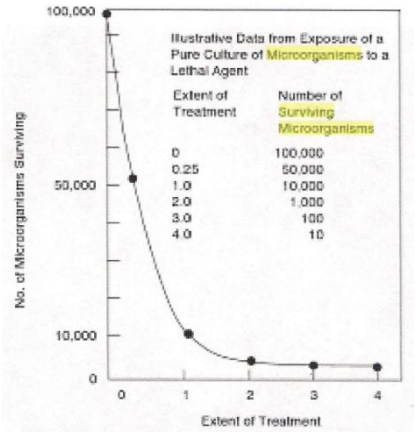


Figure 2.10
*Number of surviving microorganisms after sterilization treatment **[49]**.*

For a given treatment, the probability of survivals is determined by the number and the resistance of microorganisms and by the environment (moisture content, thermal energy and time for steam sterilization) in which they exist during treatment **[45]**.

2.4.1 Moist Heat Sterilization

Moist heat sterilization is the most efficient biological agent greatly used in pharmaceutical industry and it is a physical method as radiation and filtration (gaseous method is a chemical one) **[50]**. There exists three forms of moist heat sterilization: Autoclaving (dry saturated steam), boiling water/steam at atmospheric pressure and hot water below boiling point.

Autoclaving (*Figure 2.11*) is the method used to sterilize the CMCA mixture packed in the syringes, uses pressurized steam to destroy microorganisms. This is the most effective

method for the denaturation of nucleic acids and proteins and it had been demonstrated that it is the most hard-hitting for aqueous preparations **[46]**.

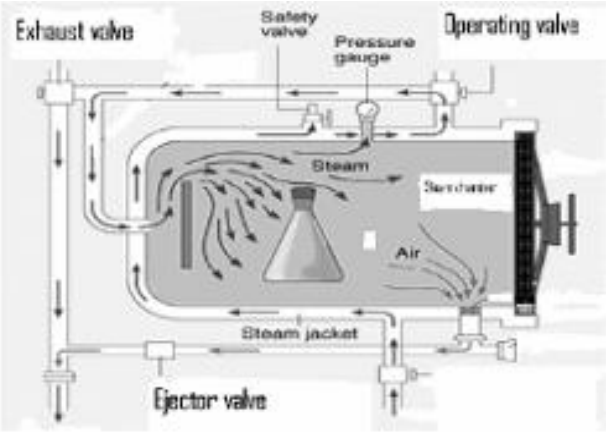


Figure 2.11
*An Autoclave **[4]**.*

A generic sterilization cycle includes heating, sterilizing and cooling phases characterized by physical parameters such as exposure time, temperature and pressure, governing the efficiency of the sterilization.

The accepted range of sterilizing temperatures is between 118 and 134°C and the *US Pharmacopeia (USP)* explains, in a footnote, that "an autoclave cycle, where specified in the compendia for media or reagents, is a period of 15 minutes at 121°C, unless otherwise indicated". Both the *European Pharmacopoeia (EP)* and the *British Pharmacopoeia (BP)* recommend a heating process at minimum of 121°C for 15 minutes as a reference condition for aqueous preparations.

The exposure timing begins when the temperature sensor (placed in the coldest spot of the chamber) reaches the set temperature; the sterilization time can be exceeded to guarantee a sterilization, also in a worst case of contamination, if the material is heat-resistant. On the other side to avoid product's degradation it is suggested to use a longer cycle at lower temperatures **[50].**

The cycle presented in *Figure 2.12* shows an initial prevacuum cycle ensuring a more effective penetration of the saturated steam in the material and avoiding the presence of cold spots **[50,51]**.

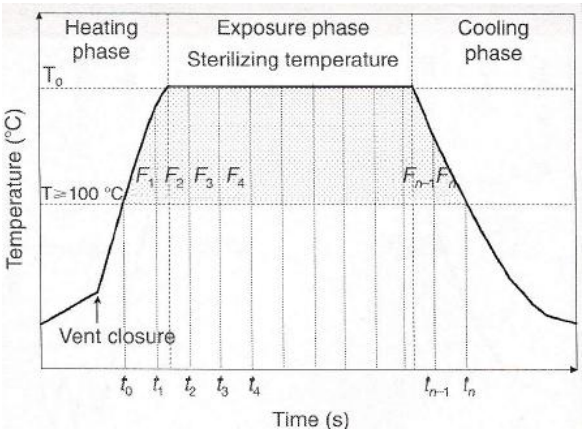


Figure 2.12
*Sterilization cycle with forced air removal **[46]**.*

The parameters usually used to identify a particular sterilization cycle and considered to evaluate the effects it has on the product are the followings:

1. D value (or decimal reduction value) represents the temperature coefficient for the lethal process and is the exposure time in minutes required to cause a 1-logarithm or 90% reduction in the population of a particular microorganism. Changing temperature the microorganism's resistance change. The smaller the D value, the more sensitive the organism is to the lethal agent.
2. Z value: defined as the number of degrees of temperature required for a 1-logarithm change in the D value.
3. F_0 value, at a particular temperature, other than 121°C, is the time (minutes) required to provide the lethality equivalent to which is provided at 121°C for a stated time. This value could be used to compare the lethality and the aggressiveness of a sterilization cycle. The greater the F_0, the greater the treatment strength.

$$F_0 = D_{121}(\log N_0 - \log N)$$

Equation 2.7
*Relationship between F_0 and the microorganisms inactivation **[10]***

.

2.4.2 Three Different Sterilization Cycles

Changing the sterilization cycle parameters, presented in Paragraph 2.4.1, the final characteristics, of the sterilized product, are influenced, three different cycles were tested in order to choose the one able to guarantee sterility and to maintain a correct mechanical behaviour of the product. The following parameters, reported in *Table 2.6,* were used by a subcontractor, to perform the test.

PARAMETER	CYCLE 1	CYCLE 2	CYCLE 3
Exposure Temperature [°C]	121,2	121,2	121,2
Temp. deviation limit (high) [°C]	1,0	1,0	1,0
Temp. deviation limit (low) [°C]	1,0	1,0	1,0
Exposure F0 [min]	15	11	11
Z value [°C]	14,1	14,1	10,0

Table 2.6
Sterilization Cycle's Specimens

Comparing the three cycles' parameters it is clear that:

- Exposure Temperature was the same for each cycle
- Cycles 2 and 3 presented a lower value of F_0 that mean that they had less exposition time than Cycle 1.
- Cycle 3 had a lower Z value than Cycles 1 and 2 so, with Cycle 3, more time was required to obtain the same mortality effect on microorganisms (in the same time the mortality value was lower **[52]**).

These considerations allow to affirm that Cycle 3 is the most conservative and Cycle 1 the most aggressive with respect to mechanical properties. To get evidence of the influence of each cycle, in the CMCA mixture, an analysis of the rheometric properties has been performed at the Industrial and Information Department of Engineering of the University of Trieste.

In the mechanical spectrum, reported below, the trends of G' and G" are indicated for each cycle and it can be seen there have been done two measures for each cycle sample in order to check reproducibility.

Considering air filling the three sterilization cycles caused similar degradation in fact the G' and G" values at 0,5Hz and 2,5Hz are similar. If the nitrogen filling is considered, it can be seen that Cycle 1 is the most aggressive and caused the major reduction of both moduli, while Cycle 3 is the most conservative and presented the biggest values of the elastic and the viscous moduli. It is important to consider that the product used in the three different cycles came from the same batch and so the initial characteristics were the same. The rheometric data confirmed that air filling is sensitive to all the three sterilization cycles considered, while nitrogen resists better to degradation presenting, after Cycle 2 and 3, values greater than the values obtained by air filling in Cycle 1, 2 or 3.

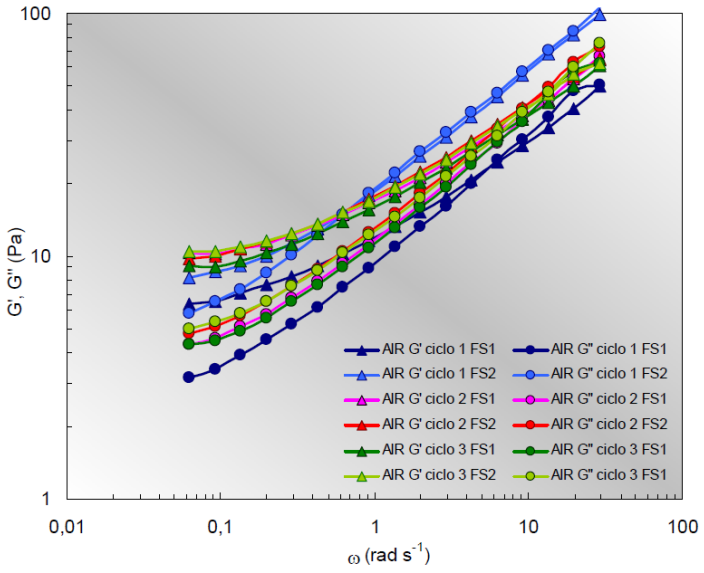


Figure 2.13

Elastic and viscous moduli of CMCA mixture sterilized with the three different cycles (O_2 filling).

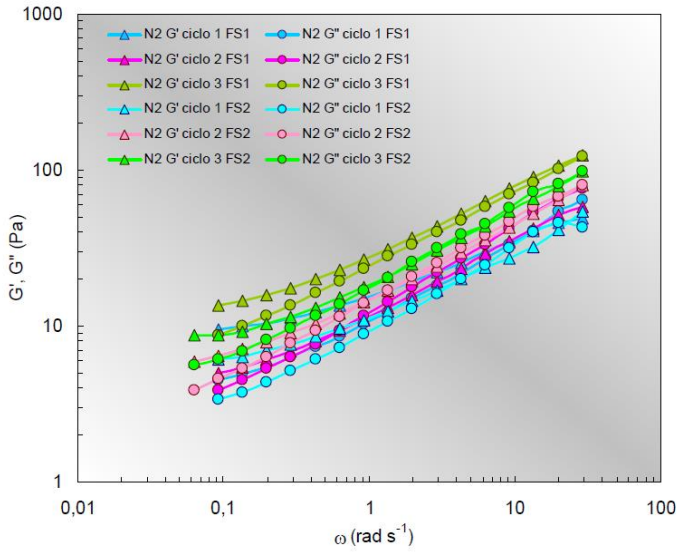


Figure 2.14

Elastic and viscous moduli of CMCA mixture sterilized with the three different cycles (N_2 filling).

2.5 PARAMETERS INFLUENCING CMCA CHARACTERISTICS

From previous investigations, it becomes clear that every process step may influence the final product's behaviour. Thus, it is necessary to understand which treatments, substances, conditions of working and storage, improve or decrease mechanical, rheological and chemical characteristics of CMCA gel and polymer. Each parameter was considered and analysed to understand how it is related to the product's characteristics and how it can modify them.

2.5.1 Sterilization Effects

Sterilization is a mandatory step that the product has to withstand: it has to be investigated how it influences viscosity. As described before, autoclaving expose the CMCA mixture to high temperature and pressure causing a decrease in viscosity. Under normal temperature conditions, the effect is reversible but longer heating and higher temperatures degrade CMC and permanently reduce viscosity **[53]**.

Pei-I Chu et al. **[51]** demonstrated that the increase of the exposure time, decreases the viscosity of the CMC gel (see *Table 2.6*).

Effect of Sterilization Time on Final Gel Viscosity

Time (min)	Average Viscosity ($n = 2$) (cps)
17	7128
19	6685
21	6586
23	5506

Table 2.6
*CMC viscosity with different exposure time **[51]**.*

They also demonstrated that the lower the starting pressure the greater is the final viscosity. There is a thermodynamic reason in fact the reaction rate is related to pressure *p* as shown in *Equation 2.8*.

$$\frac{d\ \ln K}{d\ p} = \frac{-\Delta V}{R * T}$$

Equation 2.8
Starting pressure and viscosity relationship.

where *K* is the rate constant, *p* the reaction pressure, *V* is the volume of the activated complex in the solution, *T* the absolute temperature and *R* the universal gas constant. For a bimolecular reaction, the activated complex is supposed to have smaller volume than the sum of the values for the reacting molecules; because of this, increasing the pressure, the reaction rate should increase inducing a greater degradation. The starting pressure is also directly proportional to oxygen's concentration. To demonstrate that oxygen could cause material's degradation Pei-I Chu et al. tried to perform some cycles of sterilization by autoclave, flushing the product with oxygen, nitrogen or both. What become clear was that the presence of oxygen induced a greatest degradation, than the presence of nitrogen or both oxygen and nitrogen, with the consequent highest viscosity drop. The presence of the two gasses contemporary, caused an intermediate degrading condition between the higest degradation caused by O_2 and the lowest degradation caused by N_2 **[51]**.

As demonstrated, the sterilization affects the product's properties and differences in the sterilization cycle differently influence the stability of the product. A preliminary study,

performed at the University of Trieste, of the effects of the three cycles in the trend of the product's elastic and the viscous moduli, at the different solicitation frequencies, confirmed that Cycle 1 is the most aggressive and the one which causes, not only the greater degradation at the procedure time, but also that induces a greater drop in mechanical properties in time (see *Figures 2.15* and *2.16*).

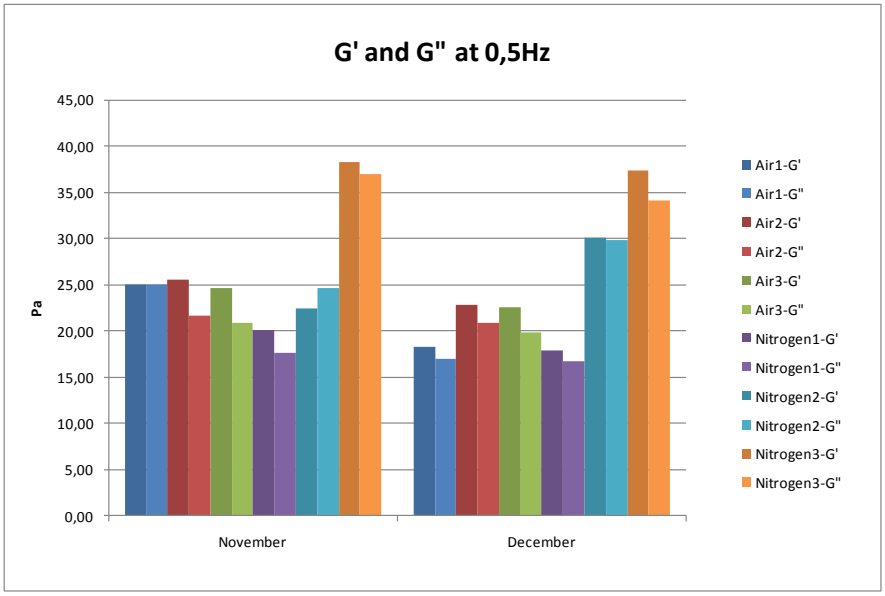


Figure 2.1
G' and G" at 0,5Hz changing in a month storage at room temperture

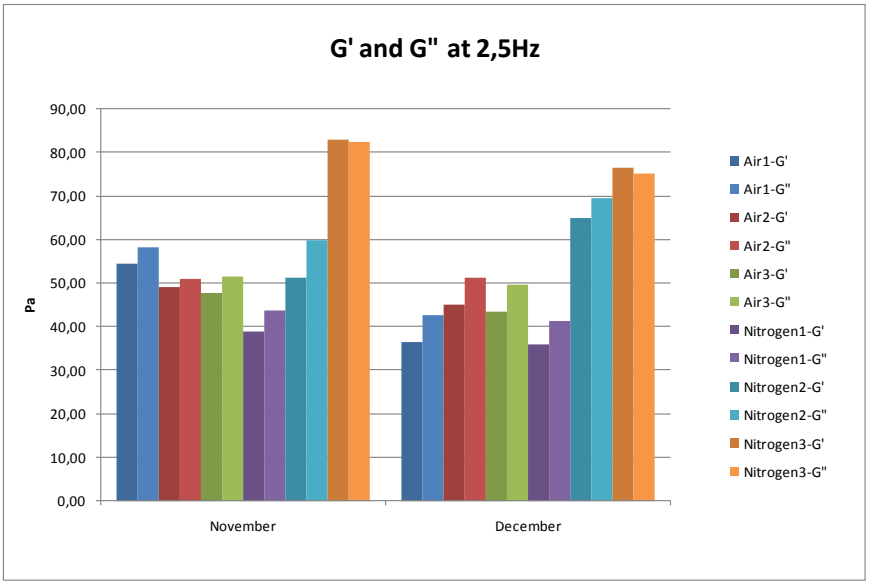


Figure 2.16
G' and G" at 2,5Hz changing in a month storage at room temperature

A more specific study on the stability of the CMCA mixture will be discussed in the next chapter in order to understand its degrading kinetic and its shelf life.

2.5.2 Other Substances and Storing Conditions Effects

There are some substances that, while added, can modify the final viscosity of the product. The order in which solutes are added is important, in fact if CMC is hydrated and the solute is then added, it has only a small effect on viscosity. However, if the solute is dissolved before CMC addition, it inhibits breaking up of crystalline areas and lower viscosities are obtained **[53]**.

The presence of NaOH influences the substitution degree (DS) of CMC so it affects the average number of hydroxyl group in the cellulose structure which are substituted by carboxymethyl or sodium carboxymethyl groups. If the NaOH concentration increases, the DS increases causing also a growth in the tensile strength of CMC films **[54]**.

Other substances tested for their effect on CMC viscosity are chelators such as EDTA (ethylenediaminetetraacetic acid) which is an autoclave stable chelator. Because of its stability at high temperature and pressure, EDTA does not lose its chelating action during autoclaving, preventing CMC viscosity loss if added before the treatment. The capacity to prevent the viscosity drop is due to the fact that chelators sequestrate metal ions that would otherwise catalyse the breakdown of the polymer-gel mixture through free radical reactions in the high heat and pressure of the autoclave **[55]**.

Methionine, methionine derivatives and small peptides comprising one or more methionine rediues or derivatives could also be added to CMC preparations but only after autoclaving because they are not necessarily stable at high temperatures. These substances interact with free radicals preventing their degrading action on CMC.

Peroxides such as H_2O_2 can adversely impact the viscosity causing degradation due to the fact that the C-H bond is vulnerable to radical mediated oxidation. The higher the concentration of hydrogen peroxide, the greater the viscosity loss in the CMC preparation is. Many anti-oxidants exists but the majority of them cannot withstand the

autoclave process. EDTA can be used to prevent oxidative, as well as autoclave, viscosity drop.

An autoclaved CMC preparation stored at 40°C for four weeks or at 5°C for six months in absence or in the presence of 400ppm of EDTA, 1,8mg/ml of methionine or both could give information on the effects of the substances presented and about their interaction. It was seen that, at 40°C without EDTA or methionine, viscosity decreased significantly in presence of H_2O_2 which concentration was of 2, 5, 20 or 50ppm. EDTA can reduce the rate of viscosity loss but could not completely prevent it storing at 40°C. Methionine was able to prevent the viscosity loss even at the greater concentration of hydrogen peroxide and the same result can be consequently obtained in the presence of both EDTA and methionine. In case of storage at 5°C, EDTA can itself prevent the viscosity loss possibly because at this temperature the rate of generation of free radicals is slower allowing EDTA to exert its sequestering activity on catalytic metals minimizing the amount of free radicals **[55]**.

The last consideration underlines the importance of a proper storage in order to maintain the desiderated characteristics and to retard aging effects. Considering storage conditions the protection from air with an airtight container prevent viscosity drop **[51,53]**. It is important also to protect against microbiological attack, to avoid prolonged exposure to elevated temperatures and exclude sunlight **[53]**.

CHAPTER 3: STABILITY AND SHELF LIFE

3.1 RHEOMETRIC ANALYSIS

The rheological tests, which provide the values of the elastic and the viscous moduli, were carried out at the Department of Industrial Engineering and Information Technology of the University of Trieste, using a rotational stress controlled rheometer equipped with a parallel plate device. The instrument controls the torque M (related to the shear stress) applied to the system, and measures the rotational velocity or angular displacement (related to the shear rate or strain, respectively). The ratio between the maximum stress and the maximum strain is the dynamic modulus G* that can be split into two components: the elastic and the viscous moduli. Ideal elastic fluids present a dynamic response to sinusoidal oscillatory shear, in which stress and strain are in phase while ideal viscous fluids are in quadrature **[58]**. For a viscoelastic material stress and strain are out of phase of an angle δ between zero and $\pi/2$. If $\tan(\delta)<1$, the elastic behaviour predominates since $\tan(\delta)$ is the ratio between the viscous modulus G" and the elastic modulus G'.

Both the stress and strain functions are sinusoidal. The following chart reports the stress and strain expressions for an elastic solid, a viscous fluid and a viscoelastic material.

SAMPLE	STRAIN	STRESS
Elastic Sample	$\gamma(t) = \gamma_0 \sin(\omega t)$	$\sigma(t) = G\gamma_0 \sin(\omega t)$
Viscous Sample	$\gamma(t) = \gamma_0 \sin(\omega t)$	$\sigma(t) = \eta\gamma_0\omega \sin(\omega t)$
Viscoelastic Sample	$\gamma(t) = \gamma_0 \sin(\omega t)$	$\sigma(t) = \gamma_0[G'(\omega)\sin(\omega t) + G''(\omega)\cos(\omega t)]$

Table 3.1
Stress and Strain expressions for elastic, viscous and viscoelastic samples.

G' and G" values can be derived from the fundamental experimental data, i.e. the maximum stress, the maximum strain and the phase shift as follows:

$$G' = \left(\frac{\sigma_0}{\gamma_0}\right)\cos\delta$$

Equation 3.1
Elastic Modulus expression.

$$G'' = \left(\frac{\sigma_0}{\gamma_0}\right)\sin\delta$$

Equation 3.2
Viscous Modulus expression.

The mechanical spectrum of a material is composed of two curves, describing the dependence of the elastic and the viscous moduli on the imposed oscillation frequency. They are obtained with a frequency sweep test (FS) in which frequency is increased or decreased within a predetermined range, keeping constant the maximum strain or the maximum stress. The experiment is performed in small oscillation amplitude mode (SAOS) to obtain information only perturbing the material structure and then characterizing its linear viscoelastic properties **[58,59]**. A schematic representation of the frequency sweep test is reported in *Figure 3.1*. Two consecutive frequency conditions are separated by a pause in order to avoid interaction effects.

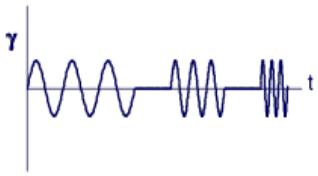


Figure 3.1
*FS Test with decreasing frequency and pauses **[58]**.*

In the case of the CMCA polymer-gel composite, the mechanical spectrum obtained from the frequency sweep test allows describing its behaviour in the whole frequency window explored and also individuating its viscoelastic responses at 0,5 Hz and 2,5Hz, which are the characteristic frequencies of the movement of the knee joint in walking and running conditions, respectively **[26]**. Comparing the mechanical spectra of the CMC raw material and the CMCA composite material, that represents the final product, it can be evinced how the manufacturing process, in its totality, changes the rheological behaviour. Focusing the attention on CMCA mixture, in correspondence of 0,5Hz, G' is between 31,0 and 45,0 Pa (average 38,4 Pa) while G" is in the interval 32,0-41,0 Pa (av. 37,0 Pa); for 2,5Hz the elastic and the viscous moduli are between 70,0-96,0 Pa (av. 83,0 Pa) and 75,0-90,0 Pa (av. 82,6 Pa), respectively. These results were obtained in a series of experiments performed on different samples of the product.

Another interesting rheological characterization of the linear viscoelastic properties of the material can be obtained from a creep-recovery experiment. The test is carried out by applying a constant stress on a material's sample to obtain the corresponding strain-time curve. In the following recovery segment the stress is removed and the strain relaxation is recorded **[59]**. The total recoverable deformation is a measure of the material elasticity and corresponds to the energy stored in the sample during the creep segment, while the non recoverable part refers to the dissipated energy related to the sample viscosity **[60]**. The recovery test is more important: a continuous shear rate condition is applied between two oscillatory time sweeps performed at 1 Hz and small amplitude oscillatory conditions. The third segment serves to evaluate if and how much both the viscoelastic moduli recover their original values, attained in the first oscillatory time sweep before the continuous shear segment.

The percentage of recovery for both moduli is calculated from the following expressions: with *Equations 1.1* and *1.2*.

$$\Delta G'_{\%} = 100 * \left(\frac{G'_{after}}{G'_{before}} \right)$$

Equations 1.1
G' percentage variation.

$$\Delta G'_{\%} = 100 * \left(\frac{G'_{after}}{G'_{before}} \right)$$

Equations 1.2
G" percentage variation.

G_{before} is the value of the considered modulus (G) attained at the end of the first segment before constant shear rate condition, and G_{after} is the value at the end of the prolonged final segment at 1Hz as shown in *Figure 3.*

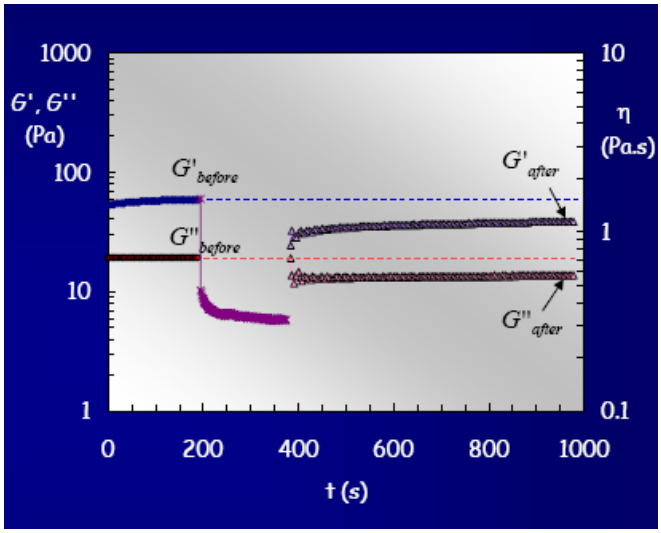


Figure 3
Example of recovery test.

Observing the test conducted on sample of CMCA mixture filled in oxygen or nitrogen and sterilized with the three different cycles, described in Chapter 2, it is possible to get a further confirmation that the filling technique, based on nitrogen as propellant gas and on the sterilization Cycle 3, presents the best recovery characteristic. The recovery test

was also carried out applying different shear rate values ($100s^{-1}$, the most aggressive, $10s^{-1}$ and $1s^{-1}$). The greater the shear rate, the lower was the recovery.

All the test described were performed at controlled temperature of 25,0°C (SD 0,1°C).

3.2 STABILITY AND SHELF LIFE

In order to get a more specific characterization of the CMCA mixture, it becomes necessary to study the stability of the material in different environmental conditions with the aim to define the proper storage procedure and to set a correct shelf life for the final product.

Real time studies are the best way to understand the real aging and the degradation of products, but it is not always possible to perform them due to the long time they take, that are not compatible with commercial requirements. In order to avoid this limit, it is possible to perform accelerated tests, in which the material is subjected to an external, more severe or more frequent, stress for a relatively short period of time.

To perform an accelerated aging test, and to understand the correspondence between real time aging and accelerated experimental time of the CMCA polymer-gel composite, a test has been designed referring to the standard guide ASTM F 1980-07 *Standard Guide for Accelerated Aging of Sterile Barrier Systems for Medical Devices* **[61]**. This standard is currently used for all Lima products to test only the resistance and the potential degradation of packaging.

The initial target was to test the packaging resistance through accelerated aging process and in the meanwhile understand any eventual modification in chemical-physical structure of the product.

This procedure is based on the assumption that chemical reactions involved in the degradation of materials follow the Arrhenius' reaction rate law: a 10°C increase or decrease in temperature of a homogeneous process results respectively in a two times or a half time change in the rate of a chemical reaction.

This concept is expressed by a Q_{10} aging factor of 2 that is a value suitable for the great part of materials for packaging such as TYVEK® or PETG but it was not appropriate to perform the test on the CMCA composite material **[61]**. In fact the accelerated test,

carried out according to standard's specifications, caused a very high degradation of the product ; in these conditions, it was not possible to predict any degradation kinetics, and, hence, to describe the degradation of the product without completely destroying it, another value for the Q_{10} has to be investigated. The description of the test and the results obtained are presented in paragraph 3.2.1 where *Figure* 3.3 shows the differences between the results and the real aging of the product. This figure clarifies why a Q_{10} value of 2 was not appropriate for the product.

A possible reason why mechanical properties vary in time is that polymers are degraded by random scission of the bonds, causing a reduction in chains length and consequently a viscosity drop. The relationship between viscosity and molecular weight is expressed by the Mark-Houwink-Sakurada equation **[62,63]**:

$$\eta = K * M^{a}$$

Equation 3.3
*Mark-Houwink-Sakurada equation **[62].***

K is a constant and *a* is a scalar which relates to the stiffness of the polymer's chains. These values are typical of the polymer analysed and they have been determined also for CMC (carboxymethyl cellulose) by Wirick M. G. in 1968 **[62]**.

The relationship between degradation and mechanical changes in a product is widely represented in literature: for example in "*Additives to Lower and Stabilize the Viscosity of Pyrolysis Oils during Storage*" by Diebold et al. **[64]** they demonstrated that pyrolysis oils contain reactive organics compounds that, during storage, interact changing physical properties as viscosity (in this particular example it increased in time). Due to the fact that this process progressed in time, they used it as a tool to obtain an aging measure; in fact they asserted that viscosity measurements are an indirect measure of the polymerization reactions that occur in the oil and the best way to measure it in polymers field **[64]**.

In 2010 it was reported **[65]**, as a good procedure to determine the stability of the analysed product with accelerated aging technique, the measures of viscosity before and after the test to determine its change that is related to the polymerisation reaction.

They also argued that a small change in viscosity corresponds to a more stable material, confirming that the material's stability is strictly related to the physical and mechanical stability in time.

Another reason why viscosity is a measure of the reaction rates is that viscosity is connected with diffusion coefficients of the molecules in mixtures **[66,67].** This is because viscosity changes in time, and thus mechanical properties changes, are therefore in relation with the rate constant of the reaction **[68]**.

The exposition to high temperature and the monitoring of consequent mechanical changes is presented as a good testing procedure in "*Accelerated Testing, Statistical Models, Test Plans and Data Analyses*" **[69]** corroborating the choice to perform an accelerated test exposing the CMCA polymer-gel to high temperature and to evaluate the corresponding drop in the elastic and viscous moduli by rheometric analysis.

FDA Guidance for Stability Testing **[63]** recommends to perform physical, chemical, biological and microbiological test before and after an aging procedure, both real time and accelerated, to be able to quantify the aging effects.

Considering these studies and results collected from different polymeric products and applications, it can be assumed that mechanical properties investigation may be a suitable way to get information on the rate of aging of the CMCA polymer-gel composite material. However, in this particular case, viscosity is not a suitable parameter for material characterization, due to the biphasic polymer and gel structure and the related viscoelastic behaviour.

In the previous Chapter, G' and G'' have been widely used for the characterization of CMCA blends, so that it can be assumed that any variation of these moduli vs. time at different temperatures may represent a proper method to evaluate material degradation.

These parameters are connected with mechanical behaviour change; chemical changes, as for example pH variations, could be taken as a measure of the product aging **[62]** but it has been seen that the CMCA composite material does not show any changes of this characteristic.

It has to be considered that G' and G" are frequency-dependents and a reference condition has to be taken into consideration: assuming 0,5 Hz and 2,5 Hz as reference frequencies, it could give the information on the field of application for the considered medical device. It is clear that the most suitable way to analyse the aging of the CMCA composite material is to consider the elastic and the viscous moduli and their changes to investigate the aging of both the polymeric and gel components. A measure of the rate of the elastic and viscous moduli drop is an indirect measure of the degradation process so it allows performing aging studies in order to understand the degradation kinetics.
Another assumption must be done before proceeding with testing: the model considered has been set considering that the degradation follows the Arrhenius' reaction rate function. This means that a 10°C increase or decrease in temperature of a homogeneous process results respectively in a two times or a half time change in the rate of a chemical reaction.

3.2.1 Q_{10} and Accelerated Test

To perform accelerated test and to find out the correspondence between real time and accelerated aging, the relationship presented in *Equation 3.4* is necessary. Desired *(RT)* is the real time aging corresponding to the accelerated aging obtained exposing to high temperature the sample for the Accelerated Aging Time *(AAT)*.

$$AAT = \frac{Desired(RT)}{AAF}$$

Equation 3.4
Accelerated Aging Time ***[61].***

AAF is the Accelerated Aging Factor that can be found with the relationship existing between the material's Q_{10} and the difference between the room temperature and the high temperature considered, as shown by the *Equation 3.5*. The Q_{10} value is a parameter connected with a change in the degradation reaction rate at two temperatures which differs for 10°C as *Expression 3.6* will show.

$$AAF = Q_{10}^{\left[\frac{T_{AA}-T_{RT}}{10}\right]}$$

Equation 3.5
Accelerated Aging Factor ***[61].***

T_{AA} is the accelerated aging temperature [°C] and T_{RT} is the room temperature [°C].
For example, if the aim is to age the product of one year exposing it at a temperature of 55°C instead of waiting a year at room temperature, the procedure, considering the standard value for the Q_{10}, is:

$$AAF = 2^{\wedge\left(\frac{55-22,5}{10}\right)} = 9,51$$

and

$$AAT = \frac{365}{AAF} = 38,38$$

To obtain a one-year-aged product exposing it to 55°C the treatment has to last 38 days.
Considering the degradation reaction for the CMCA composite material, preliminary experimental data, whose results are presented in *Figure 3.3*, suggested a value higher than two for the Q_{10} parameter. The test was performed on product's samples filled with nitrogen in glass syringes. It was used a Q_{10}=2 and exposure temperature of 55°C for 10 days, corresponding to 90 days at 22,5°C. The viscosity loss was of about 76,57% that was not corroborated by real time aging observed in 90 days in the sample analysed in which the viscosity loss was about 44,75% as *Figure 3.3* illustrates. The difference between the sample treated with accelerated aging and the one naturally aged was so great that was appreciable at sight. The values of viscosities were calculated with a vibrational viscometer at the frequency of 30Hz.
Assuming for example a Q_{10} of 2,6 the test time will correspond to 223 days giving evidence that a greater *aging factor* means that the exposition time is equivalent to a longer real time aging.

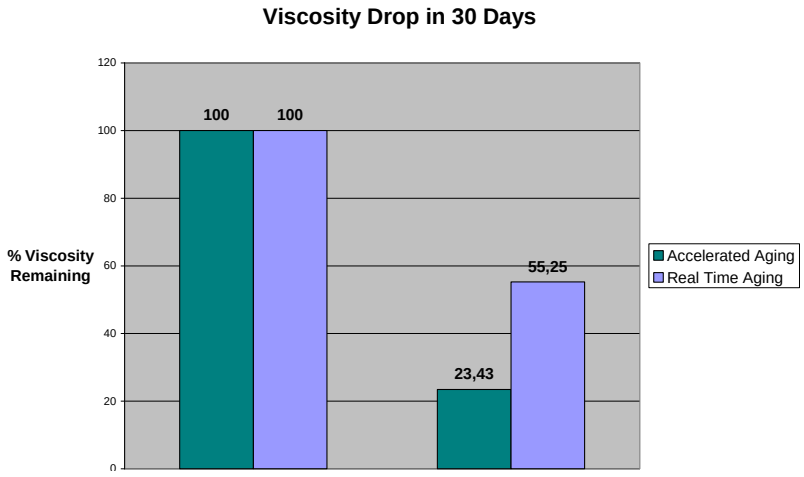


Figure 3.3
Comparison between30 days aging in Real Aging and Accelerated Aging with Q_{10}=2.

In order to find out an evidence of this hypothesis, it becomes necessary to test the material at different temperatures and to define the correct Q_{10} value able, with an additional test, to simulate the aging for the desiderated period and to evaluate the mechanical characteristics.

The Q_{10} value is connected with a change in the degradation reaction rate at two temperatures, which differs for 10°C, as the following expression shows.

$$Q_{10} = \left(\frac{k_{T_2}}{k_{T_1}}\right)^{\frac{10}{(T_2 - T_1)}}$$

Equation 3.6
Q_{10} expression.

K_{T1} and K_{T2} are the reaction rates at the two temperatures T_1 and T_2. Because of the difficulty to give a precise and correct value at the degradation reaction rate, it was developed a different procedure to evaluate the Q_{10}.

Starting from available data of real time aging in one and two months (values were collected at time zero at the beginning of the experiment, after one month and after two months), coming from the evaluation of the differences in the rheological properties it was hypothesized the worst case aging trend for the elastic and the viscous moduli characterized by a linear drop of their values.

Although it is known that degradation kinetics usually follow a parabolic trend, in this case the linear model has been preferred for two main reasons:

- during the first months the linear and parabolic trend are almost overlapping;
- the linear model represents the worst case hence the most conservative approach to set a preliminary shelf life.

The real trend and the one supposed for the study are presented in the following picture.

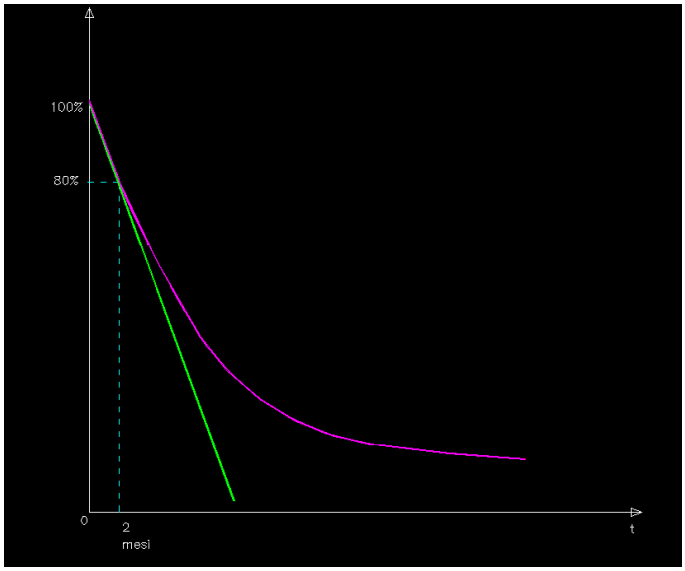


Figure 3.4
Real trend (violet) and the hypothesized trend(light green).

The real trend, in violet in *Figure 3.4*, is characterized by an initial high degradation followed by a period in which the rate of degradation becomes relatively constant and remains constant for a considerable time **[67]**. The hypothesized trend (light green) represents the worst case, in fact it supposed that the high degradation still remain through the whole life of the product analysed. In the previous picture, the period of time corresponding to two months is represented due to the fact that the hypothesized trend was developed by the use of the data coming from the real time aging in two months.

Three glass syringes, filled in nitrogen, were used to evaluate a greater aging by accelerated test. The first syringes were analysed by rheometric analysis (FS test) contemporary to the start of the exposition to high temperature (40°C and 50°C, respectively) of the other two syringes. The high temperature test lasted eight days and, at the end, the syringes were undergone to the frequency sweep test and the values of G' and G" were collected.

The temperature range chosen was not very large but there is no evidence that Q_{10} has a constant value for all the temperatures **[71]** and in the intended use of the product it will not be exposed to higher temperatures.

The percentage of the remaining Elastic and Viscous Moduli, after one and two months storage at 22,5°C, were calculated at three frequencies: 0,5 Hz and 2,5 Hz in accord with the walking and running condition respectively and 1,47 Hz to simulate an intermediate situation. These values are reported in *Table 3.2*.

The first values, at time zero for the test, was taken after one month from the product's production and G' (82,99Pa at 2,5 Hz) and G"(82,55Pa at 2,5 Hz) are very similar to the values for the human synovial fluid in the knee (at 2,5 Hz: G'=117 ±13 and G"=45 ±8) **[72]**.

SAMPLE	$G'_{0,5}$ [%]	$G''_{0,5}$ [%]	$G'_{1,47}$ [%]	$G''_{1,47}$ [%]	$G'_{2,5}$ [%]	$G''_{2,5}$ [%]
1month at 22,5°C storing	97,63	92,29	94,25	92,35	92,10	91,13
2months at 22,5°C storing	83,45	83,09	79,71	83,62	80,08	86,06

Table 3.2
Percentage Remaining of G' and G' after one or two months storage at 22,5°C at 0,5 Hz, 1,47 Hz and 2,5 Hz.

These remaining percentages of both moduli were compared to the predicted ones with the linear drop hypothesized and, in this way, the correspondence between 8 days aging at high temperature (40°C and 50°C), and the days in real time aging was found out, as *Figure 3.5* illustrates.

Eight days at 40°C caused a modulus loss (both for G' and G") of about 30-40% corresponding to 130 days at room temperature in accord with the linear prediction

(green line of *Figure 3.4*); 8 days at 50°C caused a G' and G" loss of about 40-50% as 160 days at 22,5°C.

This relationship between the number of days at high temperature and number of days at room temperature allowed finding out the value of the Q_{10}, by the use of *Expressions 3.4* and *3.5* which were calculated separately for the three frequencies and for G' and G".

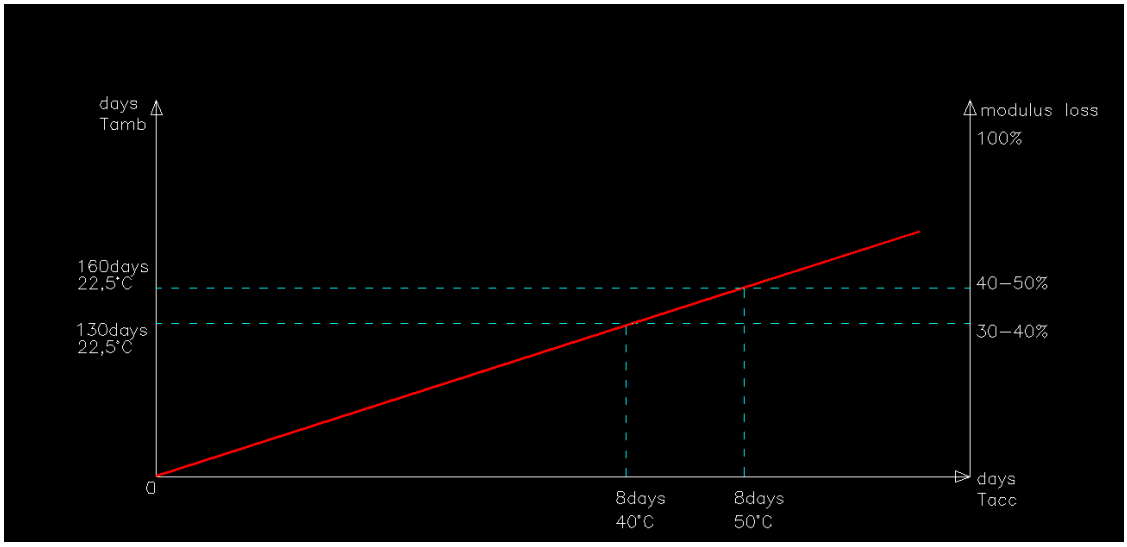


Figure 3.5
Correspondence between degradation caused by real time and accelerated aging.

No great differences were observed between the two moduli nor among the three frequencies considered; comparing the Q_{10}, obtained by the use of the data coming from the experiment conducted at 40°C or 50°C, they were not the same as it can be seen in the next chart. The average value of Q_{10} calculated for the experiment at 40°C was 4,9 and the average value for the test at 50°C was 3,06. A lower Q_{10} value means that the same accelerated test corresponds to less days of real aging: this outcome induces thinking that the product ages faster.

This very marked situation at 50°C can be easily explained considering how the aging model was created: a lower temperature (40°C) induces a lower aging and the lower the aging the lower is the difference between the real drop in the moduli and the predicted linear drop (as the *Figure 3.6* illustrates).

Q_{10}	$G'_{0,5}$	$G''_{0,5}$	$G'_{1,47}$	$G''_{1,47}$	$G'_{2,5}$	$G''_{2,5}$
40°C test	5,52	4,9	4,9	4,68	4,9	4,9
50°C test	3,23	3,03	3,03	3,03	3,03	3,03

Table 3.3

Q_{10} value calculated for each one of the three frequency analyzed, both for G' and G".

In the following picture it is possible to see two yellow areas representing the difference existing between the real aging (violet) and the hypothesized drop used for the study (light green). The areas corresponding to a remaining percentage of about 50%-60% is bigger than the one corresponding to 60%-70% giving evidence that the experiment conducted at 50°C, due to the greater degradation it induces, produces a higher aging and a higher gap from the real aging model. This is the reason why the Q_{10} value obtained from the experiment at 40°C was chosen as more representative of the real aging and anyway it still represent a worse case.

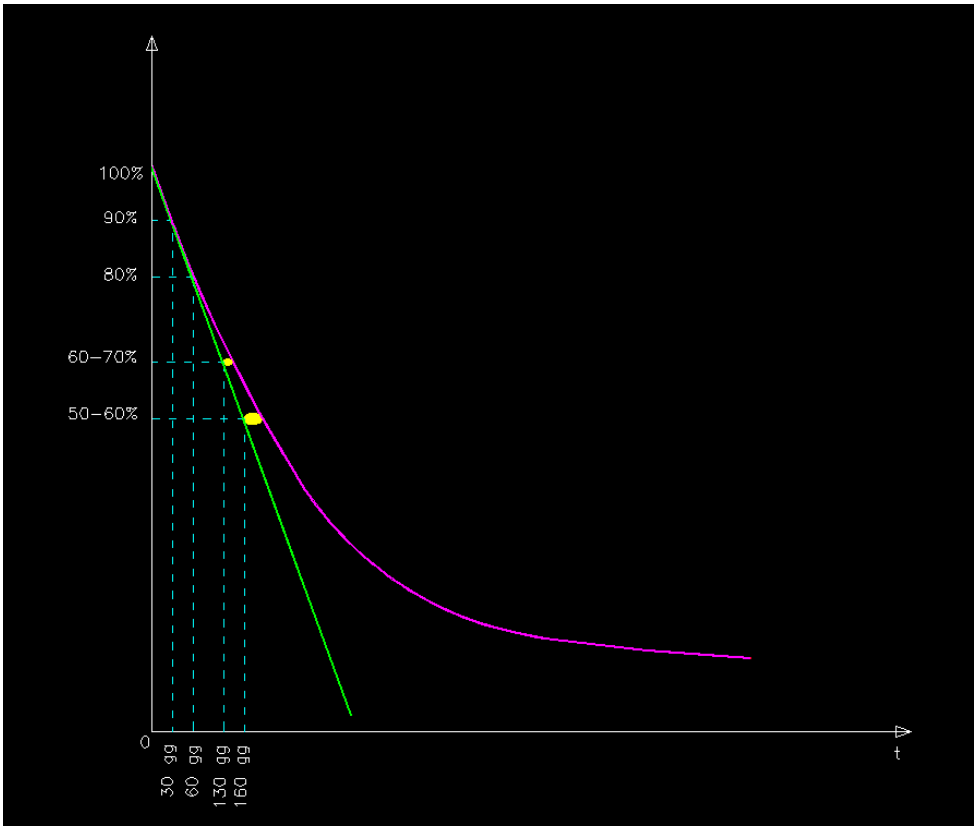


Figure 3.6

Real trend (violet) and the hypothesized one (light green) with the correspondence between aging days and the drop in the values of G' and G".

When a Q_{10} value is defined, it is possible to develop an aging experiment, exposing the product to high temperature for a precise testing time to obtain a product aged as desired.

The test shows that the Arrhenius model was not totally suitable for the CMCA polymer-gel because an increase or decrease in temperature does not result in a two times or a half time change in the rate of the degrading reaction, respectively. Further investigations should be done to verify the results obtained in more samples.

3.2.2 Storage Conditions

Storage conditions are fundamental to preserve product's characteristics and to prevent fast degradation. The adoption of glass syringes and of the automatic filling technique using the inert gas nitrogen was justified by better storage condition as illustrated in Chapter 3. In addition to these choices, the storage at 4°C was compared to the storage at 22°C to verify if a lower temperature could reduce the aging process extending the CMCA polymer-gel shelf life.

The effects on product's characteristics were analysed by rheometric analysis and the comparison between the elastic and viscous moduli in the two products, after two or three months of storage was performed.

The comparison between the remaining percentage of G' and G" after one or two months of storage at room temperature or three months at 4°C are presented in *Table 3.4*.

SAMPLE	$G'_{0,5}$ [%]	$G''_{0,5}$ [%]	$G'_{1,47}$ [%]	$G''_{1,47}$ [%]	$G'_{2,5}$ [%]	$G''_{2,5}$ [%]
3months storage at 4°C	89,10	95,07	87,75	92,38	88,69	89,39
1month storage at 22,5°C	92,10	91,13	94,25	92,35	97,63	92,29
2months storage at 22,5°C	83,45	83,09	79,71	83,62	80,08	86,06

Table 3.4
G' and G" remaining percentage after storage at 4°C or 22,5°C at 0,5 Hz, 1,47 Hz and 2,5 Hz.

The table above shows that storage of the product al 4°C reduces the degradation rate and the drop of both moduli. This is the reason why it was chosen, as the best condition for the CMCA polymer-gel composite, allowing the extension of the shelf life.

It is known that the Q_{10} value is constant for a small temperature interval but if other processes are initiated in the material, they can change the reaction rate and thus the aging rate **[71]**. The evidence is given by *Equations 3.6* and *3.7* from which it is clear that a Q_{10} = 4,97 is not adapt to represent the situation characterised by the aging at 4°C and the accelerated aging (accelerated with respect to 4°C) at 22,5°C. Applying them, it resulted that 90 days at 4°C will correspond to 4,63 days at room temperature that is very different from the real situation: in fact from the chart above, it can be seen that 90 days at 4°C induced a degradation similar to the one reached after 30 days at 22,5°C. The Q_{10} value, calculated imposing this correspondence, with the equations mentioned above, results in 1,81.

This value means that 60 days at 22,5°C correspond to 180days at 4°C: if the product is stored in fridge, after six months it will present about the 80% of the initial values of G' and G" giving evidence of a good stability over time.

In conclusion, this test highlighted that it is preferable to store the product at 4°C than at room temperature and it also allowed stating the good resistance over time of the rheological properties of the product.

CHAPTER 4: PROJECT MANAGEMENT AND MECHANICAL TESTS

The three mechanical tests performed to evaluate the wear of prosthetic implants in presence of CMCA polymer-gel dispersions, described in this chapter, are characterised by a great number of subsequent, and strictly interconnected, phases. This is the reason why a delay in one of them can prolong the whole process. The last steps, consisting in the tests themselves, were long way off the time in which they were planned.

Because of these facts, it was decided to approach the tests with the aid of project management to be able to organise activities and to estimate the time required, also in case of unexpected events.

4.1 PROJECT MANAGEMENT OF THE MECHANICAL TESTS

Project management is the discipline of planning, organizing, securing and managing resources to achieve specific goals as projects, which are temporary attempt with defined beginnings and ends **[74].**

Project management has evolved from a management philosophy, restricted to a few functional areas, into a business process. Its extension to all disciplines started with engineering but now project management resides in every profession including information systems, health care, consulting, pharmaceutical, banks and government agencies **[75]**.

By the 1990s many companies begun to realize that implementing project management was necessary due to some driving forces as competitiveness, new project development, efficiency and effectiveness. For example, if multiple simultaneous and/or large capital projects are daily present in a company, it will become important to drive to project management in order to manage them in the most efficient way. Instead, considering an organisation that heavily invests in R&D activities, the management activity is useful not only to organize the activity, but also to analyse each project and to understand if it is working good or if it is necessary to stop it**.**

Five project management methodologies exist **[75]** and are listed below:

1. *Project Management* defined as the basic principles of planning, scheduling and controlling work.
2. *Total Quality Management* which is the process aiming at assuring that the final product or the end result will meet the quality expectation of the customer.
3. *Concurrent Engineering* that is performing a work in parallel rather than in series in order to compress the project plan without incurring serious risks.
4. *Risk Management* which corresponds to the process of identifying, quantifying and responding to the risks of the project without any material impact on the project's objectives.

Only one criterion is usually chosen to get best results in the organisation of every activity, but it is also possible to integrate all of them into a single process as *Figure 4.1* shows.

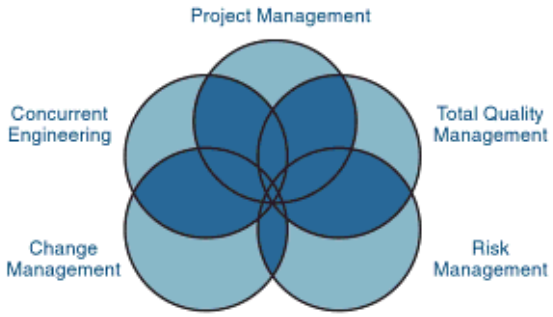


Figure 4.1
*PM methodologies **[75]**.*

The mechanical tests, described in paragraphs 4.2 and 4.3, were organized and planned with the help of Project Management to have a complete view of all the necessary steps and their interaction. The entire organization was carried on with the help of a planning software as *Figure 4.2* shows.

The following scheme reports all the process phases, the time they required and their interactions giving an idea of the time necessary to complete the tests.

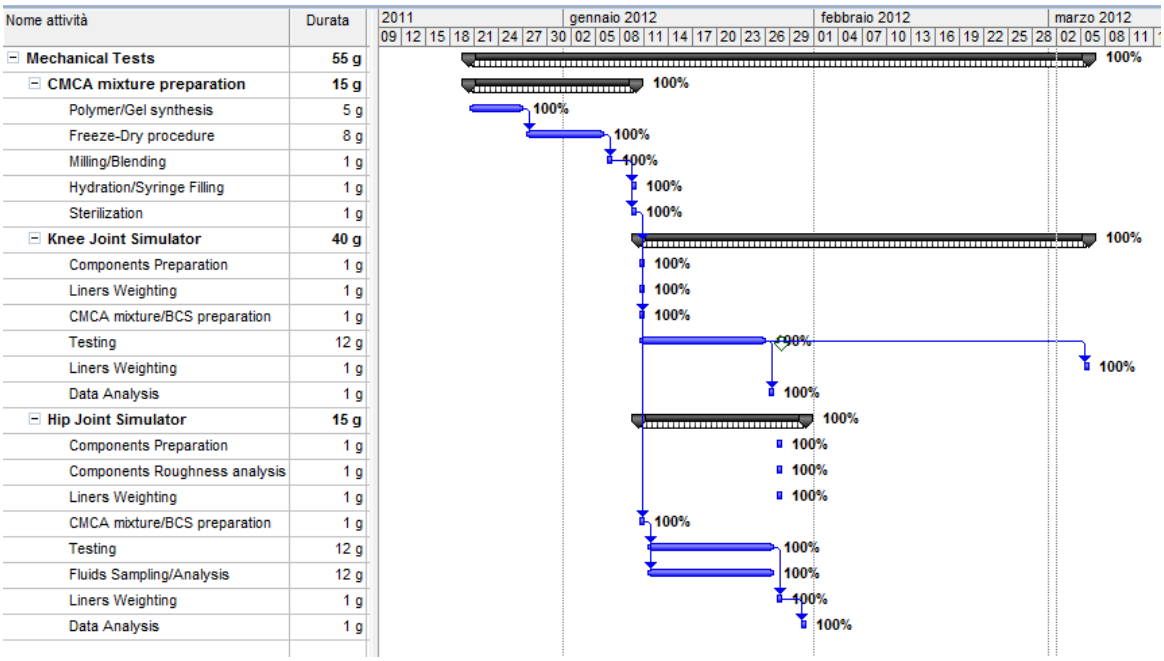


Figure 4.2
Activities Planning for Mechanical Tests.

The scheme obtained was useful to organize all the activities, prepare machines and the necessary material, and to plan the tests that have been conducted by subcontractors

like the ions test. All of these activities gave the possibility to predict the consequences of an unexpected event in terms of late. In this way it was possible to plan the work considering any adverse events giving the possibility to afford them.

4.2 KNEE JOINT SIMULATOR

This test consisted in the analysis of the wear behaviour of total knee prostheses in the presence of diluted CMCA composite material, compared to diluted calf serum as lubricants.

The second lubricant medium was composed of a solution of BCS (bovine calf serum) diluted at 25% W/W in deionized water with the addition of EDTA (7,5 g/l), to bind calcium phosphate, and Sodium Azide (4 g/l) to retard bacteria-induced degradation. This composite fluid will be termed in the following test as “serum” to mean all its compounds with the specified concentrations. The BCS has itself a total protein content of 72 g/l and this is why it has to be diluted to reach the concentration of 18 g/l in accord with the international standard **[76,77]**. Serum has been demonstrated to have the capacity to recreate “physiological wear-rates” in a manner that neither water nor saline solutions had achieved **[78]**.

In order to define the worst case, and reduce performances, the CMCA polymer-gel was diluted at 75% W/W ratio between the composite material and the calf serum. This operation reduced the mechanical properties of the fluid in which the prostheses were immersed. The CMCA mixture dilution was performed in order both to simulate the worst case for the product and the situation in which the product is injected in a joint containing synovial fluid. It is also possible that the product will be injected in a knee that has afforded arthrocentesis which are knees not containing synovial fluid; in this case the product will not be subjected to dilution.

The machine employed is a servo hydraulic four station knee joint simulator with a testing frequency of 1 Hz (admitted SD: 0,1 Hz **[76]**) able to reproduce a cyclic variation of flexion/extension angle and contact force to the interface between tibial and femoral components, simulating normal human walking. The specimens, used to create the correct movement, are defined in the *International Standard ISO 14243-1* **[76]**.

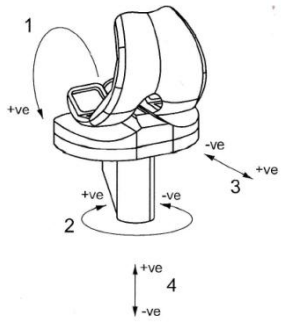


Figure 4.3
*Applied Forces on the Knee Prosthesis **[76]**.*

The applied forces are the flexion of the femoral component (number 1 in *Figure 4.3*), the tibial rotation torque (number 2), the anterior-posterior (AP) force on the tibial component (number 3) and the axial force (number 4).

The knee prosthesis used is composed of three components: the femoral component made of CoCrMo, the tibial plate in Ti6Al4V and the liner in UHMWPE (ultra-high-molecular-weight-polyethylene). Before use, all components were sterilized (the femoral component and the liner by EtO, and the tibial plate by Beta Ray). The prosthesis type used in this test is presented in *Figure 4.4.*

Figure 4.4
The knee prosthesis involved in the test

The cyclic variation of forces and angles are reported in *Figure 4.5* simulating the typical normal human walking cycle. The instrument used is provided of load cells able to verify the correct loading of the prosthetic components in accord to the defined load cycle selected.

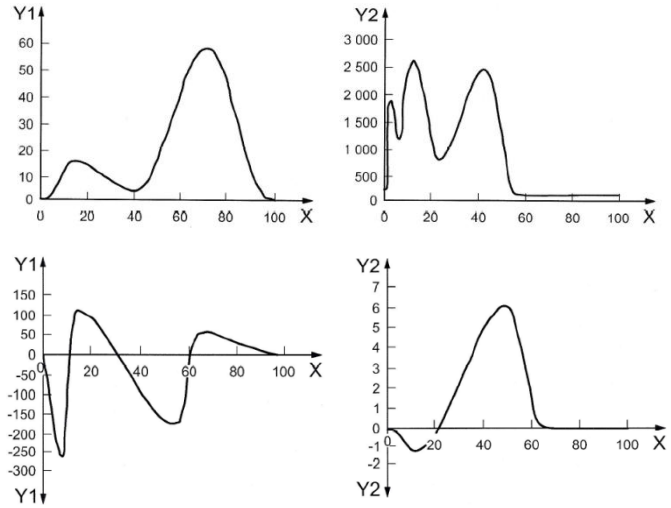


Figure 4.5
Variation during percentage of cycle time of the flexion angle (top left), axial force (top right), AP force (bottom left) and rotation torque (bottom right) ***[76]****.*

In the test, two workspaces under load were used: one with the prosthesis immersed in serum and one immersed in bovine calf serum and CMCA composite material. In addition, two other unloaded workspaces (soak control), with the same fluids, were used to evaluate material absorption. The soak controls were made of only the liner in UHMWPE, which absorbs fluid during the test (*Figure 4.6*). The absorption measure is necessary to calculate the correct value of the wear of the prosthesis, at the end of the test: in fact liners lose material but they also absorb fluid. In order to be able to carry on this control, these components had to be weighted before and after the test.

The test consisted of $1x10^6$ cycles of movement during which evaporated fluid had to be replaced with deionized water. The level sensor provided a security control: in fact if the level of the fluid goes under the limit defined, the machine stops.

To avoid excessive evaporation the containers have to be closed, as it can be seen in *Figures 4.6* and *4.7*.

Another control was given by the temperature sensor that had to verify temperature to be about 37°C, to reproduce a controlled environment simulating physiological conditions (admitted SD: 2°C) **[76]**.

Figure 4.6

S1 is the soaking sample with serum and CMCA composite;
S2 is the soaking sample with serum only.

Figure 4.7 shows the knee joint simulator with all the four stations occupied; in the analysis performed, only the central positions were used for the test, while the laterals were filled with water due to the fact that the machine needs to have all the stations occupied: even if the flexion and the rotation could be stopped in the first on the left (see *Figure 4.7*), the axial force could not and in the last position, on the right, the movement is simultaneous with the centrals.

Figure 4.7
The knee joint simulator.

The test have been stopped after 1 million cycles: then the specimens were removed, cleaned and weighted to determine the mass loss (*Table 4.1*).

The following chart reports the average weights of the liners in UHMWPE, before and after the test, obtained from a set of three measures.

SAMPLE	WEIGHT before test [g]	WEIGHT after test [g]	ABSORPTION [g]	WEAR [g]
Soaking Serum	26,211	26,212	-0,001	-
Loaded Serum	26,195	26,186	0,009	0,010
Soaking CMCA-BCS	26,101	26,102	-0,001	-
Loaded CMCA-BCS	25,914	25,912	0,002	0,003

Table 4.1
Absorption and Wear of the UHMWPE liners used in the test.

Wear and absorption values presented in *Table 4.1* are represented in *Figure 4.8*. Absorption is defined as the initial weight of the soaking subtracted of its final weight; wear is the difference between the absorption of the loaded sample and the absorption of the co respective soaking as illustrated by the following equations.

$$Absorption_{loaded/soaking} = W_i - W_f$$

Equation 4.1
Absorption for the loaded or soaking liners

$$Wear = Absorption_{loaded} - Absorption_{soaking}$$

Equations 4.2
Wear of the loaded liners

W_i represents the initial weight, before starting the test, and W_f is the final weight. In accord with these conventions, absorption and wear of the loaded liners are positive while the absorption of the soaking liners is negative.

The graph below shows that the presence of a more viscous fluid prevents the wear of the UHMWPE liner reducing material loss and its damaging; the wear rate for the UHMWPE, immersed in serum, was of 10,2 mg per million cycles while for the same component, immersed in the composite material and BCS, it was 3 mg per million cycles.

What emerges is that the solution composed of BCS and CMCA polymer-gel reduces the wear to one third providing a very effective lubrication.

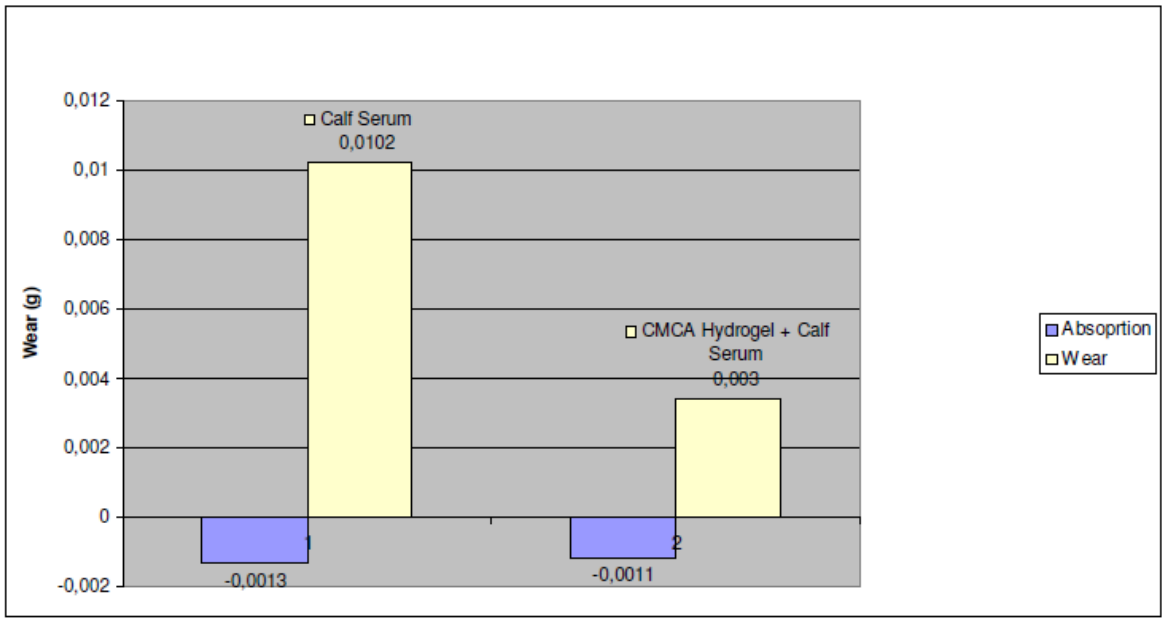


Figure 4.8
Knee Wear Test. On the left the results of the liner immersed in serum; on the right the results for the liner immersed in CMCA and BCS.

The differences in the surface of the two liners tested under load, was visible at sight. The one that was immersed in serum presented a greater damaged area and the surface was much more irregular.

These effects could be better appreciated with the picture enlargements hereinafter presented (*Figure 4.9*). On the right there is the liner tested in serum with the visible damaged area and a great presence of scratches; on the left there is the liner tested in CMCA composite material and BCS.

The test gave experimental support to the fact that the product, with its viscoelastic behaviour, is able to lubricate and protect surfaces, even under physiological loads, and maintain its action over time (1 million cycle were tested).

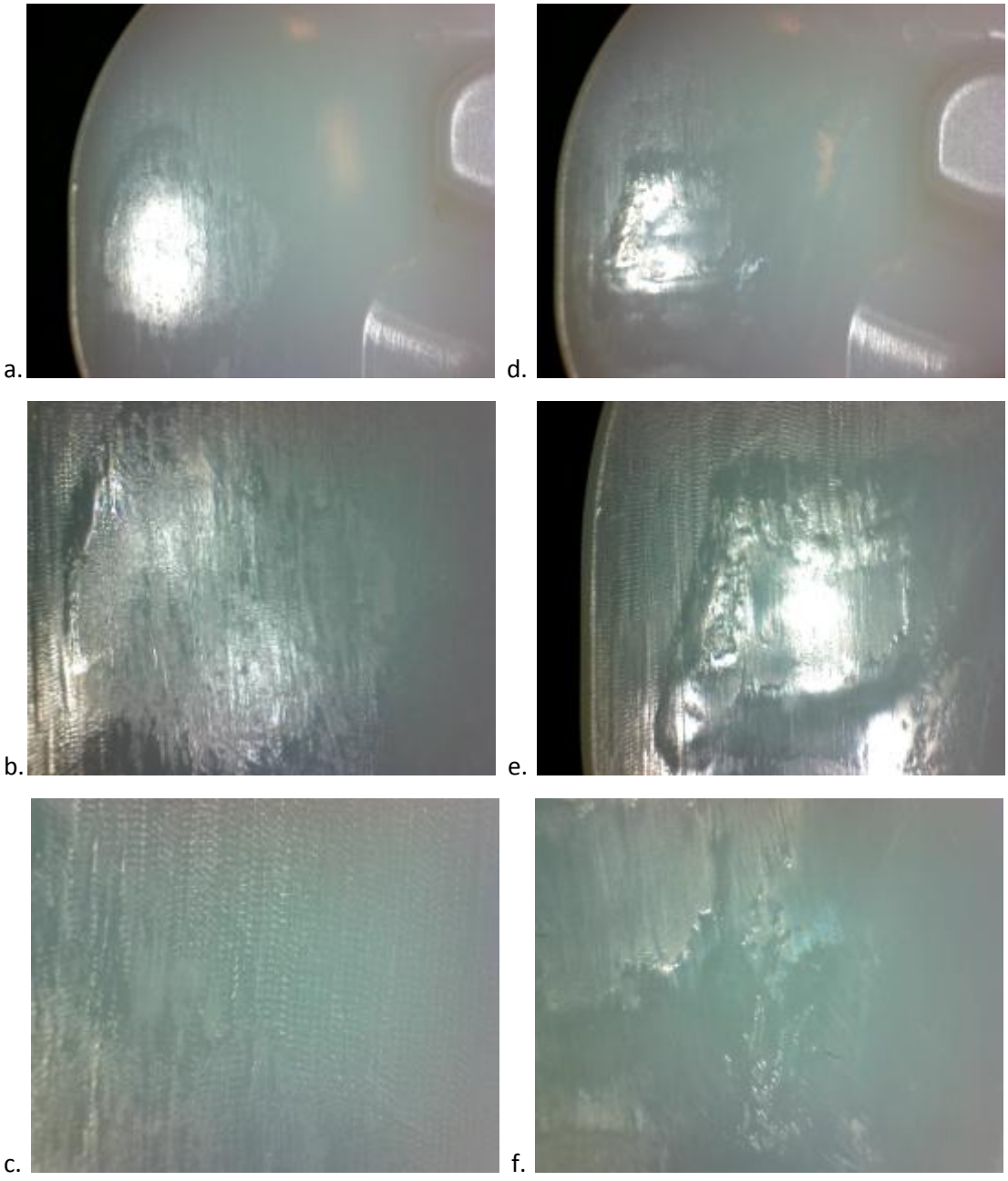


Figure 4.9
Enlarged photos of the liners used in the knee wear test:
a-b-c : Liner used in presence of CMCA-BCS;
d-e-f : Liner used in presence of BCS only.

4.3 HIP JOINT SIMULATOR

Metal on metal (MoM) total hip prostheses encountered a great use, mostly in the past, and in particular in patients younger than 65 **[79]**, due to the thought that these type of prostheses had a great resistance to wear and corrosion; according to that belief they

were also known as hard on hard prostheses **[80]**. The use of hard materials, that have been shown to have lower wear rates than metal-UHMWPE couples, took place with the aim to extend component life **[81]**. The great majority of MoM prostheses are made of CoCrMo alloys, extensively used for joint replacement due to their wear and corrosion resistance; this fact is due to a protective Cr oxide film formed on the surface that reduces corrosion and hinder metal ion release **[82]**. It has to be considered, in fact, that loads and articulations could increase corrosion rate and metal ions release.

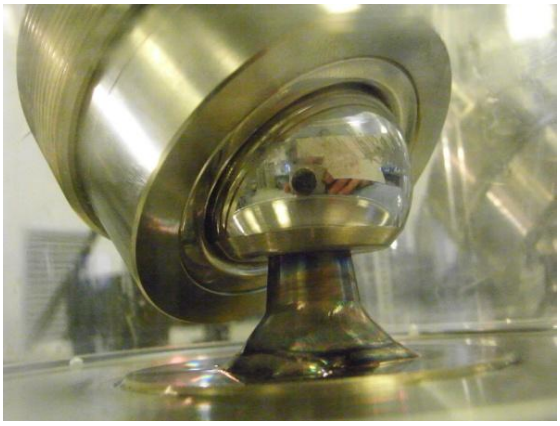


Figure 4.10
A detail of Metal on Metal coupling tested.

The reason why the production of wear particles was greatly investigated is that adverse tissue reactions are believed to be an important factor in the development of osteolysis **[83]**. There is an increasing number of reports of local tissue response mediated by degradation products, such as metal ions (usually most of them are Co ions) and wear debris, generated by wear and corrosion of Metal on Metal total hip replacements. These degradation products can cause hypersensitivity, toxicological risk and local bone resorption **[82]**. It has been demonstrated that MoM implants produce a huge number of wear particles in nanometre size, very easily absorbed by the peri-implant tissue and disseminated to other organs, which could be found in serum, urine and blood of patients with this kind of prostheses **[84]**.

The test on the hip simulator (see *Figure 4.11*) was performed in order to evaluate the effects of physiological movements on Metal on Metal total hip prostheses considering

wear and ions release. In this test two lubricating fluids were compared: bovine calf serum and CMCA mixture. The two testing fluids presented the same concentrations and the same additives used in the test performed for the knee prostheses.

This test was also performed to consider the possibility to use the CMCA polymer-gel solution to reduce side-effects in patients with MoM prosthesis and to retard the revision operation. To simulate this situation, and generally to represent the worst case, the prostheses were previously used for about 5 million cycles (a detail of the MoM prosthesis used is presented in *Figure 4.10*).

Figure 4.11
The four stations (from left to right 8-9-10-11) of the machine involved in the test.

The testing machine works at 1 Hz (SD 0,1 Hz), in accord with the International Standard ISO 14242-3 **[85]**, and the movements it reproduces are the ones hereinafter listed.

- Abduction/Adduction: motion along an axis arranged in the anterior-posterior direction through the hip joint.
- Flexion/Extension: motion that occurs about a transverse axis through the hip joint.
- Inward/Outward Rotation.

Figures 4.12 shows the movement described and the force applied by the apparatus to test the prostheses which were immersed in the lubricating media.

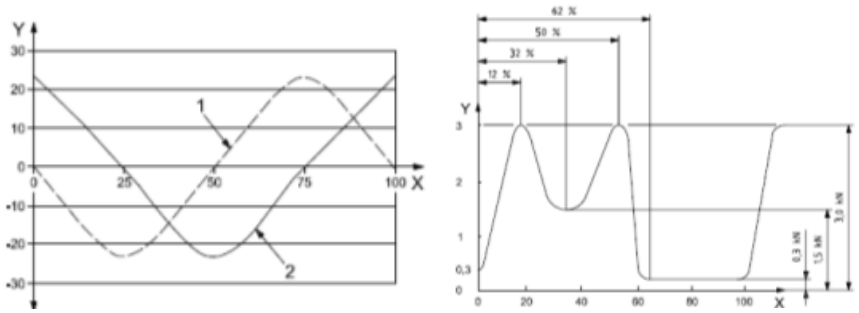


Figure 4.12

*At the top: Abduction/Adduction (1) and Extension/Flexion (2) angles changes during a working cycle **[4]**; at the bottom: Applied force on the loading axis during a working cycle **[85,86]**.*

4.3.1 Metal Ions Analysis

Samples of the testing fluid were taken at 250.000, 650.000, 950.000 cycles, in order to quantify the presence of metal ions. The volume taken was substituted with the same testing fluid, to not influence the test.

The metal analysis was centred on searching Co, Cr, Mo ions and the investigation on the differences between the two testing fluids. The test was carried on by an external laboratory and the results are presented in the chart below, with the respective standard deviation in brackets. The ion dosage has been performed by ICP (Inductively Coupled Plasma); this technique is characterized by a constant CV of 0,2. This is the reason why the greater the ion concentration, the greater the standard deviation is.

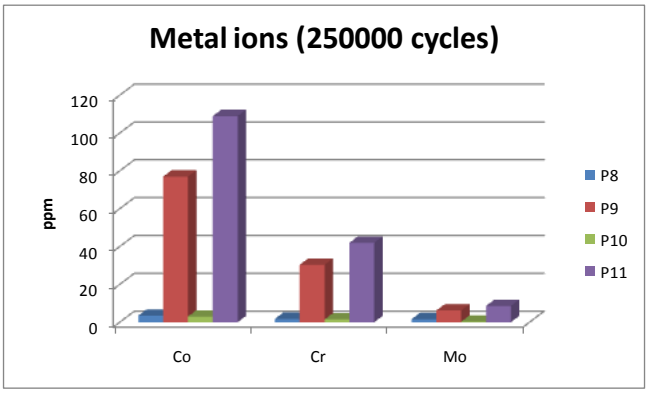
SAMPLE	Co_{250} [ppm]	Cr_{250} [ppm]	Mo_{250} [ppm]	Co_{650} [ppm]	Cr_{650} [ppm]	Mo_{650} [ppm]	Co_{950} [ppm]	Cr_{950} [ppm]	Mo_{950} [ppm]
CMCA-BCS 8	3,51 (0,71)	1,79 (0,45)	1,59 (0,33)	109 (21)	43 (11)	8,2 (1,7)	128 (23)	49 (12)	10,6 (2,2)
CMCA-BCS 9	77 (16)	30,4 (7,6)	6,2 (1,3)	114 (22)	43 (11)	8,7 (1,8)	118 (22)	45 (11)	9,9 (2,1)
Serum 10	2,98 (0,6)	1,47 (0,37)	0,259 (0,062)	5,4 (1,1)	1,55 (0,39)	0,387 (0,087)	6,5 (1,3)	3 (0,75)	0,63 (0,13)
Serum 11	109 (21)	42 (10)	8,6 (1,8)	410 (60)	156 (39)	31,4 (6,5)	488 (71)	180 (45)	39,9 (8,3)

Table 4.2

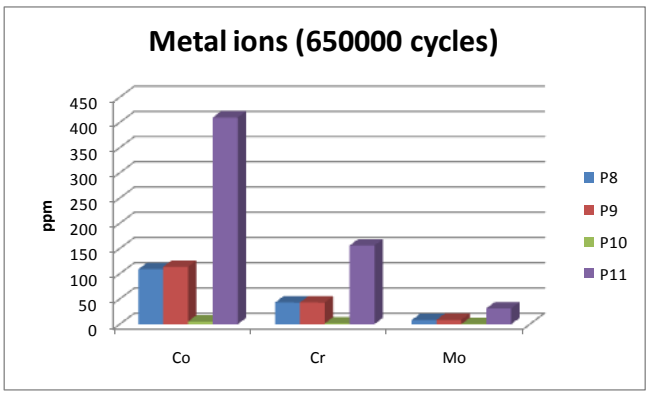
Metal Ions Concentrations in testing fluids at a different cycles.

These values are also presented in *Figure 4.13* where a., b. and c. represent the three sampling performed. It can be seen that at 250.000 cycles station 8 presents lower ion delivery than station 9 although they were both immersed in the same testing fluid. This difference disappeared in samples taken at 650.000 and 950.000, presenting a comparable level of Co, Cr and Mo. Station 10 and 11, both immersed in serum, presented very different levels of ions concentration and in particular station 10 presented the lowest values at all. This fact can be explained considering that station 10 was equipped with a prosthesis presenting the best initial condition because it was the one which was less damaged by the previous usage. The other three stations were characterised by the presence of prostheses with similar initial conditions and, comparing them, it can be seen that stations immersed in CMCA polymer-gel and BCS (stations 8 and 9) presented lower ions release than the station immersed in BCS and deionized water (station 11). These considerations are supported by the results on the wear of the metal liners and heads which are presented in paragraph 4.3.2.

a.



b.



c.

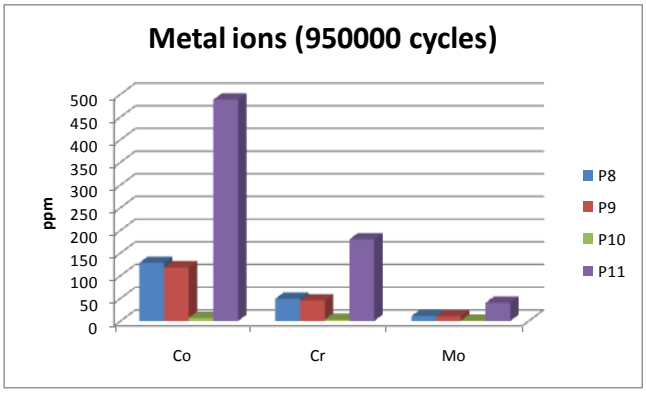


Figure 4.13
Metal ions concentrations in samples of testing fluid taken from the four stations(P8, P9, P10, P11) at 250.000 cycles (a.), 650.000 cycles (b.) and 950.000 cycles (c.)

4.3.2 Wear Analysis

The wear test, with MoM prostheses, did not need the soaking to be performed, due to the fact that metal, unlike UHMWPE, does not absorb the lubricating medium. Liners and heads were weighted before and after the test to evaluate their weight loss, caused by wear. *Table 4.3* reports the weigh at the beginning, at the end and their difference for the prosthetic components used in the hip joint simulator.

SAMPLE	WEIGHT before test [g]	WEIGHT after test [g]	WEAR [g]
CMCA-BCS station 8-head	158,135	158,107	0,028
CMCA-BCS station 8-liner	126,519	126,433	0,086
CMCA-BCS station 9-head	158,039	158,016	0,023
CMCA-BCS station 9-liner	128,050	127,988	0,062
Serum station 10-head	158,234	158,232	0,002
Serum station 10-liner	128,032	128,027	0,005
Serum station 11-head	157,460	157,100	0,360
Serum station 11-liner	127,610	127,448	0,162

Table 4.3
Wear of the CoCrMo liners and heads used in the test.

The results in *Table 4.3* are better comparable if represented in graphs as shown in *Figure 4.12*.

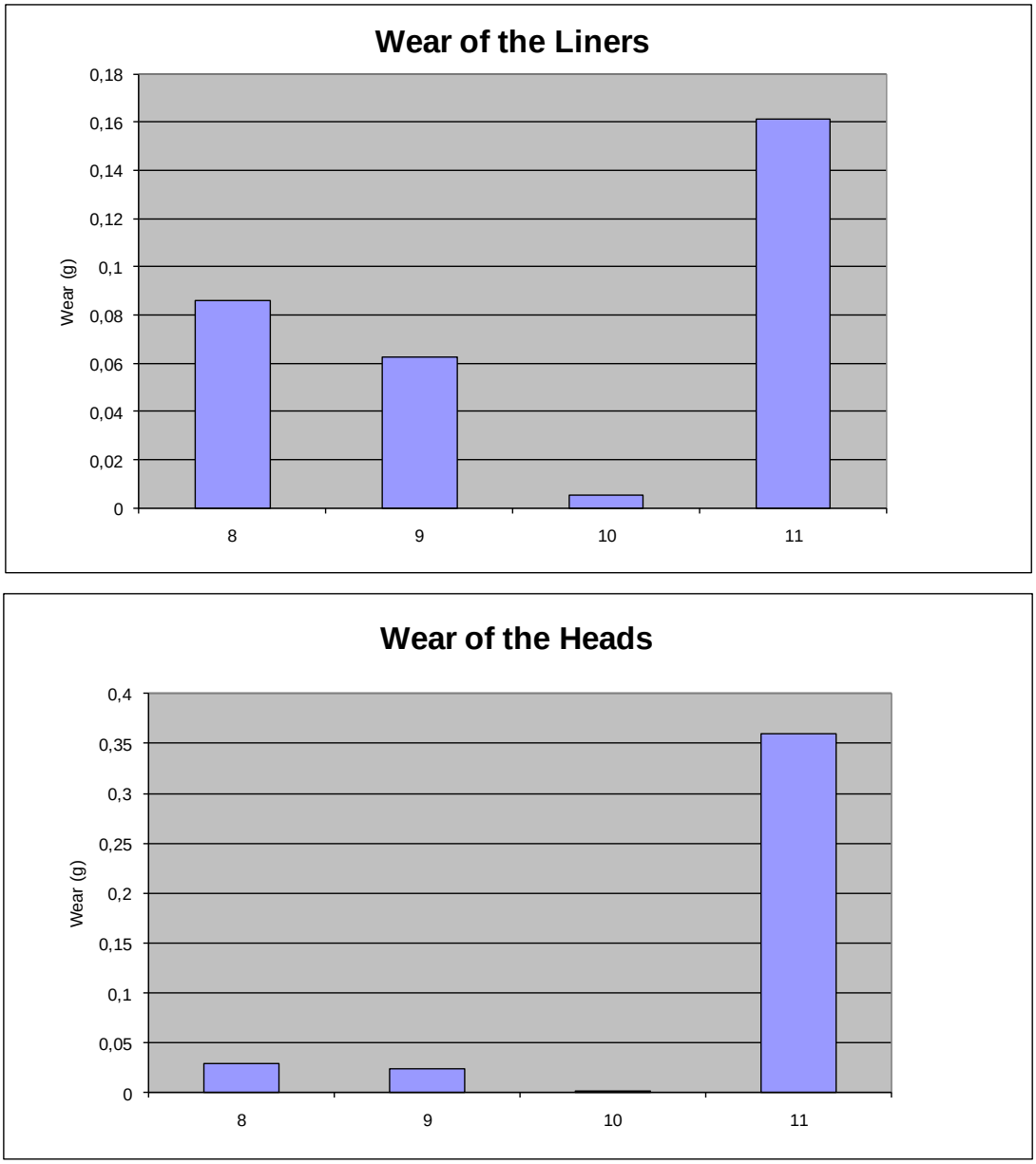


Figure 4.12
Wear of the Liners (at the top) and Heads (at the bottom) in CoCrMo for stations 8, 9, 10, 11

Station 10, which was lubricated in serum (BCS and deionized water), presented lower wear in accord with the results obtained from the metal ions analysis.

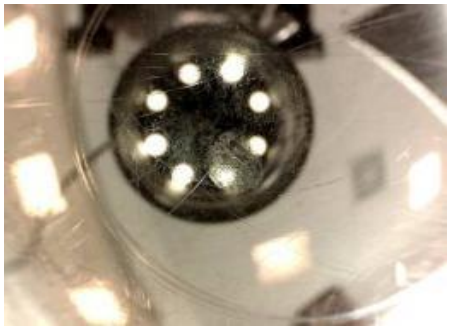
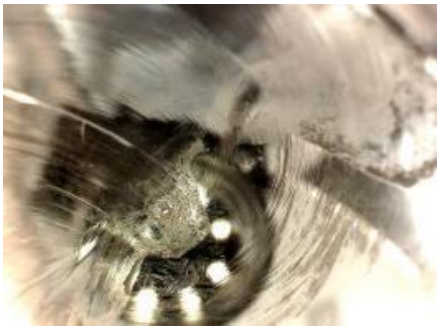
The three other stations can be better compared due to similar stating conditions; they shown that the lubrication with CMCA composite and BCS induced a lower wear, both considering liners and heads in CoCrMo. This result is the same that was obtained with the ions analysis showing that ions release and wear are connected; the reason is that

the wear, caused by the reciprocal sliding of bearing surfaces, generates particles removal and metal ions release.

The same conclusions can be obtained by the analyses of bearing surfaces. The following pictures present an enlargement of the CoCrMo heads and liners, used in the test, from which it is possible to see that station 11 (named d. in *Figures 4.13*) is the most damaged, station 10 (named c.) the less damaged, while stations 8 and 9 (named a. and b. respectively) present an intermediate level of damage because of the protection provided by the viscoelastic lubricant media in which they were immersed.

These considerations agree with the test results presented before, confirming what has been obtained with the metal ions analysis.

a.  b. 

c.  d. 

e.  *f.* 

g.  h. 

Figure 4.13
Enlarged photos of CoCrMo prosthetic heads (from a to d) and liners (from e to h)
a. Station 8 (CMCA composite and BCS), b. Station 9 (CMCA composite and BCS), c. Station 10 (BCS and deionized water), d. Station 11 (BCS and deionized water), e. Station 8 (CMCA composite and BCS), f. Station 9 (CMCA composite and BCS), g. Station 10 (BCS and deionized water), h. Station 11 (BCS and deionized water)

4.4 METAL ON UHMWPE HIP PROSTHESES

CoCrMo heads with UHMWPE liners were used to perform a wear test, on the same hip joint simulator described in paragraph 4.3, and to compare the lubrication provided by CMCA polymer-gel also with this prosthetic type.

Three stations (4, 5 and 6) under physiological load were prepared, while station 7 was used to perform the soak control. All the four stations were immersed in CMCA composite material diluted with BCS at 75% W/W.

The results upon the absorption and the wear of the same prosthetic components, in identical testing conditions except for the lubricating fluid, were given by an external laboratory. They tested four prostheses (three under load and one soaking) in BCS diluted in deionized water to simulate physiological wear behaviour and, considering the UHMWPE liners, they obtained 0,036 g (SD ±0,004) of average wear and an average fluid absorption of -0,003 g.

Before starting the test in CMCA composite, all the components were weighted and the same control was performed after 1 million cycles, at the end of the test. The following chart presents the wear for all the heads and liners and the absorption only for the UHMWPE components.

SAMPLE	ABSORPTION [g]	WEAR [g]
CMCA-BCS station 4-head	0,012	0,012
CMCA-BCS station 4-liner	0,003	0,005
CMCA-BCS station 5-head	0,001	0,001
CMCA-BCS station 5-liner	0,004	0,006
CMCA-BCS station 6-head	0,001	0,001
CMCA-BCS station 6-liner	0,003	0,005
CMCA-BCS station 7-head	-0,000	-
CMCA-BCS station 7-liner	-0,002	-

Table 4.4
Wear and Absorption for the metal on UHMWPE prostheses

Wear and absorption presented in *Table 4.4*, are calculated in accord with *Equations 4.1* and *4.2*.

Comparing to the results obtained from the test on serum, it is possible to state that a viscoelastic lubricating fluid, such as CMCA polymer-gel composite material, reduces wear; in fact, the wear rate of the liners immersed in BCS and deionized water was 35,56 mg per million cycles (SD 3,79 mg) while the one for liners immersed in diluted CMCA composite was 4,92 mg per million cycles (SD 0,14 mg).

In this test, the protective action against wear of the CMCA composite material is more evident than in the test performed in the knee simulator, showing a reduction of wear of about the 86% instead of two thirds. The prosthetic type was not the same in the two tests (knee or hip prosthesis) but the UHMWPE liners were in reciprocal movement with a CoCrMo component (femoral component or prosthetic head). Comparing the wear rate for the UHMWPE liners immersed in the viscoelastic fluid the results are very similar: 3 mg per million cycles in the knee joint test and 4,92 mg per million cycles in this test. This means that the ability to protect from wear remains in both situations.

Comparing the CoCrMo heads used in this test and the ones used in the other hip joint test, with MoM prostheses, it can be observed that the wear on MoM prostheses is slightly higher but it has to be considered that these heads were previously tested for five million cycles.

CHAPTER 5: CONCLUSIONS

This thesis work presented the characterization of the manufacturing process for the CMCA polymer-gel considering packaging, sterilization, storage and product characteristics as non pyrogenicity and stability. In addition, three mechanical tests were performed to evaluate product performances in term of lubricating and wear-protection, which are essential properties for a viscosupplementation device.

The best package conditions, characterized by glass syringe, filled under nitrogen, and contained in a plastic blister, were identified by different tests such as the IR, the pH and the rheometric analyses. It was demonstrated that material degradation was reduced with nitrogen filling in comparison with the case of the air used as propeller in the automatic filling technique. The evidence that the packaging materials did not influence chemical and physical characteristics of the product was given by pH and IR analysis.

The choice of the proper sterilization procedure has to balance two opposite needs: guarantee sterility and to not induce an excessive damage in the mechanical characteristics of the treated product. To identify the best sterilization cycle, three different cycles were designed and tested. The most conservative was chosen after the

results coming from the rheological comparison, showing the effects on the Elastic and Viscous Moduli, induced by the treatment.

The non pyrogenicity was determined by test on rabbit, according to the fifth edition of the European Pharmacopeia, that allows to find out the pyrogenicity of the material itself and/or the pyrogenicity induced by the presence of microorganisms or their parts. The test, performed on three rabbits, was successfully passed stating the non pyrogenicity of the device.

Analysing the manufacturing process, it was noted the necessity to perform two controls: the first to check if the CMCA polymer concentration is correct to continue with the following working phases; the second on the final product, to verify that the hydration procedure have been performed correctly to obtain the product with the desiderated concentration. Due to the fact that the density is related to concentration, the density test was chosen to get information on the correct concentration of the tested material and this is why a testing procedure was developed and the relationship between concentration and density was investigated.

Stability and shelf life are important characteristics to be determined while characterizing a new medical device because of the necessity to know the period of time in which it maintains adequate characteristics and performances. The product was studied giving attention to the characteristics susceptible to change in time such as the Elastic and the Viscous Moduli. They were monitored with both real time test, in two months, and accelerated test, exposing the material to high temperature (40°C or 50°C) for eight days. Preliminary accelerated tests shown that the standard parameter, to find out the correspondence between high temperature days and room temperature days, was not suitable for the CMCA polymer gel. To avoid this problem, a model of the possible drop in time of the considered characteristics in worst case condition was created, according to which the study was performed. The analysis of the values of both moduli, after the considered period of time, was performed with a rheometric study. The study allowed finding the characteristic value for the Q_{10} aging factor of the CMCA polymer-gel composite material, for the temperatures considered. The importance of this result is due to the fact that now it is possible to perform an accelerated test, at a

precise temperature (between 22,5°C and 50°C) and for a specific period of time, to simulate real aging and to investigate product's shelf life.

Storage conditions could highly influence the product's aging so a comparison between the storage at room temperature and the storage at 4°C was performed. The effect of temperature on aging and storage was evaluated by a frequent sweep test performed with a rotational rheometer, in order to investigate the G' and G" values in time at the different frequencies, with particular attention to 0,5 Hz and 2,5 Hz corresponding to the walking and running conditions. This test shown that is preferable to store the product at 4°C than at 22,5°C and allowed to affirm that the CMCA composite material presents good resistance of the Elastic and Viscous Moduli in six months storage at 4°C.

The tests were done to develop manufacturing processes and to operate a control on it, while the mechanical tests, which follows, were designed to evaluate the CMCA composite performances in situations similar to the intended use.

The first mechanical test was performed on the knee joint simulator, a machine that reproduces the knee motion with the help of a prosthetic knee. The CMCA polymer-gel was used as lubricating fluid and was diluted, with BCS, to simulate the worst case and the possible dilution that can occur while injected in a knee that has not been treated with arthrocentesis. The effects were studied comparing these consequences to the ones caused by the lubrication in serum (BCS and deionized water) giving evidence of the best lubricating properties of the CMCA composite material in term of wear reduction and surfaces protection. These conclusions were obtained by the wear evaluation given by weighting measures of the absorption and material loss in the liners and by surfaces analysis.

The second test, performed on the hip joint simulator, was designed to evaluate a new possible use for the material as a device to be injected in patients with MoM total hip prosthesis. This kind of prostheses are characterised by ion delivery with consequent arise of adverse effects. It was compared the ion delivery of four prostheses, which had worked before for about five million cycles: two were immersed in CMCA polymer-gel, diluted in BCS, and two in serum (BCS and deionized water). The relationship between wear and ion delivery was investigated to confirm that the greater the wear is, the

greater the numbers of metal ions particles is. Comparing the stations equipped with prostheses presenting similar initial wear degree, the test highlighted that the lubrication made with a viscoelastic fluid reduces wear and, because of this, reduces the ion delivery. This fact assumes great importance considering the adverse effects caused by metal particles. They could be reduced by the use of a viscoelastic lubricant medium such as the CMCA composite material. Even if the test was performed in a worst case, diluting CMCA polymer-gel with BCS, it was clear that it presented better lubricating behaviour than BCS and deionized water, used to recreate physiological wear-rates.

To obtain another confirm that CMCA polymer-gel composite material is a device able to reduce wear and prevent surface damages another test was performed. This test was conducted again on the hip joint simulator, but using a different type of prostheses. They were characterised by the presence of CoCrMo heads, as in the previous test, but the liners were made of UHMWPE. At the end of the test, after one million cycles, the wear behaviour for liners and heads, immersed in serum or in diluted CMCA mixture, was evaluated, confirming that the presence of a viscoelastic fluid greatly reduces wear.

Comparing the wear of the liners in UHMWPE used in this test and in test performed on the knee joint simulator, it was clear that the wear behaviour, in diluted CMCA composite was comparable, even in different testing conditions.

If the comparison between the CoCrMo heads used in this test and the ones used in the other hip joint test is done, it can be noticed that the wear in the first case was lower but it has to be taken into consideration the fact that, in the second case, the heads were previously used for 5 million cycles.

In conclusion, all the tests performed allowed stating that the product presents adequate characteristics to be used as a viscosupplementation device not only considering its rheological characterisation, but also its stability over time and its mechanical action in wear prevention and surfaces protection.

The pyrogenicity test concluded that the CMCA polymer-gel composite material is non-pyrogenic giving evidence of its safety.

Viscosupplementation is not the only possible use for the product: thanks to the metal ions test, it was possible to demonstrate that it could be used in patients with MoM

prostheses to reduce the metal ion release preventing the increase of adverse effects and giving the possibility to retard revision surgery.

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