Development of resorbable bioceramic bone cements towards vertebroplasty application

Sara Bardají Sierra

DOCTORAL THESIS

Title	Development of resorbable bioceramic bone cements towards vertebroplasty application
Presented by	Sara Bardají Sierra
Centre	IQS School of Engineering
Department	Bioengineering
Directed by	Dr. Salvador Borrós Gómez

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Al meu pare

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[3] No abandones nunca el amor y la verdad;

llévalos contigo como un collar.

Grábatelos en la mente,

[4] y tendrás el favor y el aprecio

de Dios y de los hombres.

[5] Confía de todo corazón en el Señor

y no en tu propia inteligencia.

[6] Ten presente al Señor en todo lo que hagas,

y él te llevará por el camino recto.

[7] No te creas demasiado sabio;

honra al Señor y apártate del mal:

[8] ¡ésa es la mejor medicina

para fortalecer tu cuerpo!

Proverbios 3: 3-8 (La Biblia)

[38] Estoy convencido de que nada podrá separarnos del amor de Dios: ni la muerte, ni la vida, ni los ángeles, ni los poderes y fuerzas espirituales, ni lo presente, ni lo futuro, [39] ni lo más alto, ni lo más profundo, ni ninguna otra de las cosas creadas por Dios. ¡Nada podrá separarnos del amor que Dios nos ha mostrado en Cristo Jesús nuestro Señor!

Romanos 8:38-39 (La Biblia)

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Sara Bardaji Sierra, 17 d'Octubre de 2018

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Abstract

Development of resorbable bioceramic bone cements towards vertebroplasty application

Increase life expectancy leads to the development of age-related diseases. One of them is osteoporosis, a progressive skeletal disease, which decreases the density of the bone mass and weakens the skeletal vertebral body of the patient. One of the most direct and severe consequences is the formation of vertebral compression fractures.

One of the minimally invasive techniques used to treat this type of fractures is named vertebroplasty. This technique has given very encouraging results, since it has shown that it improves the pain caused by the fracture, and it is able to strengthen the fractured bone. However, the materials used in this technique involve problems for the patient such as pain and necrosis a lot of others.

Therefore, the objective of this thesis is to develop a bone cement that can be injected by vertebroplasty, and that will regenerate fractured vertebral bone. In addition, the product must be biodegradable, because as the healing process progresses, the material reduces its stiffness as part of the load is supported by the regenerated tissue.

To develop this new bone cement, a copolymer combined with hydroxyapatite, zirconia and platelet-rich plasma has been selected. It has been shown to be easily injectable, and once injected, it is able to solidify quickly, without suffering cement leakage or creates necrosis in adjacent bone tissues. It also has a similar density to bone which prevents it from generating tensions that can lead to adjacent vertebrae fractures. In addition, it has an excellent resistance to compression and after the compression test, it does not present fractures and it is able of filling the entire defect created by the fracture.

Moreover, it has been shown that the addition of platelet-rich plasma gives to the material osteoinductive and osteoconductive properties. Therefore, PRP makes it bioactive. So, it is able to differentiate bone marrow mesenchymal stem cells to osteoblasts, and these osteoblasts differentiate them to osteocytes, generating bone matrix.

Finally, the biocompatibility and biodegradability of the bone cement has been verified by rat calvaria in vivo model. But the restoration of the bone has not been observed due to the limitations presented by this model. Therefore, the use of a sheep vertebra in vivo model is proposed, which is more representative of what could happen in a real patient.

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Resumen

Development of resorbable bioceramic bone cements towards vertebroplasty application

El aumento de la esperanza de vida de las personas, conlleva el desarrollo de enfermedades relacionadas con la edad. Una de ellas es la osteoporosis, una enfermedad esquelética progresiva, que disminuye la densidad de la masa ósea y debilita el cuerpo esquelético vertebral del paciente. Una de las consecuencias más directas y severas es la formación de fracturas por compresión vertebral.

Una de las técnicas mínimamente invasivas que se utiliza para tratar este tipo de fracturas se denomina vertebroplastia. Esta técnica ha dado resultados muy esperanzadores, ya que ha demostrado que mejora el dolor causado por la fractura, y es capaz de fortalecer el hueso fracturado. Sin embargo, muchos de los materiales que se utilizan en esta técnica comportan problemas para el paciente como dolor, necrosis, y un largo etcétera.

Por tanto, el objetivo de esta tesis es desarrollar un cemento óseo que pueda inyectarse mediante la vertebroplastia, y que sea capaz de regenerar el hueso vertebral fracturado. Además, el producto debe ser biodegradable, de tal manera, que a medida que la vértebra fracturada vaya creciendo, la prótesis sea capaz de biodegradarse, dejando paso al hueso que crece de nuevo.

Para desarrollar este nuevo cemento óseo, se ha trabajado con un copolímero combinado con hidroxiapatita, óxido de zirconio y plasma rico en plaquetas. Ha demostrado ser fácilmente inyectable, y una vez inyectado, es capaz de solidificar rápidamente, sin sufrir fugas de cemento ni tampoco crear necrosis en los tejidos adyacentes al hueso. Además presenta una densidad similar al hueso lo que evita que genere tensiones que pueden conllevar fracturas de vértebras adyacentes. Hay también añadir, que tiene una resistencia a la compresión excelente, y que después del test de compresión, no presenta fracturas y al mismo tiempo es capaz de llenar todo el defecto creado por la fractura.

Además, se ha demostrado que la adición del plasma rico en plaquetas le confiere al material propiedades osteoinductivas y osteoconductivas. Por lo tanto, lo hace bioactivo. De tal manera, que es capaz de diferenciar células madres de hueso a osteoblastos, y estos mismos osteoblastos, diferenciarlos a osteocitos, generando matriz ósea.

Finalmente, se ha comprobado la biocompatibilidad y la biodegradabilidad del cemento óseo mediante un modelo in vivo de calvaria de rata. Sin embargo, no ha permitido ver la restauración del

hueso por las limitaciones que presenta este modelo. Así pues, se plantea utilizar un modelo in vivo de vértebra de oveja, el cual es representativo de lo que podría suceder en un paciente real.

Resum

Development of resorbable bioceramic bone cements towards vertebroplasty application

L'augment de l'esperança de vida de les persones, porta com a conseqüència el desenvolupament de malalties relacionades amb l'edat. Una d'elles és l'osteoporosi, una malaltia esquelètica progressiva, que disminueix la densitat de la massa òssia i debilita el cos esquelètic vertebral del pacient. Una de les conseqüències més directes i severes és la formació de fractures per compressió vertebral.

Una de les tècniques mínimament invasives que s'utilitza per tractar aquest tipus de fractures s'anomena vertebroplàstia. Aquesta tècnica ha donat resultats molt esperançadors, ja que ha demostrat que millora el dolor causat per la fractura, i és capaç d'enfortir l'os fracturat. No obstant això, molts dels materials que s'utilitzen en aquesta tècnica comporten problemes pel pacient com ara dolor, necrosi, i un llarg etcètera.

Per tant, l'objectiu d'aquesta tesi és desenvolupar un ciment ossi que pugui injectar-se mitjançant la vertebroplàstia, i que sigui capaç de regenerar l'os vertebral fracturat. A més, el producte ha de ser biodegradable, de tal manera, que a mesura que la vèrtebra fracturada vagi creixent, la pròtesi sigui capaç d'anar biodegradant-se, deixant pas a l'os que creix de nou.

Per desenvolupar aquest nou ciment ossi, s'ha treballat amb un copolímer combinat amb hidroxiapatita, òxid de zirconi i plasma ric en plaquetes. Ha demostrat ser fàcilment injectable, i un cop injectat, és capaç de solidificar ràpidament, sense patir fugues ni tampoc crear necrosi en els teixits adjacents a l'os. A més a més presenta una densitat similar a l'os el que evita que generi tensions que poden comportar fractures de vèrtebres adjacents. Cal també afegir, que té una resistència a la compressió excel·lent, i que després del test de compressió, no presenta fractures i alhora és capaç d'omplir tot el defecte creat per la fractura.

A més, s'ha demostrat que l'addició del plasma ric en plaquetes li confereix al material propietats osteoinductives i osteoconductives. Per tant, el fa bioactiu. De tal manera, que és capaç de diferenciar cèl·lules mares d'os a osteoblasts, i aquests mateixos osteoblasts, diferenciar-los a osteòcits, generant matriu òssia.

Finalment, s'ha comprovat la biocompatibilitat i la biodegradabilitat del ciment ossi mitjançant un model in vivo de calvària de rata. Tanmateix, no ha permès veure la restauració de l'os per les limitacions que presenta aquest model. Així doncs, es planteja utilitzar un model in vivo de vèrtebra d'ovella, el qual és representatiu del que podria succeir en un pacient real.

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List of Abbreviations

VCF	vertebral compression fractures
VP	vertebroplasty
BK	kyphoplasty
CPCs	calcium phosphate cements
CSCs	calcium sulfates cements
HAp	hydroxyapatite
PMMA	polymethylmethacrylate
ABCs	acrylic bone cements
b-TCP	b tricalcium phosphate
IBC	injectable bone cement
UCS	ultimate compressive strength
DTS	diametral tensile strength
BMP	bone morphogenetic protein
PLLA	polylactic acid
PEG 1500	polyethylene glycol
GPC	gel permeation chromatography
hBMSCs	human bone marrow mesenchymal stem cells
ALP	alkaline phosphatase
ECM	extracellular matrix
OPN	osteopontin
OCN	osteocalcin
TGF-β	transforming growth factor-β
GFs	Growth factors
PRP	Platelet-rich plasma
ACD-A	Anticoagulant Citrate Dextrose Solution
RBCs	red blood cells
BC	buffy coat
PPP	platelet-poor plasma
a-MEM	Minimum Essential Medium Eagle Alpha Modification
PBST	phosphate buffered saline solution
MGP	matrix gla protein

BSP	bone sialoprotein

Motivation and objectives

"Sara, si un problema tiene solución, ¿Porqué te preocupas? Y si no tiene solución, ¿Porqué te preocupas?"

- *Eduardo Bardaji Ais*

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Increasing life expectancy is leading to an increase in age-related diseases, like osteoporosis, a systemic disorder that compromises bone strength and predisposes patients to an increased risk of fractures .One of the main problems related with such disease is the formation of vertebral compression fractures (VCF) (World Health Organization. 1994).

Moreover, it is a difficult disease to diagnose in the early stages. In fact, it is even difficult to specify exactly when the fracture began, and for this reason osteoporosis is often named the "silent" disease, because vertebral fractures may also go unnoticed (Bostrom and Lane 1997). Recently, a study stated that less than a third of the vertebral fractures cause enough symptoms to be diagnosed and addressed immediately by a doctor (clinical vertebral fractures). Thus, two thirds of them only cause minor pain, which results in a late diagnosis (subclinical vertebral fractures). This is a key point because a fracture of this type has very severe and durable consequences over time. So, it is important to treat the vertebral compression fractures as soon as they appear, both safely and effectively, to avoid negative short- and long-term consequences for the patient (Cooper, Atkinson et al. 1992).

The impact of the vertebral compression fractures that have been described is diverse, such as distortion in the spine (kyphosis), chronic back pain, reduced physical function with risk of immobility, decline of lung function, gastroesophageal reflux and change in appearance that may lead to which contributes to social isolation, loss of self-esteem and depression. Vertebral fracture is just the beginning of the constant deterioration of the health of the affected patients (Oleksik, Ewing et al. 2005, Suzuki, Ogikubo et al. 2008).

In fact, their health impact is far-reaching. Approximately 50% of patients who have suffered a vertebral compression fracture, develop (vertebral or not) multiple fractures throughout their lives (O'neill, Felsenberg et al. 1996; Suzuki, Ogikubo et al. 2008). Furthermore, these patients suffer a decrease in the probability of survival (Jalava, Sarna et al. 2003).

So, the fracture of healthy bone due to the fragility of damaged bone is a direct consequence. In most cases, suffering this last impact means experiencing all the other symptoms again(Oleksik, Ewing et al. 2005).

The treatments applied in these severe fractures are mainly three: conservative medical treatment, invasive surgical intervention and vertebroplasty and kyphoplasty.

On the one hand, the conservative medical treatment is based on the ingestion of drugs and includes a short period of bed rest followed by gradual mobilization with external orthoses and a hyperextension brace. These braces are usually beneficial for the first few months, until the pain is reduced. Only young patients are able to tolerate the brace, because elderly patients suffer from stronger pain and tend to require more bed rest. However, immobility predisposes patients to venous thrombosis and life-threatening complications such as pulmonary embolism. It can also lead to pressure ulcers, pulmonary complications, urinary tract infections, and progressive deconditioning. In addition, it has been reported that bone mineral density decreases from 0.25% to 1.00% per week in patients who are on bed rest (Alexandru and So 2012).

On the other hand, surgical intervention is advised when conservative therapy fails with those patients who suffer from hopeless back pain or a severe spinal deformity. However, twenty years ago, doctors observed that highly invasive operations succeeded in some cases, but for most patients, the pain and reduced mobility persisted forever. For dealing with this type of fractures, the classical open surgery with decompression and stabilization of the fractured vertebra with different kinds of metal implants often fails because of the poor quality of osteoporotic bone. Also, because of the risk of open surgery in elderly patients, these procedures have generally been limited to cases where there is concurrent spinal instability, or neurological deficit (Barr JD, Barr MS, Lemley TJ 2000) because it is difficult to achieve an adequate fixation in theosteoporotic bone(Old and Calvert 2004).

Also, invasive surgical interventions are less attractive, especially in elderly patients, due to the increase of risks like allergy to anesthesia and low bone density that involves future fractures and invasiveness (Gray, Jarvik et al. 2007).

However, minimal invasive spinal surgery techniques have evolved in the past two decades as an alternative to open surgery with decompression. Briefly, acute painful vertebral compression fractures have been targeted for treatment through percutaneous procedures termed vertebroplasty (VP) or balloon kyphoplasty (BK) (Garfin, Yuan, and Reiley 2001).

Vertebroplasty is a percutaneous injection of bone cement directly into the fractured vertebral body. In this way, the vertebra is welded to prevent fracture progress and augment the weakened vertebral body. Thus, the bone cement can stabilize and restore it to as much of its

normal weight and functional state as possible (RAY et al. 1990,Bae et al. 2010,Lieberman et al. 2001).Kyphoplasty is similar to vertebroplasty because it is a minimally invasive surgical procedure and uses bone cement to increase and stabilize the vertebrae. But, in kyphoplasty, an orthopedic balloon is inserted into the damaged vertebra to restore its structure before injecting the bone cement (Robinson et al. 2011).

These minimally invasive percutaneous procedures entail placing large spinal needles into the fractured vertebral body through a channel made in the pedicle and injecting cement under radiologic control into the fractured vertebral body. These techniques have shown that they strengthen the bone and reduce the intense pain caused by VCFs, and for now, they are a good alternative. In fact, the large number of orthopedic procedures performed each year has led to great interest in injectable cements for regeneration of bone (Grados et al. 2000). Although they have shown interesting results, there is still room for improvement, especially due to the lack of an ideal bone cement material.

A variety of cements have been developed for these applications, including ceramics, naturally derived substances and synthetic polymers. These materials demonstrate overall biocompatibility and appropriate mechanical properties, as well as promote tissue formation, thus providing an important step towards minimally invasive orthopedic procedures (Ploeg, Veldhuizen, and Sietsma 2006, Temenoff and Mikos 2000) . However, they also carry many difficulties like necrosis, injected cement leakage, inflammation, fracture of adjacent vertebrae and many others (Isador H Lieberman, Togawa, and Kayanja 2005).

Ceramic materials were the first materials used to repair bone tissue because of its biocompatibility and bioactivity (Lim et al. 2002). They have a chemical similarity to the mineral phase of bony tissue, especially in calcium phosphates (Mestres and Ginebra 2011).

Calcium sulfate cements have been used as substitutes for bone defects since 1892. But, in the 1990s, calcium sulfate was gradually replaced by calcium phosphate (CPCs), mainly hydroxyapatite. The reasons for this change were two-fold: the quick reabsorption of calcium sulfate that does not allow the complete restoration of the bone and its low resistance to the load that the vertebral body has to bear (Nilsson et al. 2004, Habib et al. 2008).

However, calcium phosphates have an additional noteworthy difficulty. They require a setting time too long to be applied in VP and KP, and it is impossible to inject them directly (Habib et al. 2008).

Due to the difficulties presented by the ceramic materials, polymers have been selected as an alternative material for VP because they allow more material design possibilities.

Most bone cements used in VP are acrylic cements based on polymethylmethacrylate (PMMA). To date, two categories of formulations of ABCs have been used in VP and KP. The first comprises the same commercially available brands that are used in cemented arthroplasties (the most frequently used being Surgical Simplex P) to which an additional amount of radiopacifier is added by the surgeon. Typically, $BaSO_4$ (to bring the loading to 20–30 wt/wt % of the cement powder) is added. The second comprises commercially available brands that are specifically formulated with a high radiopacifier concentration, two examples of which are Osteopal V28 and KyphXHV-R™ (Lewis 2006).

They exhibit high compressive strength; high mechanical strength and they cure fast which lets only a short handling time. However, they also cause infection, necrosis, injected cement leakage, inflammation and fracture of adjacent vertebrae. Moreover, these polymeric materials are not biodegradable and exhibit a strengthening factor unnecessarily high which causes fractures to the adjacent vertebrae (Baroud et al. 2003).

Due to all the problems involved in the use of single materials, different multimaterial cements have been recently proposed trying to combine the advantages of different materials (e.g.Cortoss™ Cerament™from Bone Support AB; KyphOsFS™ and ActivOs™ from Medtronic).
The new multimaterials are silica-based bioactive glasses of complex compositions. In fact, bioactive glasses possess unique properties if compared to ceramic materials such as HAp and b-TCP, as their composition can be tuned to obtain materials with tailored reactivity in the human body, ranging from a slightly bioactive behavior to a complete bioresorbability. But, sometimes leakage occurs with these multimaterial cements and it is difficult to treat fractures caused by cancer because they are very unstable (Rauschmann et al. 2010).

A complex composition also offers the unquestionable advantage of releasing ions known for their beneficial role on bone matrix mineralization (i.e. calcium and magnesium) and/or the

achievement of a proper control of local pH during ion leaching (i.e. phosphate ions), thereby avoiding cell damage due to pH variation (Vitale-Brovarone et al. 2007). Moreover, scaffold's final properties will depend primarily on the nature of the biomaterial and on the processing parameters; other interesting properties can be attained through the preparation of hybrid materials obtained by loading the scaffolds with collagen, cells or more generally biomolecules. Osteogenic cells obtained from the host through a biopsy can be multiplied in vitro and seeded onto the scaffolds before implantation.

As it can be seen, a lot of effort has been devoted in the development of bone cement compositions. However, a complete satisfactory solution is still pending. A more suitable material is needed and more work has to be done in order to find an optimal solution.
Therefore, the main objective of the thesis is finding an ideal cement to apply in vertebroplasty, so; the material have to meet the mentioned characteristics. For achieving this objective, different specific aims are proposed.

First, which is developed in the chapter one, is creating a new biodegradable and biocompatible paste with high viscosity and easy synthesis. The material has to maintain an appropriate viscosity during the injection and it has to recover its viscosity after the injection, so, it has to be thixotropic. To reach this goal a family of polyesters has been developed and their viscosity has been controlled by the length of each chain and by the reactivity of their monomers. After that, these polymers have been mixed with Hydroxyapatite to form a paste which has been complete characterized in terms of rheological characteristics and hardness to observe its thermoplastic behavior and its higher viscosity.

The second objective, developed in the chapter two, is to check that the paste is injectable within a short working time frame and it is able to fill the fracture while it avoids cement extravasations into the surrounding tissues. Also, it is necessary to check that the setting time is reduced to end the operation as soon as possible to avoid infections and to study that cement have the strength and stiffness to hold up the loads that support a healthy vertebral body and then augment and stabilize it. And finally, to analyze if the bone cement is compatible with specific needle for spinal surgery vertebroplasty. To reach this goals the assesssment of the bone cement has been done, its mechanical analysis (compression resistance) and an ex vivo test.

The third objective, developed in the chapter three, is to evaluate the osteoconductivity and osteoinductivity of the material. In other words, the bone cement should be able to stimulate in-vivo bone regeneration and establish a strong bond with the surrounding bone, with controlled resorbability to restore the functional state of it. To reach this goal the cytotoxicity of the bone cement has been checked and its capacity for differentiate Bone Human Mesenchymal Stem cells. Also, the cell differentiation markers like Alkaline Phosphatase, osteopontin and osteocalcin has been studied. Finally, an evaluation of the mineralization by staining with alizarin red has been realized and an in vivo.

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Chapter I. Development of new cement paste to apply in vertebroplasty

"Buenos días por la mañana." (no importa la hora que sea)

- Eduardo Bardají Ais

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1.1 Introduction. Injectable bone cements for vertebroplasty

As already mentioned in the section of motivation and objectives, osteoporosis is a systemic disorder that compromises bone strength and predisposes patients to an increased risk of fractures (World Health Organization. 1994).

Vertebral compression fractures (VCFs) constitute a serious health problem in the world, not only because of their high incidence but also due to their direct and indirect negative consequences on the patient's health-related quality of life and the costs to the health care system (Lindsay, Burge et al. 2005).

For dealing with this type of fractures, the classical open surgery with decompression and stabilization of the fractured vertebra with different kinds of metal implants often fails because of the poor quality of osteoporotic bone. Also, because of the risk of open surgery in elderly patients, these procedures have generally been limited to cases where there is concurrent spinal instability, or neurological deficit (Barr et al. 2000).

So, our work will be focused on synthesize and characterize a bone cement to deal with restoration of the vertebral fractures caused by vertebral compression, with minimal invasive spinal surgery techniques which have evolved in the past two decades. Acute painful vertebral compression fractures have been targeted for treatment through percutaneous procedures termed vertebroplasty (VP) or balloon kyphoplasty (BK)(Garfin, Yuan et al. 2001).

The minimally invasive percutaneous procedures entail placing large spinal needles into the fractured vertebral body through a channel made in the pedicle and injecting of cement under radiologic control into a fractured vertebral body. These techniques have shown that they strengthen the bone and improve the intense pain caused by VCFs, and for now, they are the best choice. In fact, the large number of orthopedic procedures performed each year has led to great interest in injectable cements for regeneration of bone(Grados, Depriester et al. 2000).

A variety of cements have been developed for these applications, including ceramics, naturally derived substances and synthetic polymers. These materials demonstrate overall biocompatibility and appropriate mechanical properties, as well as promote tissue formation,

thus providing an important step towards minimally invasive orthopedic procedures (Temenoff and Mikos et al. 2000). However, they also carry many difficulties like necrosis, injected cement leakage, inflammation, fracture of adjacent vertebrae and many others(Lieberman, Togawa, and Kayanja et al. 2005).

Due to the importance of the bone cement choice, it is necessary to discuss how the bone cements have evolved over time and which have been their results. And with this perspective, we are going to develop a new composition.

There are three types of injectable biomaterials in vertebroplasty: calcium sulfates and calcium phosphates cements, acrylic bone cements and multimaterial cements (Heini and Berlemann 2001).

1.1.1 *Calcium sulphates cements (CSCs) and Calcium Phosphate Cements (CPCs)*

Ceramic materials were the first materials used to repair bone tissue because of its biocompatibility and bioactivity (Lim, Brebach et al. 2002). They have a chemical similarity to the mineral phase of bony tissue, especially in calcium phosphates (Mestres and Ginebra 2011).

$CaSO_4$, also known as “plaster of Paris”, has a long clinical history for use as a bone graft substitute in various skeletal sites, the use having been first proposed by Dreesmann in 1892 and developed by Peltier in 1961 (Peltier 1961). However, in the original form, the recrystallization of plaster of Paris after it is mixed with water is random, and the crystalline structure contains many defects. More recently, surgical-grade CSCs have been developed, with the powder constituent being calcium sulfate hemihydrate. When mixed with a diluent, the powder is converted to calcium sulfate dihydrate, producing a paste or putty with a solid or partially solid structure; that is:

$$CaSO_4 \cdot 0.5H_2O + 1.5\ H_2O \rightarrow CaSO_4 \cdot 2H_2O$$

When used as an injectable bone cement (IBC), surgical-grade CSC inhibits fibrous tissue in growth, creates a slightly acidic environment that encourages angiogenesis and osteogenesis and, as the cement dissolves, bone forms, thereby allowing the void occupied by the cement to be replaced by new bone(Larsson and Hannink 2011). Depending on the volume and

location, surgical-grade CSC filler resorb in vivo mainly by dissolution, generally within about 2 months. One widely used commercially available brand is MIIG X3(Urban et al. 2004). It is a calcium sulfate hemihydrate which, when mixed with water, forms a paste that hardens in about 5 minutes. Its ultimate compressive strength (UCS) and diametral tensile strength (DTS), determined after curing in ambient laboratory air for 24 h, are 96.4 +- 5.9 and 16.0 +- 0.2 MPa, respectively(Baroud et al. 2003).

Although calcium sulfate cements have been used as substitutes for bone defects since 1892, in the 1990s calcium sulfate was gradually replaced by calcium phosphate (CPCs), mainly hydroxyapatite. The reasons for this change were two-fold: the quick reabsorption of calcium sulfate that does not allow the complete restoration of the bone and its low resistance to the load that the vertebral body has to bear (Habib et al. 2008; Nilsson et al. 2004).

There are many different ways of categorizing CPCs, one being the rate of resorption. In this category, CPCs may be divided into two types: apatite and brushite. Depending on the initial composition, apatite cements form different forms of apatite as the end-product; for example, calcium-deficient hydroxyapatite and carbonoapatite. Apatite cements degrade more rapidly than hydroxyapatite, although their degradation rate is still regarded as being slow and some formulations (such as tetracalcium phosphate-based ones) experience an increase in strength with time in vivo and are biocompatible (although inflammatory reactions have been reported in cases when the cement does not set) (Yuasa et al. 2004). Brushite cements are more degradable than apatite cements, resorb very quickly and suffer a rapid decrease in strength in vivo (although the mechanical properties of the healing bone increase as bone in growth occurs), and are biocompatible (although inflammatory reactions have been reported in some cases) (Theiss et al. 2005).

A CPC hardens through a slow exothermic reaction (thus preventing the attainment of high curing temperatures), during which the cement does not shrink. The main drawback of a CPC is its lack of macroporosity, which means that fast bone ingrowth does not take place and the cement degrades layer by layer from the outside to the inside. Two commercially available brands are Norian SRS and BoneSource™. Norian SRS, an apatitic mineral medium-viscosity cement, is sold as a reactant pack containing the powder mixture and the mixing liquid (Na_2HPO_4 solution). In vivo, the Norian SRS paste sets to form dahllite (carbonated calcium phosphate apatite) via an isotherm crystallization reaction(Goodman et al. 1998).

Dahllite is similar to the mineral phase of bone in terms of crystallinity and chemical composition. Histological analysis has indicated that, over time, dahllite is subjected to creeping substitution and remodeling in a manner that is similar to that observed in human bone; that is, via osteoclastic resorption.In vitro, BoneSource™ is fully converted to HAp via crystallization within 24 h. The cement has a microporous structure (volumetric porosity of about 5–10%), sets in about 7 min, and reaches a mean UCS of about 26 MPa within 24 h. One experimental CPC formulation is a brushite cement composed of -TCP, MCPM, and $Na_2H_2P_2O_7$, with the last-mentioned constituent being added to control the cement's setting time (Baroud et al. 2003; Ison et al. 1994).

However, calcium phosphates have an additional noteworthy difficulty. They require a setting time too long to be applied in VP and KP, and it is impossible to inject them directly (Habib et al. 2008). Finally, it can be said that, although calcium sulfate and phosphate cements carry some difficulties, both are bioactive materials also capable of stimulating bone regeneration. Hence, they are good candidates so far (Nilsson et al. 2004). For this reason, we will have them present during the design of the new formulation.

Due to the difficulties presented by the ceramic materials, polymers have been selected as an alternative material for VP because they allow more material design possibilities.

1.1.2 Acrylic Bone Cements (ABCs)

Most bone cements used in VP are acrylic cements based on polymethylmethacrylate (PMMA). To date, two categories of formulations of ABCs have been used in VP and KP. The first comprises the same commercially available brands that are used in cemented arthroplasties (the most frequently used being Surgical Simplex P) to which an additional amount of radiopacifier is added by the surgeon. Typically, $BaSO_4$ (to bring the loading to 20–30 wt/wt % of the cement powder) is added. The second comprises commercially available brands that are specifically formulated with a high radiopacifier concentration, two examples of which are Osteopal V28 and KyphXHV-R™ (Lewis 2006).

They exhibit high compressive strength; high mechanical strength and they cure fast which lets only a short handling time. However, they also cause infection, necrosis, injected cement leakage, inflammation and fracture of adjacent vertebrae. Moreover, these polymeric

materials are not biodegradable and exhibit a strengthening factor unnecessarily high which causes fractures to the adjacent vertebrae (Baroud et al. 2003).

1.1.3 *Multimaterial cements*

Due to all the problems involved in the use of single materials, different multimaterial cements have been recently proposed trying to combine the advantages of different materials (e.g.Cortoss™ Cerament™from Bone Support AB; KyphOsFS™ and ActivOs™ from Medtronic).

Cortoss™ is low viscosity cement that is a good example of the enhanced features of these combined cements. Some features of Cortoss™ include: (1) the use of a non-volatile liquid monomer, that after mixing(2), it has a consistency of toothpaste and stays that way until it polymerizes quickly, in a matter of seconds; (3) a low polymerization exotherm, of about 63°C; (4) a modulus that is close to that of cancellous bone; (5) good bioactivity; and (6) allows the development of a cement–bone interface that strengthens over time, with bone apposition occurring at that interface without any fibrous interposition(Boyd et al. 2008; Lewis 2006).

Other new multimaterials are silica-based bioactive glasses of complex compositions. In fact, bioactive glasses possess unique properties if compared to ceramic materials such as HAp and b-TCP, as their composition can be tuned to obtain materials with tailored reactivity in the human body, ranging from a slightly bioactive behavior to a complete bioresorbability.

A complex composition also offers the unquestionable advantage of releasing ions known for their beneficial role on bone matrix mineralization (i.e. calcium and magnesium) and/or the achievement of a proper control of local pH during ion leaching (i.e. phosphate ions), thereby avoiding cell damage due to pH variation. Furthermore, it has recently been discovered that the dissolution products from bioactive glasses exert a genetic control over the osteoblast cycle, and more specifically silicon has been found to be the ion that contributes most to the mineralization of bone and to gene activation(Vitale-Brovarone et al. 2007).

In addition, glasses do not melt at a constant temperature but soften as the temperature increases, which is an advantage from the conformational point of view. Different methods can be used to prepare a scaffold. Polymeric sponges possess an open, trabecular structure that can be used as a template for a ceramic replica through impregnation of the sponge with slurry of ceramic powders and a subsequent thermal treatment. This procedure to soften

glasses can be successfully used to attain a good sintering of the ceramic particles while maintaining a sufficient viscosity and thus avoiding the risk of collapsing of the trabecular structure during the thermal treatment (Ramay and Zhang 2003).

Moreover, scaffold's final properties will depend primarily on the nature of the biomaterial and on the processing parameters; other interesting properties can be attained through the preparation of hybrid materials obtained by loading the scaffolds with collagen, cells or more generally biomolecules. Osteogenic cells obtained from the host through a biopsy can be multiplied in vitro and seeded onto the scaffolds before implantation.

In addition to osteogenic cells production, glass–ceramic scaffolds can be also used as delivery vehicles of growth factors such as bone morphogenetic proteins (BMPs) that transform the host precursor cells into bone matrix producing cells. In fact, BMPs cannot be successfully used by themselves since they quickly diffuse and disperse from the injection site due to their low molecular weight (Vitale-Brovarone et al. 2007).Given the importance of the material being bioactive, it will also be considered to create bone cement that is capable of releasing growth factors that favor bone maturation.

Although a lot of effort has been devoted in the development of bone cement compositions, a complete satisfactory solution is still pending. For instance, leakage sometimes occurs with these multimaterial cements and it is difficult to treat fractures caused by cancer because they are very unstable (Rauschmann et al. 2010).

So, once analyzed all the limitations that the products developed until the moment have, all the required essential features that are needed for bone cement in vertebroplasty are studied. It is important to understand them perfectly in order to satisfy them.

The bone cement must meet a multitude of characteristics. The first one is that it has to be ease-of-handling. This first property is the most important for clinical use of any biomaterial. The cements should easily be prepared at the operating stage to facilitate surgeon surgery (Temenoff and Mikos 2000). Therefore, viscous properties must be balanced between two needs. The need of the material to remain at the site of injection to prevent leakage of bone cement. And also, the need of the surgeon to easily manipulate its placement to fill successfully the gap created by the fracture (Lee, Oh et al. 2012). It is necessary to complete the last need before the hardening process begins, but avoiding the risk of extravasation

(Laredo and Hamze 2005). Also, taking into account that the bone cement is applied by vertebroplasty, the bone cement should be injectable through needles or cannulas of a certain size to avoid open surgery (Phillips, Pfeifer et al. 2003).

In other words, the material should set in several minutes to minimize the length of the procedure while allowing surgeons enough time for placement before the biomaterial cure. Also, if the setting reaction involves a temperature change, the increase or decrease should be as small as possible to reduce damage to the surrounding tissues (Frayssinet, Gineste et al. 1998).

The setting time, the period that the product takes to harden, is determinant to the clinical procedure. Therefore, it is important to dispose of sufficient time for the material to be delivered but also to use the shortest time to close the defect. The surgeon needs these two characteristics to operate successfully (Deusser, Sattig et al. 2011).

Therefore, for the new generation we propose a solution with paste consistency, to remain at the site of the injection to prevent leakage. At the same time, the material would fill the gap created by the fracture. In addition, it must not heal inside the body to avoid high temperatures that cause cell death.

Also, biocompatibility is an imperative characteristic in new material. It should have the ability to be delivered with an appropriate host response in their specific application. This means that the material must not provoke an unresolved inflammatory response or demonstrate extreme immunogenicity or cytotoxicity. This characteristics must be accomplished for the intact material, for the degradation products and for any of its unreacted components(Thomson, Wake et al. 1995).

Sometimes, the biocompatibility is achieved through a sterilization process of the product to prevent infection. However, the sterilization method must not interfere with the bioactivity of the material or alter its chemical composition which could, in turn, affect its biocompatibility or degradation properties (Deusser, Sattig et al. 2011).Obviously, the new formulation proposed will be biocompatible once synthesized, avoiding sterilization processes that can vary their properties.

A biodegradable support material would be ideal too. The product reduces stress; allows the fracture to heal completely and the need for a second surgery to remove the fixation device is eliminated (Klein, Driessen et al. 1983).So, It is important that the rate of tissue formation be coupled to the degradation rate, so that the load-bearing capabilities of the tissue are not compromised (Thomson, Wake et al. 1995).

The polyesters are formed by monomers linked by ester bonds. This type of polymers are degraded by enzymes present in the organisms life, so they can be degraded in several biotic environments (Leja and Lewandowicz 2010).

Different factors affect the polyesters biodegradability:
-the molecular mass; the increase in molecular mass reduces its biodegradability
-the crystallinity
-the mechanical factors; they are not predominant during the biodegradation process, but they can activate or even accelerate biodegradation (Lucas et al. 2008)
-hydrolysis is another way by which polymers can be degraded. However, hydrolysis depends on parameters such as water activity, temperature, pH and time.

The biodeterioration of thermoplastic polymers is through two mechanisms; erosion of the surface and by deeper erosion (bulk erosion). In the case of deep erosion, the mass of the polymer decreases drastically as it loses important fragments of the material and consequently the molecular weight changes due to the links break. This phenomenon is caused by substances such as water,acids, bases, transition metals, radicals, or radiation, but not by enzymes. They are too large to penetrate the polymer matrix (Lucas et al. 2008).

On the other hand, in the superficial erosion, there is no big change in the polymer matrix molecular weight, but the enzymes provoke a matter loss in the polymer matrix. If the diffusion of chemical substances through the material is faster than the polymer bonds excision, the polymer undergoes bulk erosion. If the broke of the links is faster than the chemical substances diffusion, the degradation process occurs mainly on the surface of the matrix.

The most common erosion enzymes are hydrolases (depolymerases). For example, three amino acids are used in this reaction mechanism; aspartate, histidine and serine. Aspartate interacts with histidine ring to form a hydrogen bond. The histidine ring in that orientation

interacts with the serine. Histidine acts as a base and deprotonates serine to generate a very nucleophilic alkoxide group.

So, this group attacks the ester bond, because alkoxide group is a better nucleophile than an alcohol group, leading to the formation of a terminal alcohol group and an acyl-enzyme complex. As a result, water attacks the acyl-enzyme bond to produce a carboxyl end group and release the enzyme. This arrangement of serine, histidine and aspartate is referred to as the catalytic triad (Lucas et al. 2008).

Polycaprolactone is an example of that process and degrades in two stages. The first stage consists of the ester cleavage by non-enzymatic hydrolysis, autocatalyzed by carbon terminal groups of the polymer chains. When the molecular weight is reduced to 5000, the second stage starts with a decrease in the excision rate of the chain and weight loss begins due to the great diffusion of oligomeric species. The polymer becomes prone to fragmentation, or there is enzymatic surface erosion, or, the phagocytosis contributes to the absorption process (Leja and Lewandowicz 2010).

Polylactic acid is another example. The chains split results in the formation of carboxylic endings groups with acidic nature which improve the rate of hydrolysis. This mechanism is called autocatalysis and causes polyester matrices to present greater erosion (Edlund and Albertsson 2003).

As it has been seen before, biodegradable polyesters are very interesting in biomedical applications, where the application is temporarily, as in the case of sutures, in bone prostheses, scaffolds, and in drug release matrices. Polymers derived from aliphatic diacid monomers are also important from an ecological point of view, since they are green polymers, and they are easily disposable as waste. In this sense, we have chosen as the basis of the new formulation polyester because of its biodegradability.

Except for PLLA, which shows slow degradation, most of the polymers degrade in 5-8 weeks. While this time of degradation is sufficient for surgical suture and for implants in the field of ligaments repair, this rate of degradation is too high for temporary implants in the field of orthopedic surgery which function lasts between6 and 12 months (Claes 1992).

Last but not least, the mechanical properties of the injectable cement must be as similar as possible to healthy bone mechanical properties. As well as providing proper support in the early stages of healing, graded load transfer is needed later in the process for creation of replacement tissue that is identical to the original (Yaszemski, Payne et al. 1996). The mechanical properties that should be more considered are compression, tension and torsion, compressive properties (especially for replacement of cancellous bone) and tensile properties for cortical bone (Yaszemski, Payne et al. 1996).

After all this analysis, the first step is synthesizing a product that can come close to the ideal described material. Our laboratory developed a thermoplastic polymer synthesized with biocompatible monomers (described in pharmacopeia) (Borrós and Horna, 2014).This polymer is the beginning of our study to develop a new approach to injectable new multimaterial for VP that complies with the requirements described above.

So, in this first chapter a development of the synthesis of the initial product will be done, modifying the monomers and some conditions of the synthesis to obtain better reactivity and better rheological properties. Also, a characterization of the material will be realized. As it will be observed, these first tests will give us promising results as discussed below.

1.2 Materials and Methods

After observing the results of the synthesis of the thermoplastic polymer developed in our laboratory (Borrós and Tomas, 2014), the properties of the final product were improved by modifying the monomers and the reaction conditions. In this way, the total reactivity was increased and rheological properties of the final material were more appropriate for the final application of the material.

1.2.1 Synthesis and characterization of the polymers

a. Synthesis of Polyester Polyol

The synthesis of this polymer was carried out in two stages. In the first stage, the hard segment (called prepolymer) was synthesized with 1,8- octanediol (Sigma, O3303) and glutaric acid (Sigma, G3407) as monomers.

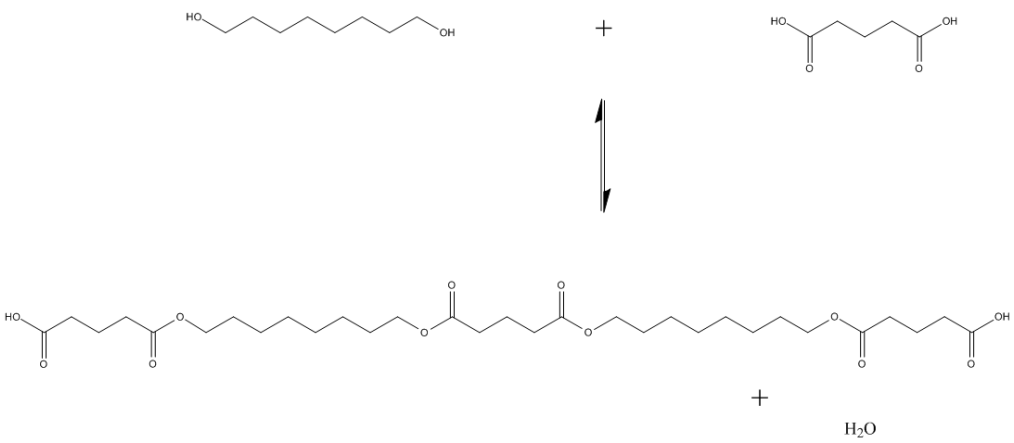

Figure I - 1: Reaction 1

5.5g of 1, 8- octanediol and 6g of glutaric acid were added in a molar ratio of 1: 1.2 respectively in a 100ml balloon, and they reacted in a microwave (CEM Discovery Microwave) in the synthesis conditions shown below:

-Power: 100W

-Temperature: 120ºC

-Time: 1h

-Compressed air as a refrigerant

-Without vacuum

-Magnetic stirring

An excess of glutaric acid was added. The reason was to end all the prepolymer chains in acid and to promote the reaction of the prepolymer with the PEG 1500 (Figure 1).

In the second step, 7.8g of polyethylene glycol 1500 (Sigma, 10783641001) were added in a molar ratio of 1:1 between the prepolymer and PEG 1500. The experimental conditions were the same as in the first stage.

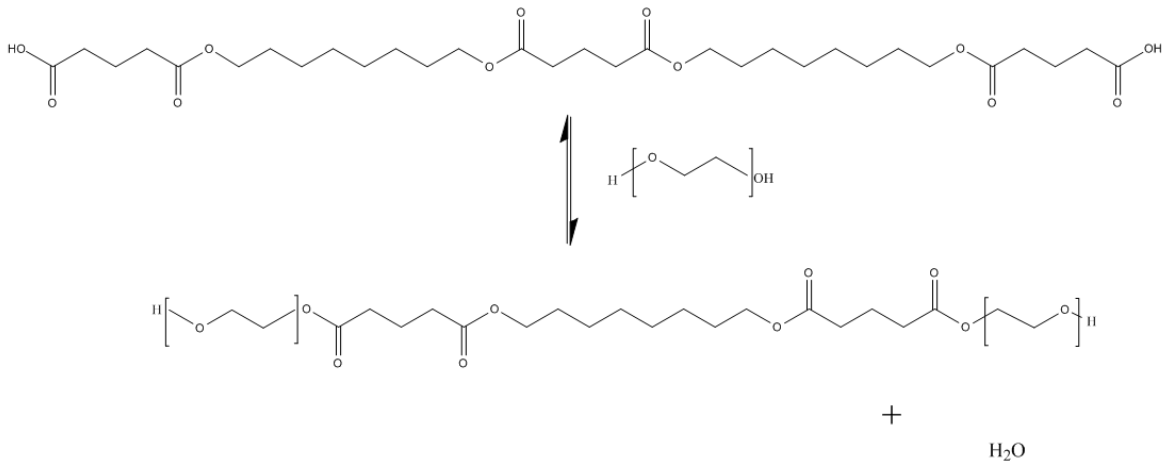

Figure I - 2: Reaction 2

17.3 grams were obtained. The product was a white paste which was used without purification process.

A1. Polyester Polyol and Hydroxyapatite

Once formed the polyester polyol, a different percentages of Hydroxyapatite (25%, 50% or 100%) were added. After adding the Hydroxyapatite (Sigma, 289396), the product was mixed in a Speed Mixer with 7 periods of 1 minute at 3000rpm.

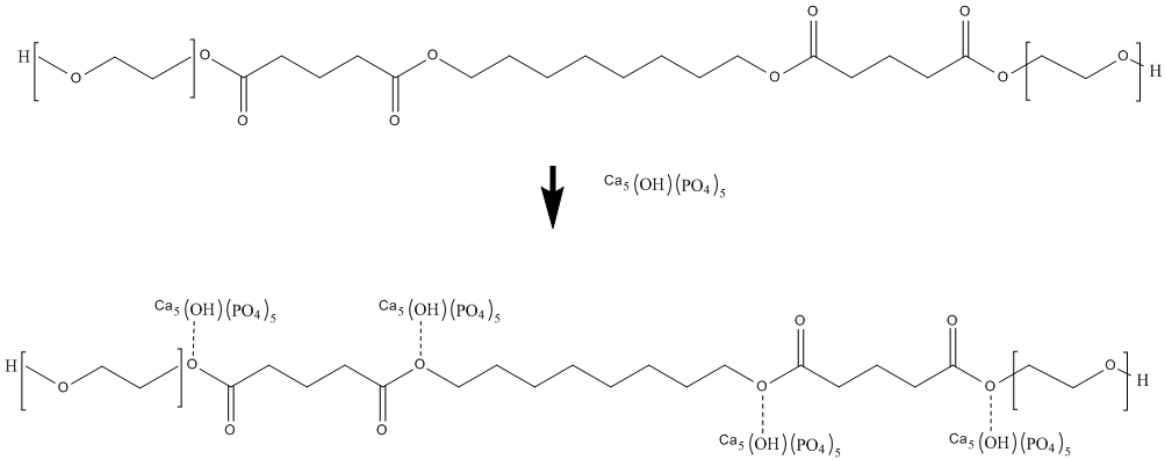

Figure I - 3: Interaction between the hydroxyapatite hydroxyl and the ester oxygen group of the polyester polyol

To form a paste with 100% of HAp; 50% ofHAp and 50% of polyester polyol were mixed. For example, to obtain 34.5 grams of the final product, 17.3 grams of the polyester polyol and 17.3 grams of the HAp were fused.

To form a paste with 50% of HAp; 50% of HAp and 100% of polyester polyol were mixed. For example, to obtain 25.9 grams of the final product, 17.3 grams of the polyester polyol and 8.6 grams of the HAp were fused.

b. Synthesis of Polyester Polyol with Glutaryl Chloride

Glutaryl Chloride Distillation

Before starting the synthesis, glutaryl chloride (Sigma, G4608) was distilled to remove all impurities generated. The distillation was performed with vacuum, during 3-4 hours at 130ºC.

In this second synthesis, glutaric acid was replaced by glutaryl chloride. The synthesis of this polymer was also performed in two stages. In the first stage, the hard segment (called prepolymer) was synthesized with 1.8-octanediol and glutaryl chloride.

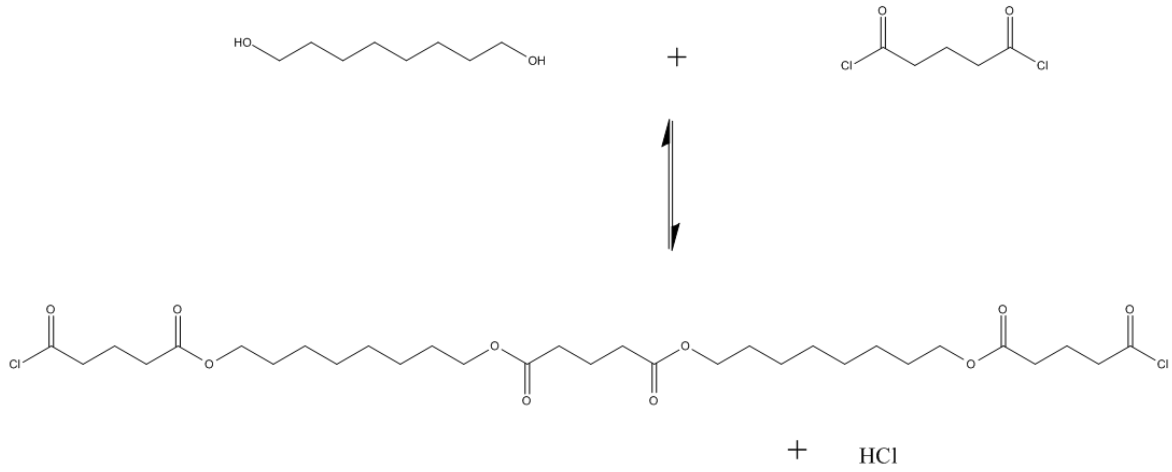

Figure I - 4: Reaction 3

1.8g of 1,8- octanediol and 2.6g of glutaryl chloride were added in a molar ratio of 1: 1.2 respectively in a reaction tube. The reaction was carried out in a silicone bath at 70ºC, for 1 hour, with magnetic stirring.

In the second step, 2.6g of polyethylene glycol 1500 were added in a molar ratio of 1:1 with the prepolymer. The experimental conditions were the same than in the first stage.

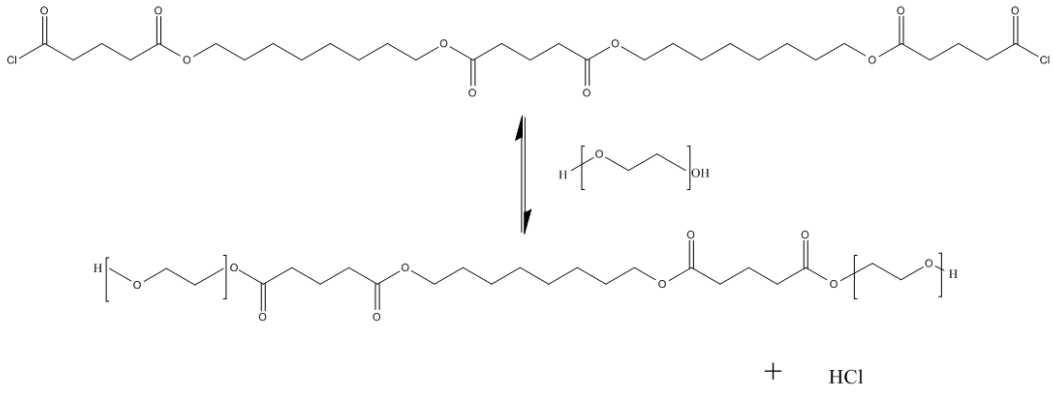

Figure I - 5: Reaction 4

A soda trap was connected to the reaction to capture all the free hydrochloric acid. Once the second reaction was finished, the plate was turned off. After 10 minutes, 0,5mL of methanol (Sigma, 322415) were added, and the reaction took 10 minutes. The methanol excess was added to end capping the free acid chloride chain ends.

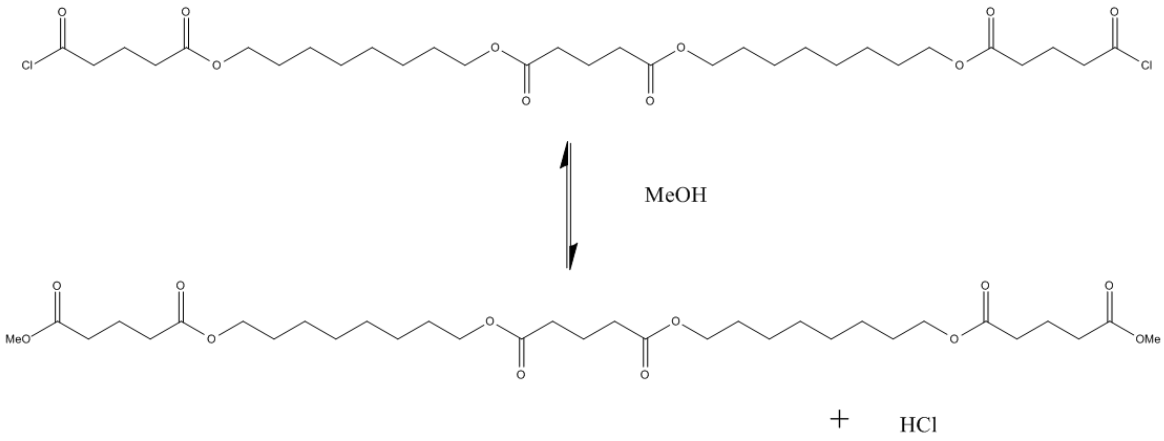

Figure I - 6: Reaction 5

Finally, the product was washed once with ether and twice with water. 5.2 grams of product were obtained before the cleaning process and 2g after washing it.
The final product was a white granulate.

B1. Polyester Polyol with Glutaryl Chloride and Hydroxyapatite

When the polymer was formed, 50% of Hydroxyapatite was added. After adding the bioceramic product, the mix was heated to 50ºC to facilitate the mixing process and the homogeneity of the sample. The reaction scheme was the Figure 3. More than 50% of HAp generates brittleness in the paste. 2.91 grams of product were obtained. The final product is a white paste.

c. Synthesis of Polyester Polyol with 1.8 moles of Glutaryl Chloride

In this case, the final polymer has the same chemical structure as polyester polyol, but the molar ratio of glutaryl Chloride respect to 1,8-octanediol is 1:1.8 instead of 1:1.2.

The synthesis of this polymer was also performed in two stages. In the first stage, the hard segment (called prepolymer) was synthesized with 1.8g of 1.8-octanediol and 3.8g of glutaryl chloride. The reaction was realized in a silicone bath at 70 ° C for 1h. The reaction scheme was the Figure 4**Error! Reference source not found.**.

In the second step, 2.6g of polyethylene glycol 1500 were added in a molar ratio of 1:1 with prepolymer. The experimental conditions were the same as in the first stage. The reaction scheme was the Figure 5.

Once the second reaction was completed, the plate was turned off. After 10 minutes, 0.65 mL of methanol were added, and the reaction took 10 minutes. In this case, the added MeOH quantity was higher than in polyester polyol because the added glutaryl chloride quantity was higher too.

Finally, the product was washed twice with ether and twice with water to remove impurities and short chains successfully. 6.5 grams of product were obtained before the cleaning process and 2.9g after washing it.

The final product was a white granulate.

C1. Polyester Polyol with 1.8 moles of Glutaryl Chloride and Hydroxyapatite

When the polymer was formed, 50% of Hydroxyapatite was added. After adding this bioceramic product, the mixture was heated to 50ºC to facilitate the mixing process and the homogeneity of the sample. The reaction scheme was the Figure 3.
4.3 grams of product were obtained. The final product was a white paste.

d. Synthesis of the Polyamide

The synthesis of this polyamide was also performed in two stages. In the first stage, the hard segment (called prepolymer) was synthesized with 1.8- diaminooctane (Sigma, D22401) and glutarylchloride.

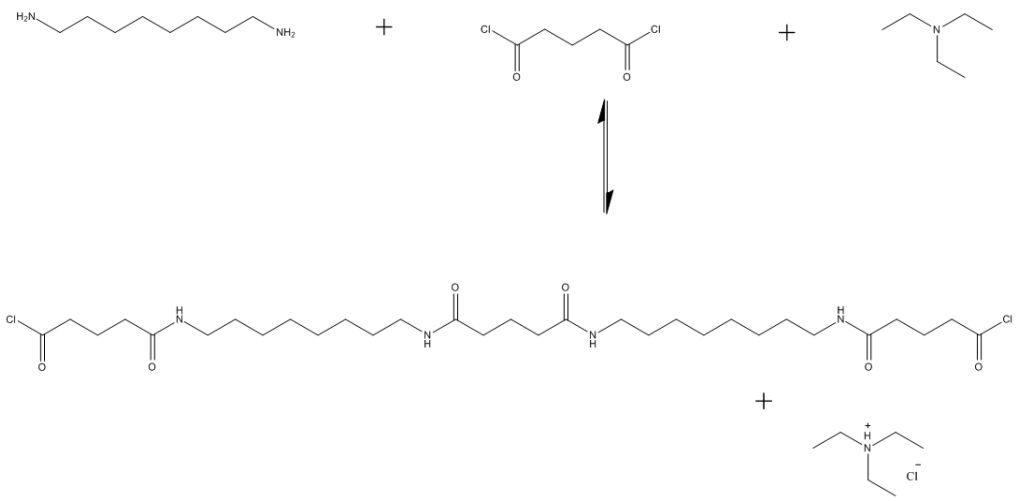

Figure I - 7: Reaction 6

In a 100ml balloon 1.8g of 1.8-diaminooctane were added and dissolved in dichloromethane (Sigma, 270997). The balloon was cooled in an ice bath. Then 1.9mL (2.6g) of glutaryl chloride was added dropwise. Finally, 4.2 ml of triethylamine (Sigma, T0886) were added dropwise and slowly too. All the steps were performed with magnetic stirring throughout the reaction. After 30 minutes, the ice bath was removed and the reaction was stirred at room temperature for two hours.

In the second step, 2.6g of polyethylene glycol 1500 were added in a molar ratio of 1:1 with the prepolymer. The experimental conditions were room temperature and magnetic stirring for 2 hours.

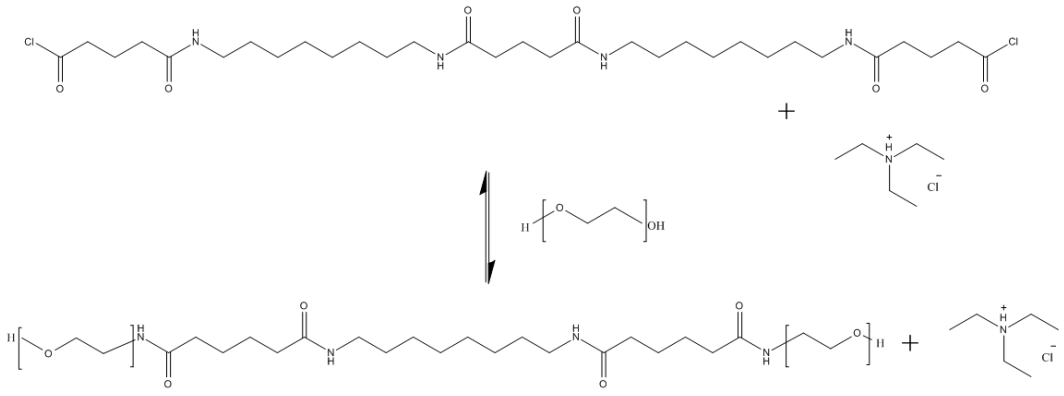

Figure I - 8: Reaction 7

Finally, the product was washed with ether and water twice. 6.1 grams of product were obtained before the cleaning process and 3.1g after washing it.

D1. Polyamide and Hydroxyapatite

It was impossible to mix the polymer with Hydroxyapatite because the polyamide does not flow at any temperature. Polyamide was discarded.

1.2.3 Rheological properties

The rheological experiments of all polymers were realized to choose one product as a candidate, depending on its viscosity. As already mentioned, in this particular application, the maximum viscosity is sought, so the material is able to withstand the mechanical load.
All the rheological assays were made with a rheometer AR 550 (TA instruments). A steel plate with 20 mm was used as a measuring geometry for all the tests and all the assays were done with 800 μm GAP, considering that the normal force exercised on the sample does not exceed 0.1 N.

a. Flow Experiments

A1. Viscosity vs Temperature

First, the sample was subjected to a temperature ramp from 25°C to 60°C, monitoring the viscosity (flow resistance of the material) to observe the variation of the material viscosity respect the temperature.

A2. Shear stress vs shear rate at 43ºC

A flow curve was realized for each sample applying a shear stress ramp from 0 to 20.000 Pa at 43°C.

A3. Shear stress vs shear rate at 46 ° C

In this case, the test was performed in the same conditions but at 46°C instead of 43°C. Polyester polyol with glutaryl chloride only flowed from 46 degrees.

b. Thixotropy

The aim of this experiment was to check if the fluid would be thixotropic. So, the samples were subjected to increasing shear stress over a period of time. After that, a constant tension was maintained for 30 seconds, and finally the tension was decreased until the starting point.

B1. Thixotropy test of polyester polyol with glutaryl chloride and HAp at 46 ° C

In a first step, a ramp of shear stress from 0.63 Pa to 1000Pa was performed during 1 minute. In the second step, the value of the shear stress was remained constant (1000Pa) and the shear rate increases slightly, during 30 seconds. And finally, a ramp of shear stress from 1000 Pa to 0.63 Pa was performed during 1 minute. This experiment was realized at 46 ° C.

B2. Thixotropy test of polyester polyol with glutaryl chloride and HApat 50 ° C

In this case, the test was performed at the same conditions but at 50 °C instead of 46 °C.

c. Oscillatory experiments

C1. Variation of storage modulus (G ') and loss modulus (G' ') respect of the temperature

This experiment was realized in oscillatory mode. In this test a temperature ramp was applied from 50°C to 25°C; decreased 3°C/min, strain was 1% and frequency was 1 Hz. The last two were fixed.

The reason why these two parameters were fixed was because the material was thermoplastic. When the strain was fixed at 1% (strain in which the material structure will not break but otherwise it does cause flow), the amplitude was inherently fixed to which the oscillation test was performed.

Moreover, the frequency was set at 1 Hz because it was the frequency at which oscillatory experiments are usually performed when working with thermoplastic materials (Arán Aís 2000).

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1.4 Results and discussion

a. Polyester polyol characterization

Polyester polyol is a polymer developed in our laboratory. It is synthesized with biocompatible monomers (described in pharmacopeia). However, it presents weak rheological properties despite the chain length of the monomers (Borrós and Tomas, 2014). So, the synthesis of the product is reproduced and the reasons why is weak at rheological level are analyzed carefully.

The synthesis of the formed polymer is analyzed by GPC to extract information about the formation of the product. The GPC of polyester polyol is compared with the GPC of PEG 1500 to verify that PEG 1500 has properly reacted with the prepolymer.

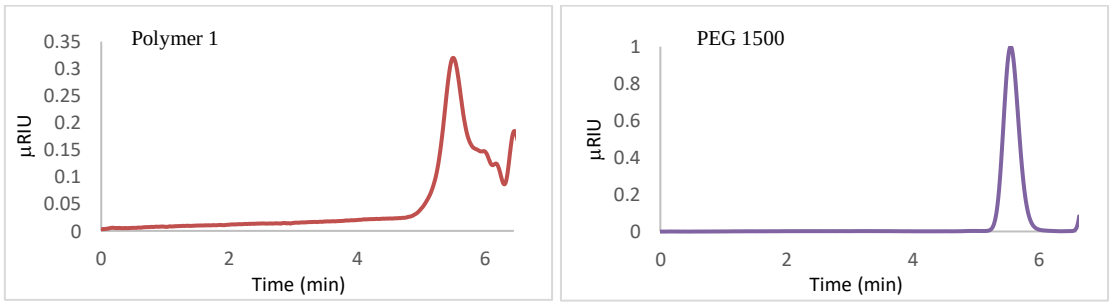

Figure I - 9: GPC of polyester polyol and PEG 1500

The two graphs show a marked peak from minute 5 to minute 6. Probably PEG 1500 has not properly reacted with the prepolymer formed by glutaric acid and 1,8-octanediol.Therefore, in both GPC the peak is basically represented by PEG 1500.

The reason why PEG 1500 has not reacted with the prepolymer is bascially the low reactivity of the prepolymer chain ends. Glutaric acid is not sufficiently reactive to bind with PEG 1500 effectively and the final polymer is not formed.

To increase the reactivity of the prepolymer chain ends, the glutaric acid of the first reaction is replaced by glutaryl chloride, which is more reactive with all active hydrogen (e.g polyols).

The chemical reason why glutaryl chloride is more reactive than glutaric acid is because the chloride of the glutaryl chloride is better leaving group than the hydroxyl of the glutaric acid.

So, the chloride nucleophilic substitution is easier than the hydroxyl nucleophilic substitution to form the ester of the polymer. In conclusion, to improve the reactivity of the prepolymer with polyethylene glycol, the glutaric acid is replaced by glutaryl chloride to form the polyester polyol chains.

However, the homogeneity of the reaction is also important and the conditions are slightly varied too. The reactions are realized in microwave (CEM Discovery Microwave). In a microwave, power and temperature are applied at the same time, so probably the reaction is heterogeneous because it is difficult to form long chains of polymer when the reaction is not under control.

In seeking to overcome both limitations, the microwave is replaced by a silicon bath at 70ºC. With the silicon bath the reaction only receives temperature (not power) and this temperature is distributed homogeneously throughout the reaction.

Therefore, this argumentation leads to change the design of the polymer synthetic procedure. The use of microwave is changed by a traditional wet chemistry procedure using an activated version of the glutaric acid (glutaryl chloride) after a completed pre-purification under vacuum distillation. However, the product rheological properties are studied to observe the influence of HAp and extract more conclusions.

A1. Rheological properties of polyester polyol

Flow Curve

Polyester polyol meets certain requirements such as being biocompatible because of its monomers. However, its rheological properties are weak. For these reason different percentages of HAp are added in the polyester polyol, to check if its viscosity improves noticeably.

As it has been stated before, its viscosity is important because the bone cement must be able to support the mechanical load that supports a vertebral bone without fracture.
Once the polyester polyol is analyzed by GPC, it is mixed with different percentages of Hydroxyapatite using a Speed Mixer (see materials and methods) because HAp increases the viscosity of the final paste (Borrós and Thomas, 2014). So, the ability of the polymer to form a

thermoplastic paste with good rheological properties is evaluated using a complete rheometric analysis.

The temperature at which all the samples are flowing is determined by a temperature vs viscosity ramp.

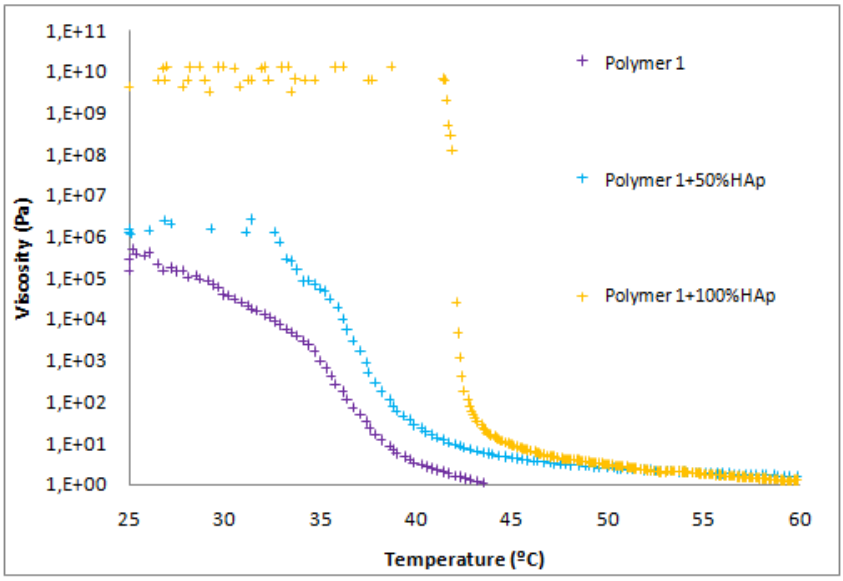

Figure I - 10: Temperature vs Viscosity ramp

The temperature at which the polyester polyol + 100HAp begins to flow is 43 ° C. Then, the flow curves are performed at 43ºC because polyester polyol + 100HAp is the more viscous product in the graph.

Also, the polymer presents a thermoplastic behavior as it can be checks in figure 10, when it is mixed with HAp or when it is alone. It is interesting to say that the product can be considered thermoplastic because at 37 degrees it is able to solidify to withstand the load that the vertebrae supports, and at a higher temperature it is able to be injectable by the surgical technique of vertebroplasty. Next, the flow curves of each sample are realized.

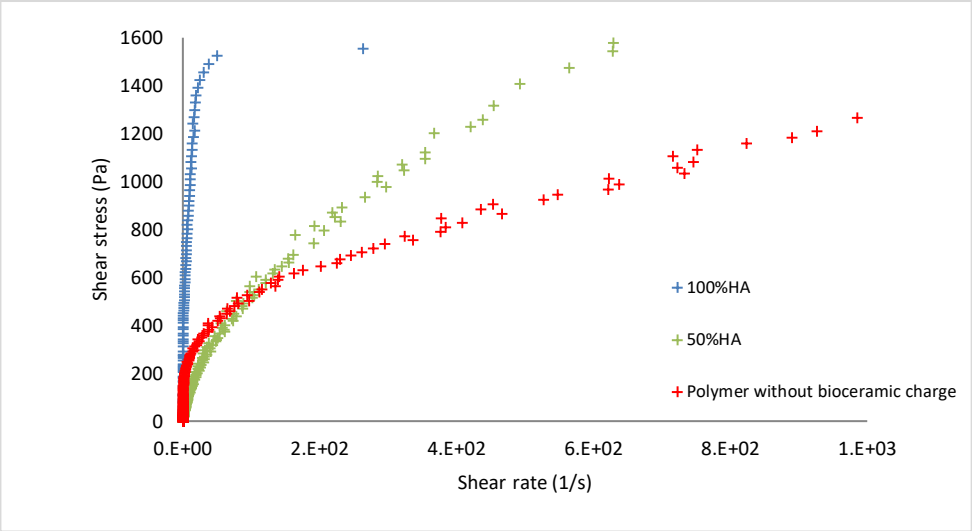

Figure I - 11: Flow curve of Paste 1, Paste 0,5 and Polymer 1 at 43ºC

The presence of the bioceramic in the polymer increases the product viscosity. Then, the higher the percentage of HAp in the polyester polyol is, the more shear stress is necessary to apply to flow it at the same shear rate (Borrós and Thomas, 2014).

Considering the application of this product, it is important to look for the maximum viscosity. The paste has to bear all the efforts that the vertebra receives, knowing that the vertebra is weak because of the fracture. So, initially, the vertebra leaves more load on the prosthesis (Claes 1992). In this sense, the material designed would present high viscosity at the beginning of the healing process, but when the new bone grows and the prosthesis is subject to more shear stress, the material viscosity decreases while the new formed bone takes its place. This behavior is expected from polyester polyol + 100%HAp, which is pseudoplastic; a decrease in its viscosity is produced when shear rate increases.

Polyester polyol + 100%HAp is a candidate for the moment, but it is interesting to replace glutaric acid for glutaryl chloride, and to analyze the rheological properties of the final product to see if they improve .The idea is to make the synthesis more reproducible, controlling the polymer molecular weight. So, the prepolymer has to react in a controlled way and generate copolymer chains.

So, if the chains are longer than in polyester polyol formed with glutaric acid, it will be possible to remove impurities from the polyester polyol formed with glutaryl chloride through washes with water and organic solvents avoiding the product dissolution.

b. Polyester polyol with 1.2 glutaryl chloride + HAp and polyester polyol with 1.8 Glutaryl Chloride + HAp

The synthesis of polyester polyol has some differences from the synthesis of polyester polyol with glutaric acid. Firstly, glutaric acid is replaced by glutaryl chloride for its reactivity. Also, the reaction between polyester polyol with glutaric acid is not performed in the microwave (120ºC, 100W), because glutaryl chloride is highly reactive. So, the reaction occurs more homogeneously when it is performed only with temperature in a silicon bath.

Later, when the PEG 1500 is added and the second reaction is completed, a quantity of methanol is added in excess because maybe there are a few chains without reacting with PEG 1500 (ending in acid chloride). This step is important because if chains end in acid chloride, this acid chloride is hydrolyzed to acid and this causes acidity in the media. Because of the application of this product a neutral pH is required (Hong et al. 2006).

Finally, the polyester polyol with glutaric acid is washed with ether and water. The aim of the cleaning process is to eliminate: the short chains of prepolymer that are not linked with PEG 1500 or linked with MeOH, some impurities of the glutaryl chloride and free PEG 1500.

Once the product is washed and dried in the freeze dryer, its GPC is performed.

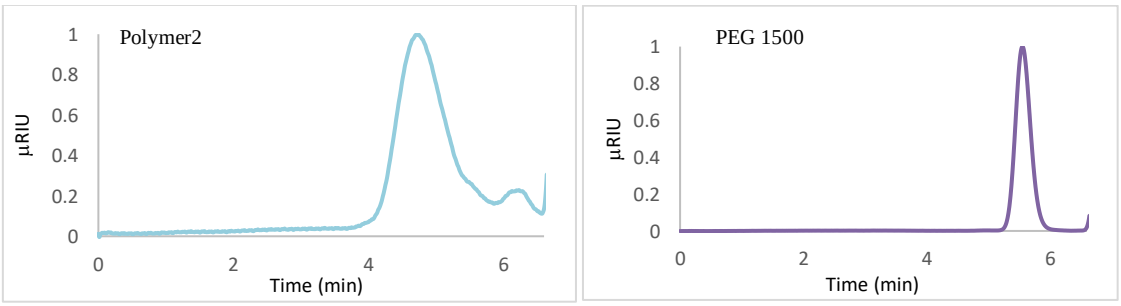

Figure I - 12: GPC of polyester polyol with 1.2 glutaryl chloride and PEG 1500

A pronounced peak between minute 4 and minute 5.7 is observed. This peak probably corresponds to the polyester polyol with glutaryl chloride. Between minute 5 and minute 6 any peak is observed; therefore, there are no free PEG 1500 in detectable amounts by GPC. As expected, glutaryl chloride has reacted better than glutaric acid with PEG 1500.

Finally, polyester polyol with glutaryl chloride is mixed with 50% of HAp to obtain the highest possible viscosity, because only accepts 50% maximum of HAp since more quantity produces brittleness in the paste.

At this point, another new polymer is synthesized because it is interesting to create a product which has 100% of hydroxyapatite - polymer interaction. The idea is to get a product with a high limit of fluidity. The reason why it has to flow easily is because once the injection starts, the surgeon have not to find problems with the injection, which has to be homogeneous and without too much effort. However, once the product is injected, it has to be viscous enough to stay in the place where it has been injected and able to support the weight of the vertebra.

Polyester polyol with 1.2 glutaryl chloride is almost the same as polyester polyol with 1.8 glutaryl chloride, but with different molar ratio in the synthesis of the prepolymer. In this case, 1.8 moles of glutaryl chloride are added instead of 1.2, for 1 mole of 1,8-octanediol. In polyester polyol with 1.2 glutaryl chloride, the formed chains are longer but the total number of chains is lower. Polyester polyol with 1.8 glutaryl chloride has more quantity of chains that can interact with hydroxyapatite but the chains have less length, for the excess of glutaryl chloride.

Despite two of them present the same experimental conditions, polyester polyol with 1.2 glutaryl chloride has higher viscosity than polyester polyol with 1.8 glutaryl chloride because of its structure but the first one interact less with HAp than the second one.

Therefore, it is important to check which factor is more determinant for the final viscosity of the product; the hydroxyapatite quantity or the length of the chains. Polyester polyol with 1.8 glutaryl chloride requires more washes as polyester polyol with 1.2 glutaryl chloride because more moles of glutaryl chloride are added. So, there are more impurities and they are more difficult to remove.

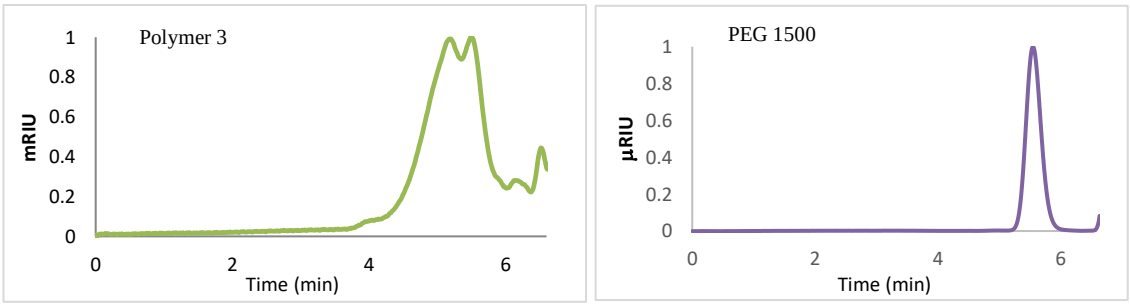

Figure I - 13: GPC of polyester polyol with 1.8 glutaryl chloride and PEG 1500

The GPC of polyester polyol with 1.8 glutaryl chloride presents two peaks between minute 4,8 and minute 5,8. However, the peak between minute 5 and minute 6 coincides with the peak of PEG 1500 although the product has repeatedly been washed and the PEG 1500 excess has been removed.

So, maybe the reason why two peaks are observed is the formation of two different polymer chains. Possibly there are some chains formed by polyester polyol with 1.8 glutaryl chloride prepolymer and PEG 1500 and chains formed by polyester polyol with 1.8 glutaryl chloride prepolymer and methanol. The two of them have their influence in GPC test.

Polyester polyol with 1.2 glutaryl chloride with HAp is generated with polyester polyol with 1.2 glutaryl chloride + 50% of HAp and polyester polyol with 1.8 glutaryl chloride with HAp is generated with polyester polyol with 1.8 glutaryl chloride + 50% of HAp. Finally, more quantity of HAp produces brittleness in both cases.

A2. Rheological properties of polyester polyol with 1.2 glutaryl chloride and polyester polyol with 1.8 glutaryl chloride + HAp

Flow Curve

Although both polymers only interact with a maximum of 50% of hydroxyapatite, rheological analysis of both of them is done to compare the results. Polyester polyol with 1.2 glutaryl chloride is the one with the longest chains, so probably it is the most viscous. But, it is important to check it.

So, the different results obtained with the flow curves of each of the samples are shown:

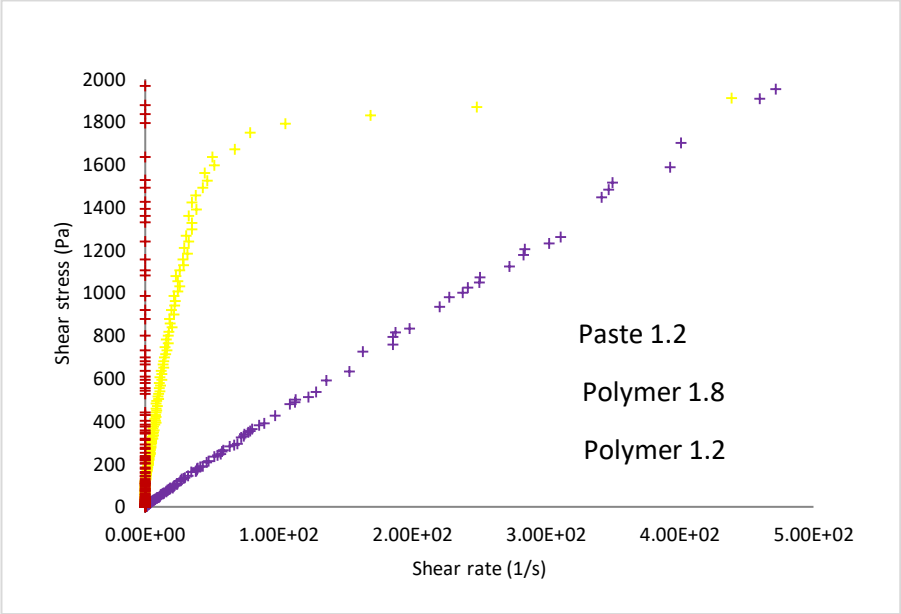

Figure I - 14: Flow curve of polyester polyol with 1.2 glutaryl chloride, polyester polyol with 1.8 glutaryl chloride and polyester polyol with 1.2 glutaryl chloride + HAp at 43ºC

Polyester polyol with 1.8 glutaryl chloride is the most flowing sample with less shear stress applied at 43ºC. So, it is the least viscous too. In addition, polyester polyol with 1.2 glutaryl chloride is the one which has the longest chains and a higher viscosity than expected. When the prepolymer chains are longer, viscosity is increased because more effort is required to flow a long chain rather than flow a shorter chain.

In fact, polyester polyol with 1.2 glutaryl chloride is so viscous that its flow curve is not observed at these shear stress values. It is necessary to apply more shear stress to observe the flow curve of the polyester polyol with 1.2 glutaryl chloride.

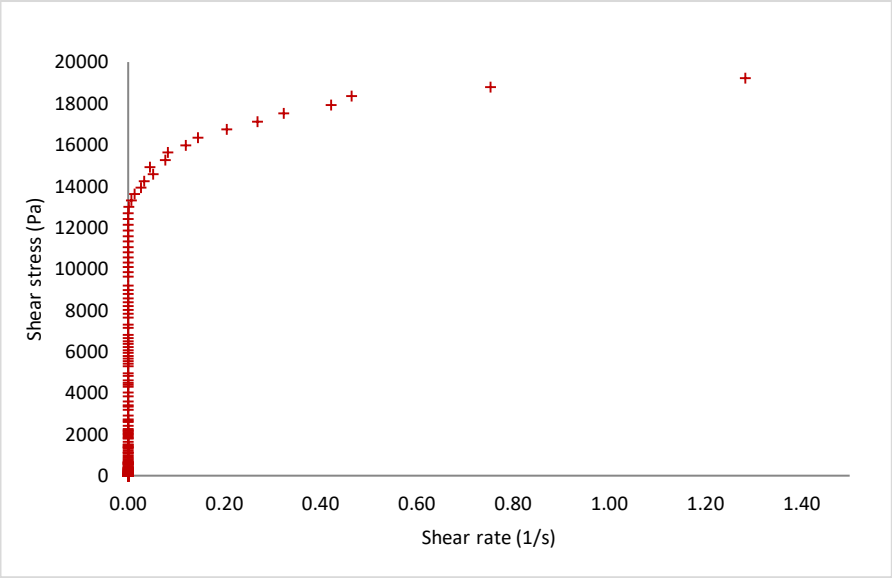

Figure I - 15: Flow curve of polyester polyol with 1.2 glutaryl chloride at 43ºC

Polyester polyol with 1.2 glutaryl chloride is so viscous that the flow curve of polyester polyol with 1.2 glutaryl chloride with HAp has to be performed at 46ºC instead of 43ºC. At 43ºC, the product is located below its yield point.

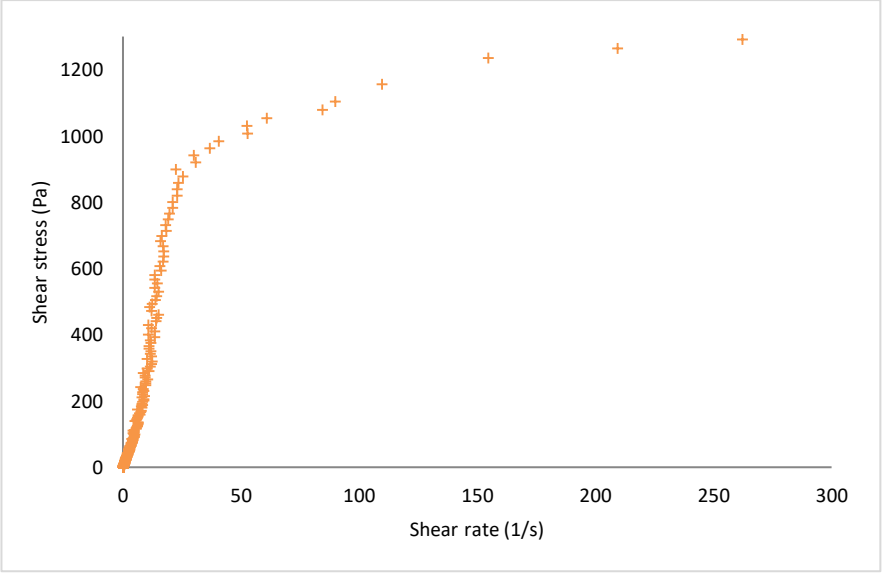

Figure I - 16: Flow curve of polyester polyol with 1.2 glutaryl chloride at 46ºC

Finally, polyester polyol with 1.2 glutaryl chloride with HAp is selected to work with because it is the one with higher viscosity.

However, in this application it is important for the hydrolysis time to be adequate as the prosthesis must support the mechanical load of the fractured vertebra while it heals. So, the hydrolysis time has to be long enough for the prosthesis to be in the bone until it begins to reconstruct. Although polyester polyol with 1.2 glutaryl chloride is a good candidate, another new polymer is developed to increase the polymerhydrolys is time.

c. Polyamide

An Amide bond is much harder to break than an ester linkage, and for this reason the polyamide is selected. The molar ratio between 1,8-diaminooctane and Glutaryl Chloride is 1: 1.2 respectively. An excess of Glutaryl Chloride is added to end all the prepolymer chains in acid chloride. This last step is to promote the reaction of the prepolymer with PEG 1500 to form the polyamide.

In the first stage of the reaction, an ice bath is used because the reaction is exothermic and it is necessary to low the temperature. Also, it is important to note that the function of triethylamine is reacted with hydrochloric acid which is liberated from the acid chloride.

If there is free hydrochloric acid which is easily protonated, it can react with 1,8-diaminooctane. Then, the ammonium salt (NH_4Cl) is formed, and polymerization doesn't run.
Regarding the amount of triethylamine, 2 equivalents per one mole of the acid chloride have to be added because glutaryl chloride has two chlorides per molecule.

After the first stage, prepolymer and PEG 1500 are mixed in a molar ratio of 1: 1.2 respectively. The reaction is only tested at room temperature because polyamide does not flow as we will see later, and therefore it is discarded for this application. So, more variables have not been tested.

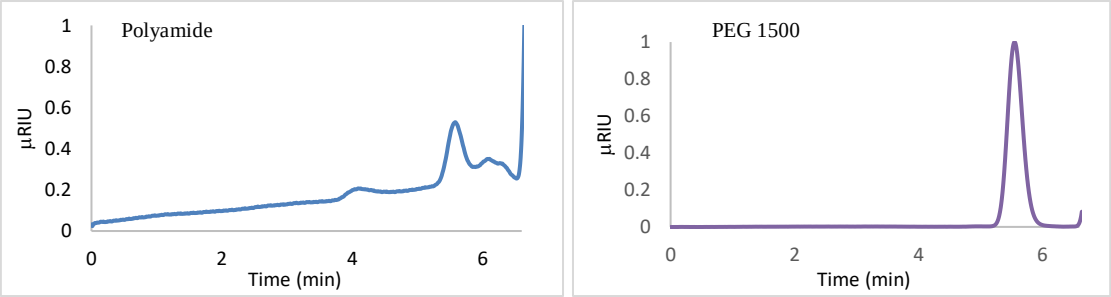

Figure I - 17: GPC of Polyamide and PEG 1500

A marked peak between minute 5 and minute 6is observed. Probably, a significant amount of PEG 1500 has remained from the second reaction. Finally, between minute 5.8 and minute 6.4, there are two small peaks very close. Maybe these two peaks belong to the polyamide which has been unbound with PEG 1500.

Polyamide + %HAp

Polyamide does not flow at any temperature, so it is impossible to mix it with Hydroxyapatite. Polyamides are discarded as a possible candidate because they do not flow, and the first objective of this thesis is to synthesize a thermoplastic paste.

B2. Thixotropy of polyester polyol with 1.2 glutaryl chloride and HAp

After selecting the polyester polyol with 1.2 glutaryl chloride+ 50% HAp for its promising results, its rheological properties are studied more deeply. Thixotropy is a very important property of the material and is required in the product to be applied in vertebroplasty because it is necessary for the paste to find good conditions of injectivity (shear stress vs compression).

In this technique, the material is injected by syringe and it is important to see the behavior of the selected material while it is being injected and after the injection. The rheological properties of the paste must be the same before and after the injection. At the beginning of the injection, the shear stress applied increase during 30 seconds, but after that, maximum shear stress is applied during 30more seconds. Once the material is injected, it is important to note if the rheological properties exhibited by the material are the same as before the injection or on the contrary, the rheological properties decrease, and the paste loses its viscosity.

So, the thixotropy experiment consists of three phases. In the first phase, a viscosity ramp is applied to the product from 0 to 30 seconds to observe its pseudoplastic behavior.

In the second stage, the material is left for 30 seconds at maximum viscosity. The aim is to observe if the material gets to hold viscosity at that point (measured like shear rate), or if on the other hand, its shear rate is falling over time. This stage is performed considering the injection time that the surgeon needs to apply the product (application time), and the behavior of the material respect to the constant pressure.

Finally, in the third stage, shear rate (or the viscosity of the paste) decreases until the initial value and it is observed if it decreases as a thixotropic material (fluid in which viscosity decreases with time) or not.

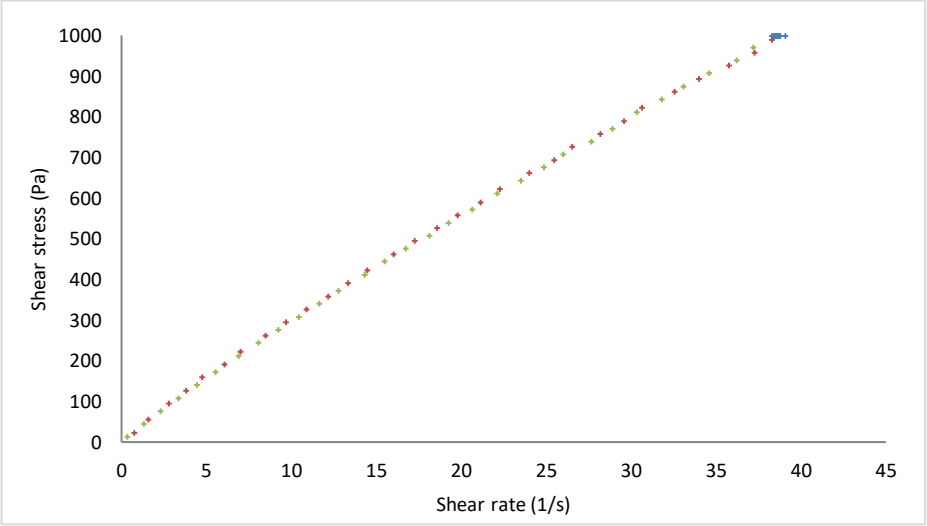

Figure I - 18: Thixotropy assay of polyester polyol with 1.2 glutaryl chloride + HAp at 46ºC

The first thixotropy test is performed at 46ºC, but in this case is necessary to apply more temperature to observe its thixotropic behavior. The shear stress applied is lower than in the flow curve, so the temperature has to be higher for the paste to flow.

In fact, in thixotropy assay it is impossible to apply much shear stress. During the second stage, the material is left up to a maximum constant shear stress during certain period, and the material structure breaks. For these reason, in the first stage of the thixotropy assay, the maximum value of shear stress is lower than 20000 (in contrast to the flow curve). So, the rupture of the material structure is avoided.

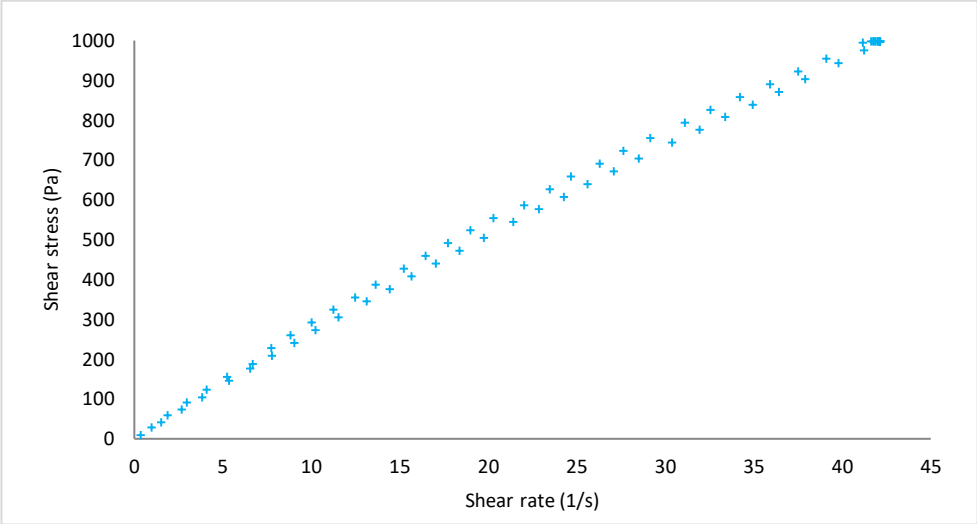

Figure I - 19: Thixotropy assay of polyester polyol with 1.2 glutaryl chloride + HAp at 50ºC

The second thixotropy test is performed at 50ºC and the viscosity of the material is measured. The bigger the remaining area is between the curves, the greater is the degree of thixotropy.

So, the thixotropic behavior of the paste is observed more clearly at 50° than at 46°C. In the first stage, where a continuous shear stress ramp is applied from 0 to 1000Pa, shear rate increases (material flows). Then, in the second stage, where a constant maximum shear stress is applied for 30 seconds, the material remains within the same shear rate. And finally, in the last stage, the shear stress decreases at the same time as shear rate.

In conclusion, polyester polyol with 1.2 glutaryl chloride with HAp is an appropriate candidate because presents a clear thixotropic behavior with controlled hysteresis, which is needed in a material that is applied in vertebroplasty.

C1. Experiments in oscillating mode.Variability of new materialconsistency (G 'and G' ') against the Tª

Finally, the viscoelastic properties of the polyester polyol with 1.2 glutaryl chloride+ HAp are characterized by a study of the variation of the elastic modulus (G') and loss modulus (G'') versus temperature. It is decisive to determine from what temperature the material has a higher viscous component (G '') than elastic component (G'). From that point, the material

ceases to behave like an elastic material, and it begins to behave like a plastic material, and from that point, the material begins to flow.

The storage volume Modulus (G') and loss modulus (G'') are represented as a function of frequency.

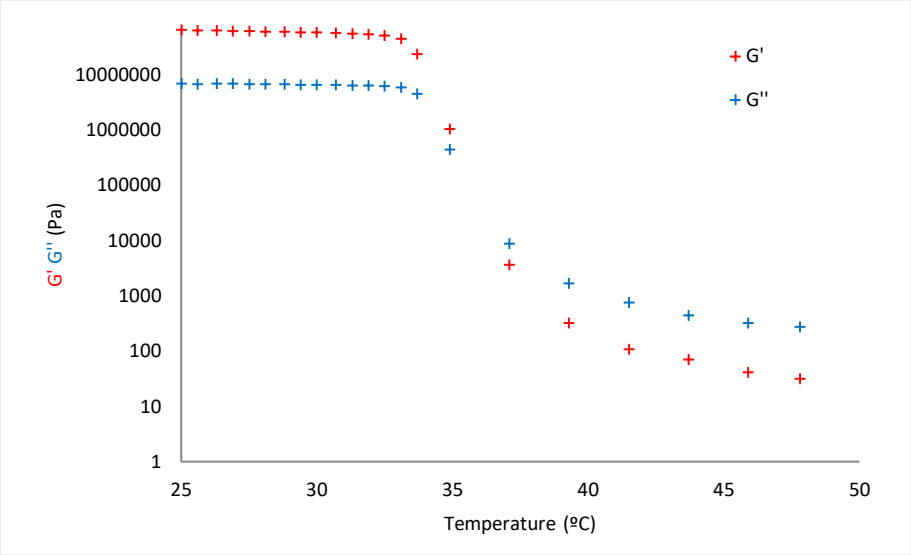

Figure I - 20: G' and G'' respect to the temperature of polyester polyol with 1.2 glutaryl chloride + HAp

The product can be injected from 37.1°C and applying 8598 Pa, which is when it starts to flow. This rheological test is indicative regarding the injectability of the paste. Also, it is important to keep in mind other factors such as the length of the syringe, the LPR, among others (Habib, Baroud et al. 2008,Bohner and Baroud 2005).

1.5 Concluding remarks

New family of biodegradable and biocompatible polyesters has been developedand their viscosity can be controlled by the length ofeach chain and by the reactivity of their monomers. Two compositional parameters have been studied specifically: The ratio Polyester/Polyethylenglycol and the amount of Hydroxyapatite.

The pastes have been complete characterized in terms of rheological characteristics and hardness with shear stress. The best combination of polyester polyol with glutaryl chloride and Hydroxyapatite has been investigated in order to characterize its rheological characteristics and to study the modification of viscosity in front of temperature in order to establish an injectable formulation.

Then, the polymer which reacts more effectively and has the best rheological properties for this application has been selected. Polyester polyol with 1.2 glutaryl chloride, the selected polymer, has been mixed with HAp and the formed paste exhibits a good thermoplastic behavior and higher viscosity.

Next, in the chapter two, the mechanical properties of the selected paste are analyzed to ensure that it is really able to hold the mechanical compression load that healthy vertebral body supports, and then, augment and stabilize it after the compression test. So, it has the strength and stiffness enough.

Also, the injectability of the polyester polyol with 1.2 glutaryl chloride and HAp is checked within a short working time frame and if it is able to fill the fracture while it avoids cement extravasations into the surrounding tissues.
In addition, it is studied if the setting time is reduced to end the operation as soon as possible to avoid infections. And finally, it is analyzed if the bone cement is compatible with specific needle for spinal surgery vertebroplasty.

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Chapter II. Assessment of the new formulation

“Cap problema.”

- Eduardo Bardají Ais

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2.1 State of the art and assessment of the new material

As it has been stated, the present study focuses on developing injectable bone cement for vertebroplasty, which is a minimally invasive surgery that has been introduced for the medical treatment of vertebral compression fractures. Specifically, it goals are to augment the weakened vertebral body, stabilize it and restore it to as much of its normal height and functional state as possible (Mathis, Barr et al. 2001).

Until now, polyester polyol with glutaryl chloride and HAp, the pseudoplastic paste that has been worked with has shown promising results. It exhibits a thermoplastic behavior, a higher viscosity and adequate viscoelastic properties.

The selected formulation will be optimized throughout this chapter to comply the main requirements for injectable bone cement. It will be easily injectable, it will has an adequate viscosity during and after the injection, high radio-opacity, suitable mechanical properties and working and setting times compatible with the surgical procedure (Deramond, Wright et al. 1999).

In biomedical implants and devices is necessary to measure and evaluate if the needs that demand the prosthesis are being covered. So, the vertebroplasty material design will be done in this chaper two. Next, the assessment required in bone cement will be developed.

The first characteristic is biodegradability. Most used materials for these surgeries are biodegradable. It is interesting as a second operation to remove the implant is not necessary because it biodegrades over time and the charge transfer occurs in a progressive way. This is an advantage for the patient and reduces the operating cost of treating injuries.

Moreover, the biodegradation is interesting from a mechanical point of view. An injured connective tissue such as a fractured bone needs the protection of the surgical implant to allow restoration. During the healing process, the implant reduces progressively its protective function and transfers gradually more load to the tissue, stimulating a faster healing and accelerating the process of remodeling.

These two phenomena can be seen in figure II-1. On the one hand, the implant degradation that gradually loses its function. And on the other hand, the tissue strength increases during the healing process.

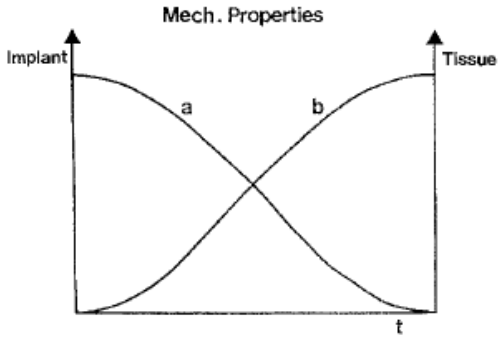

Figure II - 1: Optimal degradation characteristic of an implant for tissue protection (a) and improvement of tissue strength during the healing process (b)(Claes 1992)

Initially, the fractured bone has no strength and the material carries the entire load. Therefore, the implant must have the highest mechanical properties at that time. As the healing process progresses, the material reduces its stiffness as part of the load is supported by the regenerated tissue. Moreover, this reduction in rigidity allows a better load transfection to the bone.

When bone healing is over, the material loses its mechanical function. In this sense, it is important to underline that the most important limiting factors for the application of biodegradable implants are their mechanical properties. Additionally, the mechanical properties are also very important for bone cements in vertebroplasty because their use affects the stiffness in vertebral body and the load transfer in adjacent vertebra (Berlemann, Ferguson et al. 2002; Laredo and Hamze 2004).

These two factors may lead to an adjacent vertebral failure (AVF) which is a frequently observed post-surgery complication of percutaneous vertebroplasty.

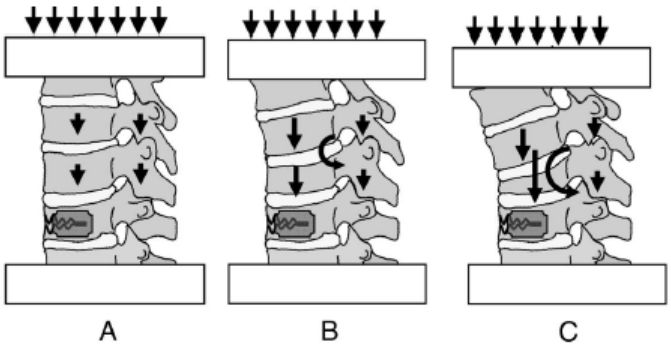

Figure II - 2: Load transfer in adjacent vertebra (Chiang, Wang et al. 2009)

Clinical studies showed 12% to 24% of patients suffered subsequent fractures post vertebroplasty within 1 year. Also, 41% to 67% of the subsequent fractures occurred in the adjacent vertebra of the treated (augmented) vertebra. The fracture rate of adjacent vertebra is 3 times higher than the non adjacent vertebra(Chiang, Wang et al. 2009).

Therefore, it is essential to consider the mechanical properties of vertebral trabecular bone (elastic modulus), where the injected cement is inserted (which varies from 109 to 327 MPa in the area) (Helgason, Perilli et al. 2008). This would be the target elastic modulus of any bone cement intended to be used in verterbroplasty.

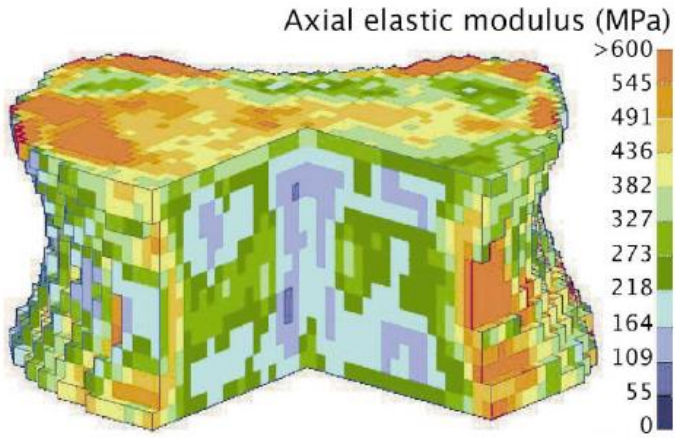

Figure II - 3: Distribution of axial elastic modulus within a lumbar (L1) vertebral body (Crawford, Cann et al. 2003)

Therefore, it will be tested if our bone cement is biodegradable to not generate tensions inside the vertebra, and to avoid adjacent vertebral failure. Also, its elastic modulus has to be between 218-300 MPa.

Another problem associated with the mechanical properties of the implant is injectability. Bone cement must be injectable because it is an essential property in minimally invasive clinical applications as described below.

However, bone cements may show different problems when they are injected. For instance, most of the materials already described in the tesis suffer a phenomenon named liquid-phase migration. This effect, produced due to the poor stability of the injectable formulation, leads to an excess of liquid (mainly water) in the injected cement. The phenomenon is clearly related with the pressure needed for the injection. Even though the forces required for injecting the material are very reasonable and the cement can be manually applied by doctors, a filter-pressing phenomenon can occur leading to a dramatical increase of the injection force needed, which complicates manual injection.

To solve this problem, an increase in the ratio liquid/particles has been proposed in the final formulation. However, this approach has shown a decrease of the mechanical properties of the final cement due to the formation of highly porous cements, mechanically weak (Habib, Baroud et al. 2008,Bohner and Baroud 2005).

Another feature related with the filter-pressing problem is time dependence. Although high injection speed reduces the liquid migration and improves the cement homogeneity, the reduction of observation time during the minimally invasive medical intervention, increases the patient risk(Habib, Baroud et al. 2008).

Two other solutions have been proposed to overcome the filter-pressing problem: to increase the viscosity of the liquid mixture and to reduce the permeability of the particles. With these solutions, the capability of the liquid mixture to pass through particles is reduced, so the filter-pressing problem too. Nevertheless, 100% injectability is not reached. This implies that filter-pressing occurs, as stated, evenatvery small forces(Bohner and Baroud 2005).

Other factors influencing the filter-pressing problem are the syringe gauge (the smaller, the less filter pressing), and the use of acannula (cannula increases filter-pressing).

Also, the injection has to be homogeneous (without separation of the two components) and with a proper viscosity, enough to be easily injected through a vertebroplasty needle but avoiding the risk of cement leakage from the vertebral body.

As can be seen, the bone cement injectability is one of the main problems presented by vertebroplasty materials. For this reason, during this chapter, we do need to emphasise the importance of this phenomenon. In fact, injectability is the first property that we will be studied.

Another important characteristic that materials used in vertebroplasty must have is easiness-of-handling. This property is key for any biomaterial intended for clinical use. Cements should be easily prepared at the operating theatre to facilitate surgery (Temenoff and Mikos 2000).

Therefore, viscous properties must be balanced between two needs: the need of the material to remain at the site of injection to prevent leakage of bone cement and the need of the surgeon to easily manipulate its placement to fill successfully the gap created by the fracture(Lee et al. 2012).

It is necessary to complete the last need before the hardening process begins, while avoiding the risk of extravasation (Laredo and Hamze 2005). Obviously, the working (which includes mixing and injection) and setting times should be compatible with the surgical procedure, to ensure a slot in which the cement is still injectable and a rapid hardening when the cement is in situ. In this sense, it is worth to indicate that vertebral cement must be completely set at the end of the surgical procedure, to allow the immediate mobilization of the patient after the treatment.

Also, if the setting reaction involves a temperature change, the increase or decrease should be as small as possible to reduce damage to the surrounding tissues (Deusser, Sattig, and Boger 2011; Frayssinet et al. 1998).

Finally, another essential requirement for vertebral cements consists in having a suitable radio-opacity degree to ensure its visualization under radioscopic guidance, both during the positioning inside the vertebral body in the percutaneous injection procedure (in order to observe any possible leakage) and during the subsequent follow-up checks on the patient.

In order to impart radio-opacity to the injectable composite material, radio-opaque powders, such as $BaSO_4$, are usually added. But this radio-opaque agent would represent a further

dispersed phase which would cause the worsening of miscibility, injectability and mechanical properties, especially bending strength and dynamic fatigue performance, of the bone cement.

However, Zirconia is an optimal radiopacifier. The first proposal of the use of zirconium oxide for medical purposes was made in 1969 and concerned orthopedic application. ZrO_2 was proposed as a new material for hiphead replacement instead of titanium or alumina prostheses. The in vivo evaluation of $ZrO_2$was reported with no adverse responses. Moreover, these first studies were largely carried outin vivo because in vitro technology was not yet sufficiently advanced(Manicone, Iommetti et al. 2007).

Since 1990, in vitro studies have also been performed in order to obtain information about cellular behavior towards zirconia. In vitro evaluation confirmed that ZrO_2 is not cytotoxic. Mutagenicity was evaluated by Silva and by Covacci, and both reported that zirconia is not able to generate mutations of the cellular genome.

In addition, Zirconia has mechanical properties similar to stainless steel. Its resistance to traction can be as high as 900-1200 MPa and its compression resistance is about 2000 MPa. Also, cyclical stresses are tolerated well by this material. (Manicone, Iommetti et al. 2007).

So, in this chapter, polyester polyol with glutaryl chloride and HAp homogeneity throughout injection will be proved and a mechanical analysis will be realized to ensure that the product is going to support the mechanical load of the vertebra. Finally, an ex vivo assay will be done to ensure that the bone cement is compatible with the surgical intervention.

2.2 Materials and Methods

2.2.1 Homogeneous injection

In order to easily test the injectability of the cement, a qualitative test was carried out. It consisted on preparing the cement by mixing the powder phase with the polymer and deposits the polyester polyol with glutaryl chloride and HAp into the syringe. Then, the syringe was heated to 50ºC in the syringe warmer during 25 minutes. A syringe warmer device was acquired to be used in future in vivo experiments too.

Figure II - 4: Syringe warmer

After warming, the cement was injected. The qualitative evaluation of the injectability consisted on observing the amount of cement remaining into the syringe: the more material was left, the less it was injectable.

Also, the quantitative evaluation was measure through the components quantity of the mix along the injection. So, the bone cement was cut into pieces weighing 0.06 grams. Next, every piece was analyzed.

First, hydroxyapatite and zirconia were separated from polyester polyol with ethanol solvent, and the sample was centrifuged. The bioceramic was at the bottom of the falcon tube, and the polymer was in the upper part of the falcon tube suspended in the solvent. Finally, the bioceramic was deposited in a Freeze Dryer for 24 hours and weighed.

2.2.2 Ex vivo

This experiment was produced at Veterinary Medicine Department, School of Science and Technology, University of Évora.

Ex-vivo studies were carried out in 3 ovine lumbar vertebrae (L4, L5 and L6), from 1 mature Merino sheep, previously frozen at -20ºC for 48h and then stripped off soft tissue and disarticulated. Two defects were created bilaterally in the vertebral bodies with a dorsolateral cortex entrance. The chosen access point was between the pedicles and transverse processes of each vertebra, considering that the instrumentation of the pedicle always carries the risk of fracture to the pedicle and the risk of vertebral foramina disruption; these risks were doubled with a bipedicular approach. Moreover, with this approach only a limited access angle was possible and defects would be smaller and not connected (Lucena, Oliveira et al. 2014).

An osteo introducer system (Kyphon, Medtronic Spine LLC, Portugal, Ref. T05E) was used. A blunt osteo introducer stylet (ø 3,5mm) inside a cannula (ø 4 mm) was placed between the pedicles and transverse processes of each vertebra to access manually the cortical bone of the ovine vertebrae and to perform the entrance hole. Then the blunt osteo introducer stylet was removed and a precision manual drill (ø 3,35 mm) was advanced inside the cannula with an orientation of 30º-50º regarding a transverse plane and 0º-30º regarding the frontal plane, towards the center of the cranial hemivertebrae. Bone debris were removed from the defects (Lucena, Oliveira et al. 2014).

After the preparation of the vertebrae, the syringe with the cement bone was heated to 50ºC in the syringe warmer during 25 minutes. A vertebroplasty needle was applied to the unplugged syringe and then the cement was injected with a uniform velocity into the defect. Finally, the vertebra was analyzed by X-ray.

2.2.3 Rheological properties with zirconia as a radiopaque agent

The rheological experiments of all polymers were realized with a rheometer AR 550 (TA instruments). A steel plate with 20 mm was used as a measuring geometry for all the tests. And 800 μm GAP was worked with, considering that the normal force exercised on the sample does not exceed 0.1 N.

Flow Experiments

Shear stress vs shear rate at 46ºC

A flow curve was realized for polyester polyol with glutaryl chloride and HAp + 30% of Zirconia. Then, the curve was studied applying a shear stress ramp from 0 to 20.000 Pa at 46°C.

Oscillatory experiments

Variation of storage modulus (G ') and loss modulus (G' ') respect of the temperature

This experiment was realized in oscillatory mode. The conditions of this test were; temperature ramp from 50 ° C to 25 ° C with a decreased of 3°C/min. Strain was 1% and frequency was 1 Hz, and they were fixed.

The reason why these two parameters were fixed was because the material is thermoplastic. When the strain is fixed at 1% (strain in which the material structure will not break but otherwise it flows), the amplitude in which the oscillation test is performed is inherently fixed. Moreover, 1 Hz is the frequency at which oscillatory experiments are usually performed when working with thermoplastic materials. If the frequency is higher, the material structure breaks because there is too much flow. So, it is impossible to measure the material response as a G 'and G'' (Arán Aís 2000).

2.2.4 Mechanical analysis

One of the fundamental requirements for our formulation is to be mechanically similar to the living material on which it will come into close contact. That means our cement must show a compressive strength comparable to cancellous vertebral bone, about 15 MPa.

The compressive properties of the formulation were tested because ASTM F2224-09 (Standard Specification for High Purity Calcium Sulphate Hemihydrate or Dihydrate for Surgical Implants) was found as a reference. But for flexural properties no standards were found and for these reason they were not tested.

Three different cement compositions were tested. The new formulation was compared to the commercial control Cerament®, from Spine Support and to Spineghost, a competitor.

All the materials were tested in dry conditions after its injection and were tested with a load cell of 5 kN and with a speed of lowering of the load maintained constant at 1 mm/min.

2.2.5 Polymer Synthesis

This synthesis was based on chapter 1 of the thesis. It is a larger scale adaptation of the procedure described in the previous section. The reason why it is aimed to synthesize more quantity of product is for the subsequent ex vivo and in vivo experiments to be carried out.

The synthesis was carried out in two stages. In the first stage, 18.4g of 1.8-octanediol and 20 ml of distilled glutaryl chloride were added in a molar ratio of 1: 1.2 respectively in 1L reactor. They reacted in the synthesis conditions shown below:

First stage:
-Temperature ramp: from 10 ºC to 70ºC
- Time: 20 min
- Inert atmosphere (Argon)
- Mechanical stirring

Second stage: synthesis of the hard segment
-Temperature: 70ºC
- Time: 60 min
- Inert atmosphere (Argon)
- Mechanical stirring

After these two stages, 25.9g of polyethylene glycol were added to synthesize the soft segment.

Third stage: synthesis of the soft segment
-Temperature: 70ºC
- Time: 60 min
- Inert atmosphere (Argon)
- Mechanical stirring

A soda trap was connected to the reaction to capture all the free hydrochloric acid. Once the second reaction was finished and the temperature was at 25ºC, a methanol excess was added to end capping the free acid chloride chain ends.

Finally, the product was washed with ether and water. 21 grams of product were obtained before the cleaning process and 7.1g after washing it.

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2.3 Results and discussion

2.3.1 Homogeneous Injection

First of all, the degree of injection of the new formulation is analyzed, because it is very important that the entire product is injected, without leaving paste inside the syringe. So, the phenomenon of filter pressing has to be avoided.

Figure II - 5: Syringe

The Injectability (denoted as "Inj%") is the percentage value calculated by dividing the extruded volume (or mass) by original volume (or mass):

$$Inj\% = 100\% \frac{Massextrudedfromthesyringe\ (g)}{Totalmassbeforeinjection\ (g)}$$
$$= 100\% \frac{Volumeextrudedfromthesyringe\ (mm^3)}{Totalvolumebeforeinjection\ (mm^3)}$$

Equation II - 1: Injectability

In this case, the mass extruded from the sringe (g) is the same that total mass before the injection, and the volume extruded from the syringe (mm^2) is the same as the total volumne before injection (mm^2). So, the product is 100% injectable.

The injection temperature is fixed at 50ºC, and the mechanical properties of the materials are tested and evaluated at 37ºC, showing a good performance regarding the vertebroplasty application. A video is available describing the whole injection procedure developed.

C:\Users\Sara\Dropbox\Tesi Doctoral_Sara\Thixotropic Paste.mov

The video shows the setting time and the working time only take a few minutes. However, commercials cements like Cerament (Ehrenborg et al. n.d.) or under study materials like Spine Ghost (Dadkhah et al. 2017) have a setting time and a working time that take one hour approximately.

These characteristics are very important for two reasons. On the one hand, the setting time has to be short because if the patient spends a lot of time with the wound exposed, there will be a very high risk of infection.

On the other hand, the working time has to be short too including the elimination of pain for the patient after the implantation. In this sense a lot of work is done on the injectability of the material, because the formulation at 37ºC has a good rheological properties for the vertebrae, and It is able to pass from 50ºC to 37ºC in a few minutes. So, its mechanical recovery is very fast and the patient can recover his mobility as soon as possible.

Once the product is already injected, it is important to consider the uniformity of the extruded cement along the injection and avoid the heterogeneity of the paste, canceling all the separation forces. Although compositional changes are not too important, the rheological properties of the cement are influenced very significantly by this phenomenon (Habib, Baroud et al. 2008).

For these reason, the quantity of HAp is analyzed for each 0.06 grams of cement bone:

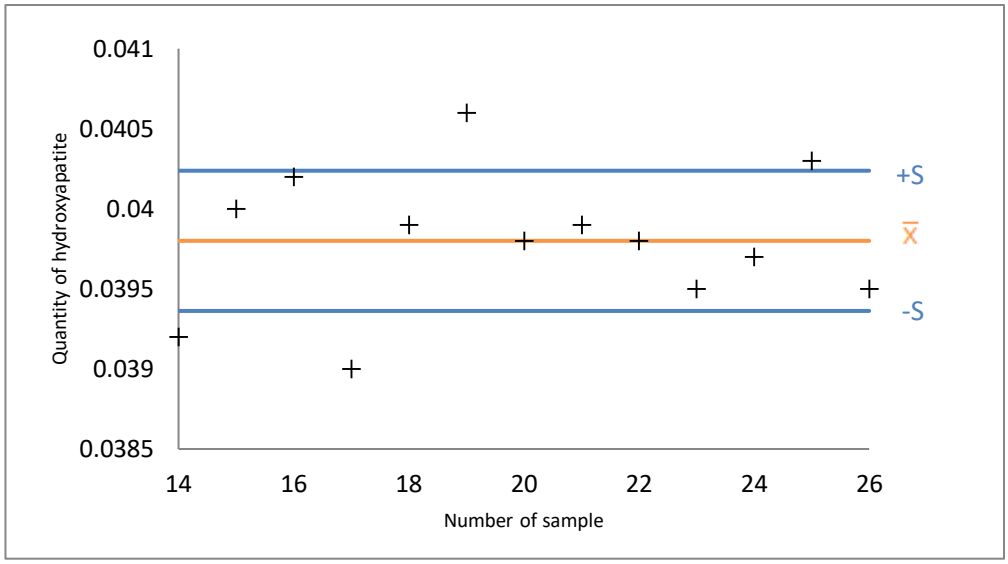

Figure II - 6: Quantity of hydroxyapatite for each 0.06 grams of bone cement

As shown in the graph, values are not far from the average value generally. So, the dispersion degree of data regarding the average is not very high. The amount of HAp is homogeneous throughout the injection. So, no accumulation of bioceramic inside the syringe is observed.

These data lead us to conclude that rheological properties of the paste do not vary before or after injection because the amount of bioceramic remains constant. So, the bone cement maintains its properties before and after the injection.

2.3.2 Ex vivo test and X-ray observation

Until now, the injection of the formulation has been optimized to apply the paste in large quantities without losing its rheological properties and without suffering the phenomenon of filter pressing. But in vertebroplasty technique, it is necessary to check if the product is compatible with the complete procedure. For this reason, an ex vivo assay is performed, to analyze the compatibility of the bone cement with the medical procedure.

Prior to live surgery experiments, the development of an animal vertebral bone defect model and feasibility evaluation of the model with bioactive injectable bone cement is needed. Sheep is becoming the animal of choice for testing vertebral implants because this specie is readily

available and shows great homogeneity when selected for age, breed, and sex. Due to similar volume and size of its vertebrae compared to humans it is possible to use identical implants and instruments as performed under clinical conditions. The biomechanical behavior of ovine lumbar spine was found to be qualitatively similar to human specimens (Lucena, Oliveira et al. 2014).

Ex-vivo tests have been done in partner University of Evora facilities to asses the injectability of the materials in sheep vertebra with specific designed defect. In fact, a protocol of the injectability of the formulation has been established.

Initially, the syringe with the cement bone is heated to 50 ºC in the syringe warmer during 25 minutes. After that, the cannula is inserted in the vertebrae defect. The product of the syringe is injected inside the cannula and it is pushed through the cannula into the defect.
Next, the pictures of the experiment performed with the thermoplastic paste are presented.

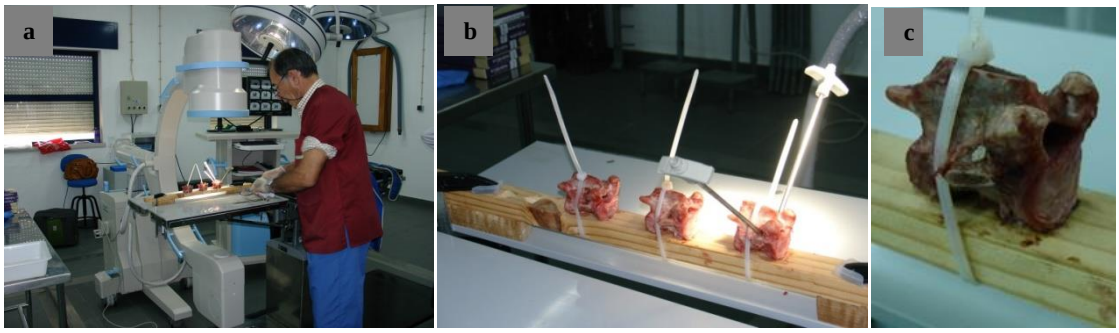

Figure II - 7: a) The surgeon inserting the product into the cannula b) The cannula inserted into the bone defect to inject the bone cement c) New formulation injected into the bone defect

Figure 8 c) is a detail of a L4 vertebra where it is possible to see the hole produced by the defect filled using paste. Also, the surgeon can insert the product during 3-4 minutes while he observes the filling by X-Ray and the formulation become hard only in 3-4 minutes too. Any extravasation is observed and it is able to fill the gap of the bone defect completely. So, at the moment, the new formulation is a good candidate because it is easily manipulated by surgeons.

After the promising results of the first step in the ex vivo experiment, the study finishes using microtomography to ensure that the distribution on the material within the vertebra defect is homogenous, without pores (reported by partner Evora).

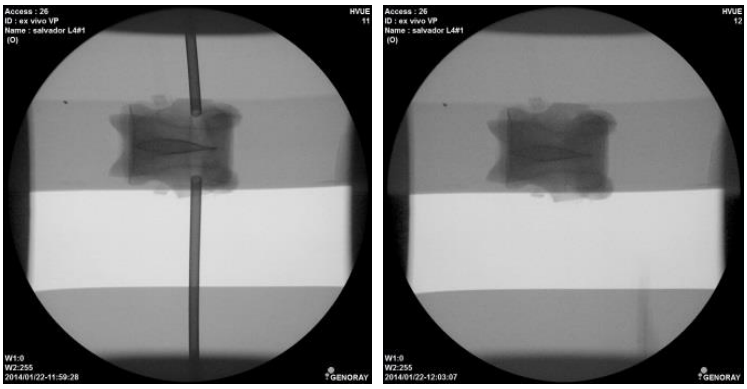

Figure II - 8: Analysis by X-ray.Left; the fracture without the paste. Right; the fracture filled with the paste

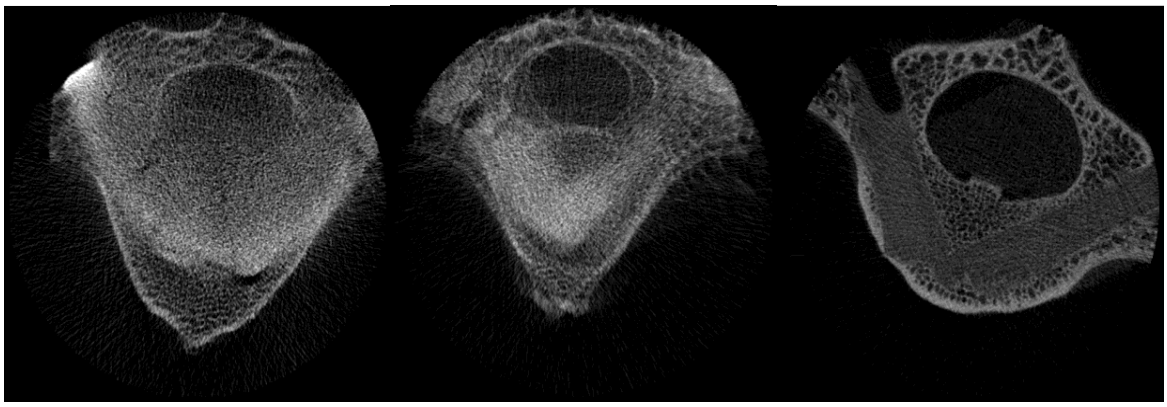

Figure II - 9: Micro CT imaging post injection. Cerament, Competitor and our product

Although does not present any extravasation and it is able to fill the gap of the bone defect completely, the same does not happen with the other two bone cements. Cerament is a commercial product that is used in vertebroplasties, and Spine Ghost is a material in development. Both are unable to fill the gap left by the fracture in the vertebra. The reason is that they heal by setting process inside the vertebra, and they contract leaving an empty space in the fracture (Rauschmann et al. 2010). In fact, our product does not heal inside the vertebra, but on the contrary, it polymerizes utside the vertebra, that is, before being introduced into it.

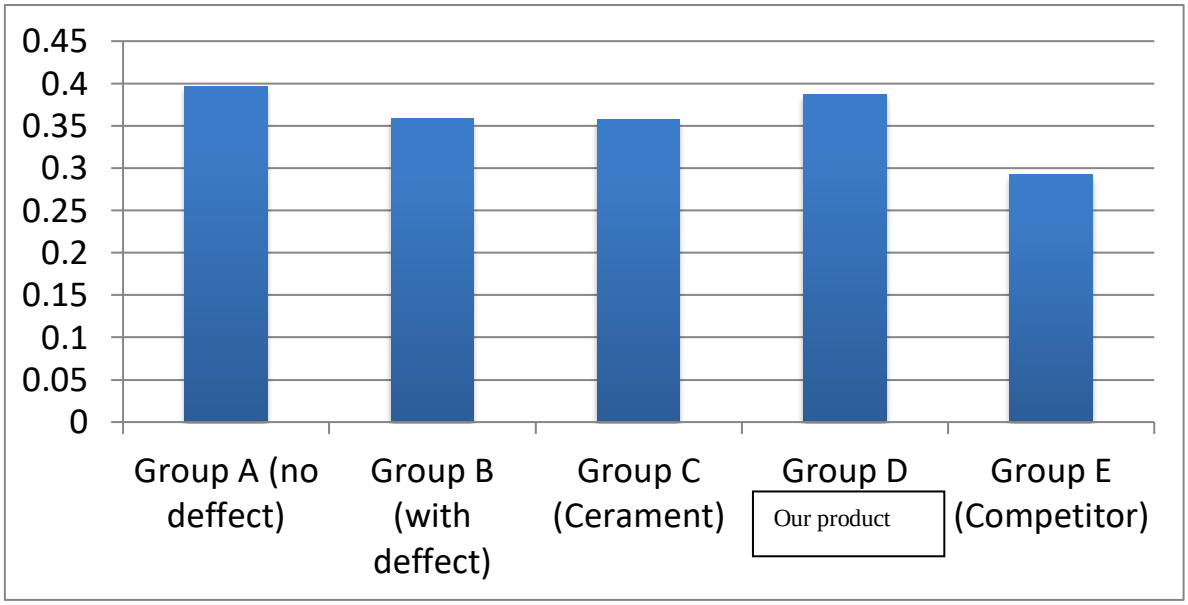

Figure II - 10: Density

As may be seen, the density of our bone cement is very similar to the bone density with no defect. This bone cement property is appropriate because when the bone cement density is very different from the fractured bone density, stress is generated in the fractured vertebra that contains the bone cement. This means that the fractured vertebra leaves its tension in the healthy adjacent vertebra, and the latter is fractured by the tension that supports (adjacent vertebral fracture).

So, it is very positive that our bone cement density is very similar to healthy bone density, because it is important to avoid internal tensions. However, it is difficult to differentiate it from bone by X-ray because our bone cement density is too similar to the bone density, as observed at the next images:

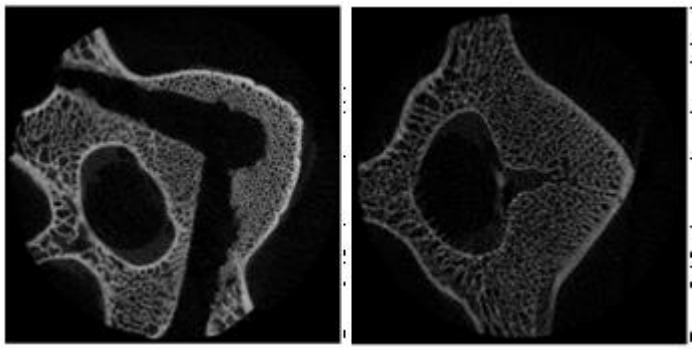

Figure II - 11: Micro CT imaging. Vertebra with defect, Vertebra filled with bone cement

So, our product does not have enough radiopacity. As a response, a new formulation is developed using ZrO_2 as radiopaticity agent. In vertebroplasty intervention, the bone cement is injected in the vertebra with a syringe as commented, and all the process is observed by X-ray. Therefore, it has to be radiopaque.

Zirconia is a good candidate as a radiopaque agent. It has been proved that with 20% of this radiopacifier, the radiopacity occurs in polymers like PMMA.

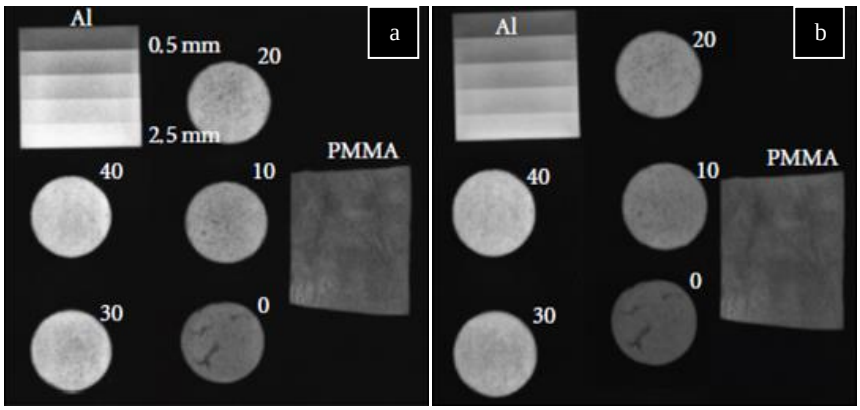

Figure II - 12: X-ray image comparing pCPC samples containing 0 to 40% ZrO2, with a commercial PMMA cement (Vertebroplastic) and an aluminium wedge with 0.5 to 2.5mm in thickness. The X-ray opacity was measured at 1mAs, with 40 kV (a) and 80 kV (b)

Also, the biocompatibility and osseointegration of ZrO_2 have been widely demonstrated in vitro and in vivo. Some authors have observed that it has no cytotoxic effects on fibroblasts, and additional studies have shown that Zirconia does not induce cellular genome mutations. In

fact, no adverse responses were observed after the insertion of ZrO_2 implants into bone or muscle at in vivo models (Åberg, Pankotai et al. 2011).

In conclusion, the new formulation with 30% of Zirconia is radiopaque and applicable with the surgical method of vertebroplasty. So, if the product proves to be biocompatible and it has good mechanical properties, it is ready to go to the clinic stage.

Finally, 30% of Zirconia is chosen as the right amount of the radiopaque agent without altering the mechanical properties and the rheology of the paste significantly.

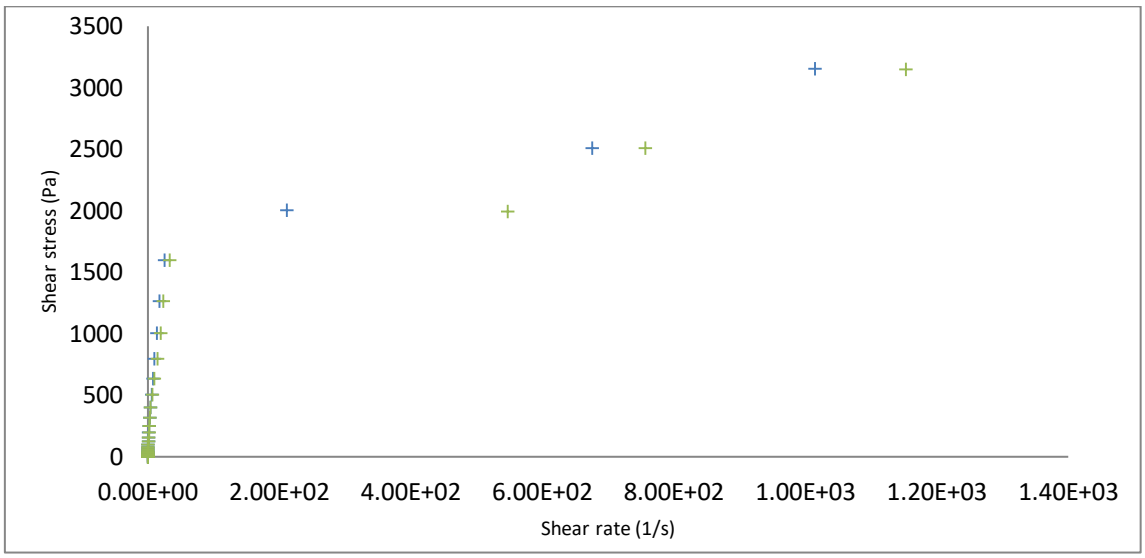

Figure II - 13: Shear rate vs shear stress of paste with 30% of Zirconia (blue) and paste without Zirconia (green)

The flow curves of product with 30% of Zirconia and product without Zirconia are very similar between them and they have a pseudoplastic behavior too. So, there is a decrease in viscosity while the shear rate increases in both products.

Also, the samples are characterized with a study of the variation of the elastic modulus (G') and viscous modulus (G'') front temperature. They have the same behavior in terms of viscoelastic properties too.

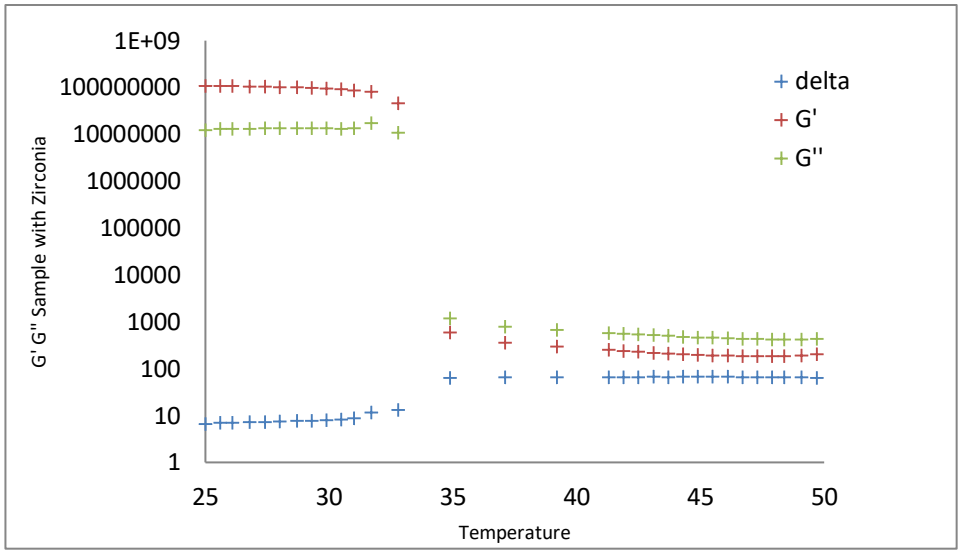

Figure II - 14: G' G'' of our product with 30% Zirconia respect to the temperature

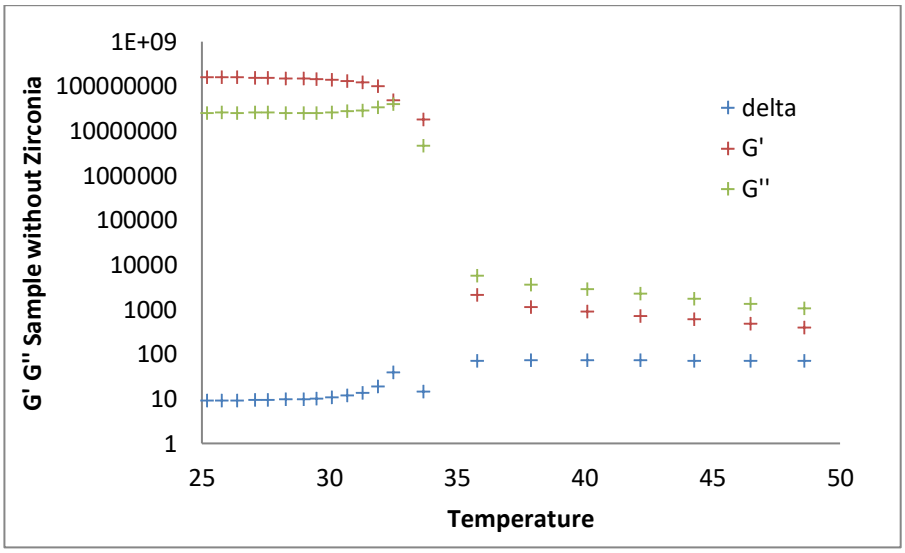

Figure II - 15: G' G'' of our product respect to the temperature

Zirconia does not change the rheological properties of the final product. Therefore, ZrO_2 is incorporated it into the formulation of our bone cement.

2.3.3 Mechanical analysis

As already mentioned in other sections, the injected prosthesis must be able to withstand the entire mechanical load that vertebra supports usually. For this reason, a compression test of the material is realized after the ex-vivo test.

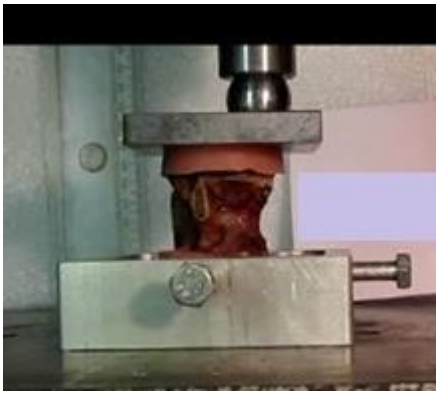

Figure II - 16: Compression test of the bone cement

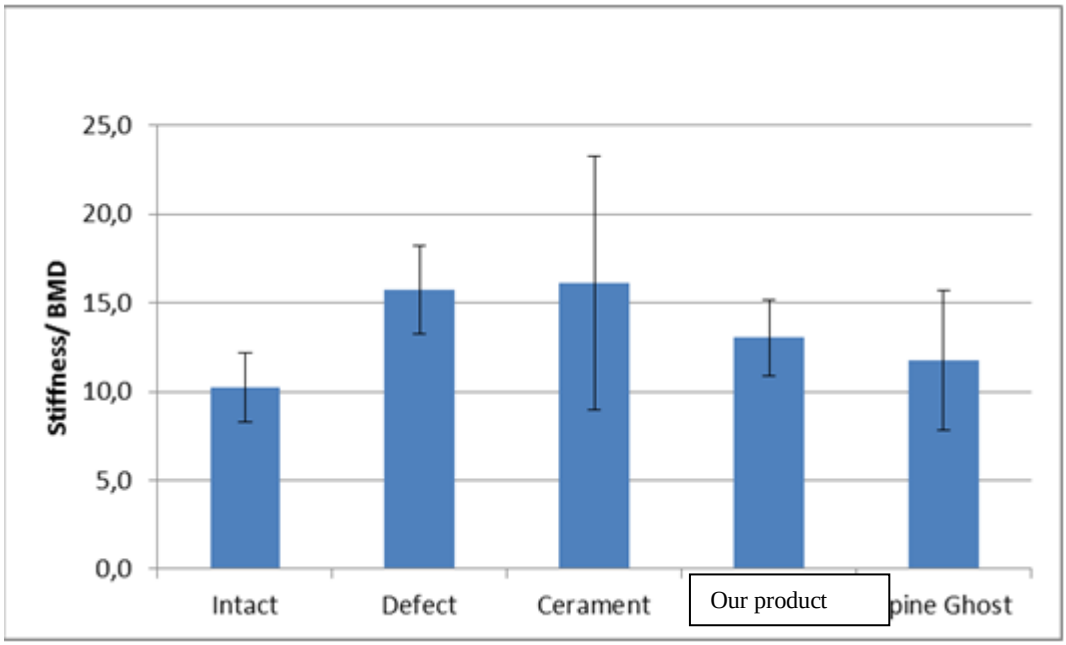

Figure II - 17: Normalized Stiffness

As may be seen in the graph, our product has a similar compression resistance to the intact bone and to the bone with defect. Also, its standard deviation is very low compared to the other bone cements. So, our bone cement has an optimum compression resistance to bear the compression tension that supports a fractured vertebra.

2.3.4 Post- mechanical testing

The mechanical integrity of the vertebrae after the compression assay is tested too. It shows no fractures after the assay.

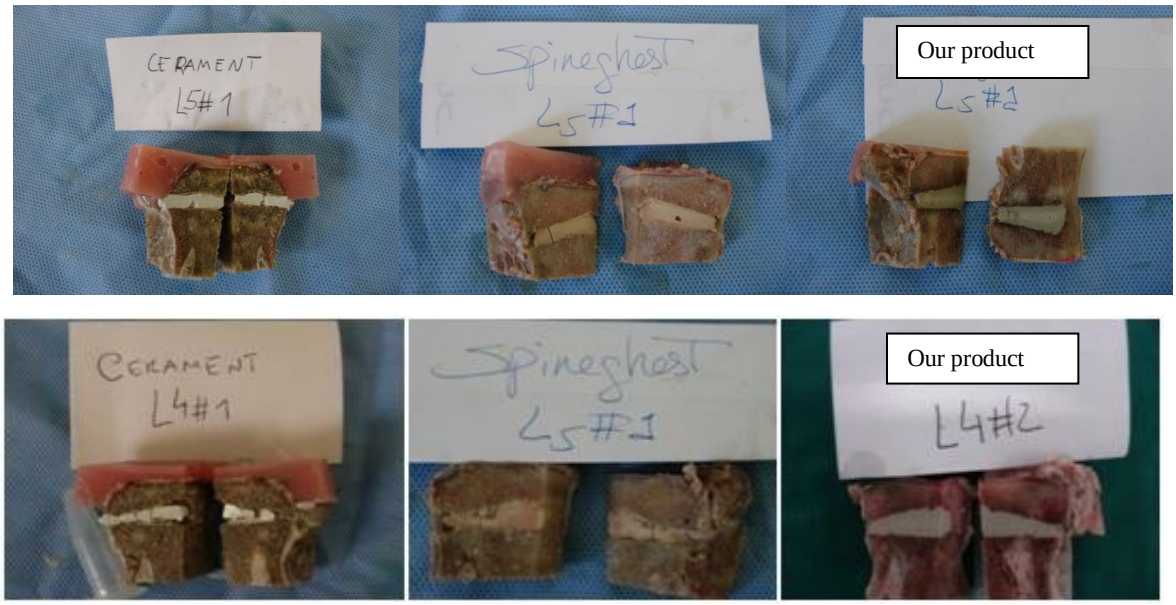

Figure II - 18: Post-mechanical analysis of Cerament,Spineghost and our bone cement. Fractures

Our bone cement does not present any fracture in contrast to other two cements. Spine-Ghost and Cerament present a significant lower compressive strength respect to our product, both in dry conditions. So, the new formulation is significantly more resistant to compressive stress than the Spine-Ghost and Cerament because it does not present fractures.

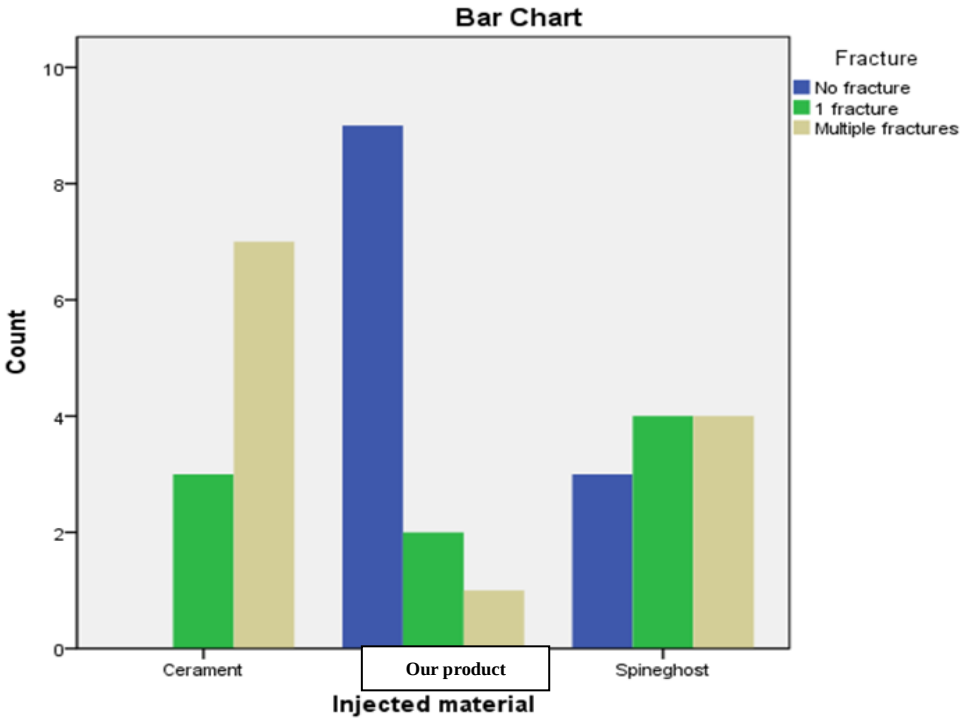

Figure II - 19: Post-mechanical analysis of Cerament, Spineghost and our product. Fractures

Several experiences have been made of each bone cement. Our product is the one that presents more samples with no fracture (9), only two samples with one fracture, and one sample with multiple fractures. The other bone cements present less encouraging results as seen in the graph.

Also, the defect filing after the compression assay is tested. As shown in the image, our bone cement is the one that best fills the defect.

Figure II - 20: Post-mechanical analysis of Cerament, Spineghost and our product. Defect filling

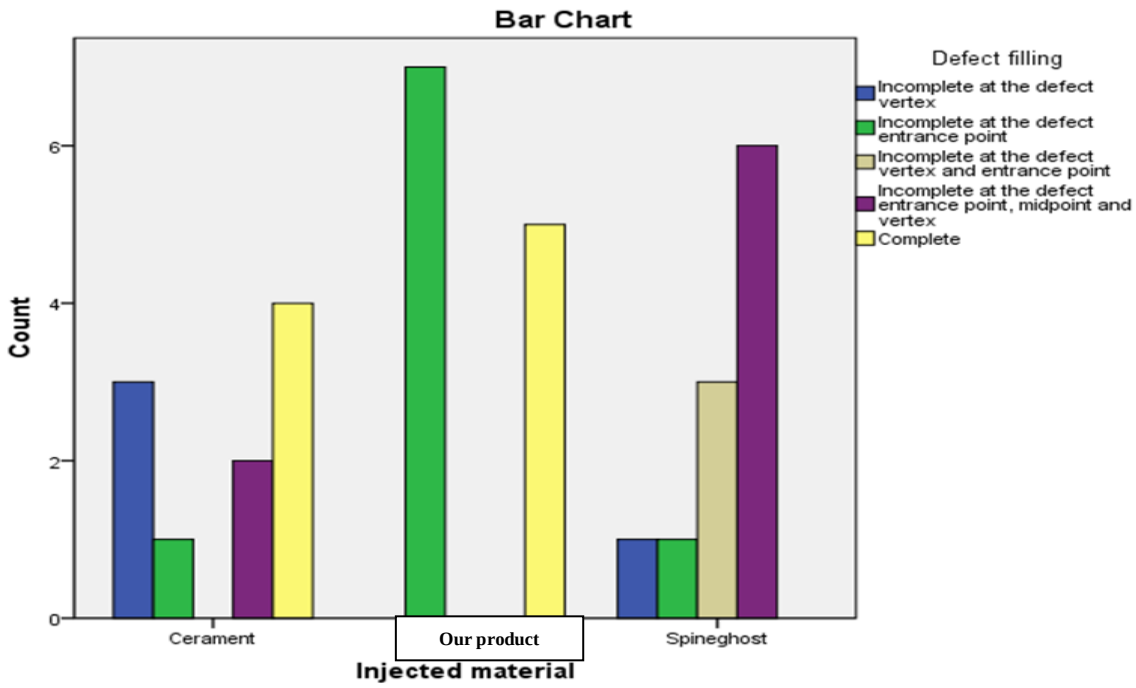

Figure II - 21: Post-mechanical analysis of Cerament, Spineghost and our product. Defect filling

Several experiences have been made of each bone cement. Our paste is the one that presents more samples with complete filing (6) andcorrect filing except at the defect entrance point (7). The other bone cements present worse results as seen in the graph.

The reason why our bone cement presents six experiences with correct filing except at the defect entrance point is the radiopacity. When these tests were performed, zirconium oxide had not yet been added. So, as the surgeon did not see the bone cement completely clear, he did not know exactly when the defect was full and he stopped to injecting it too soon. Then, he stopped injecting the cement before the defect was completely full. Probably, if this experiment had been performed after adding the zirconium oxide, all the defects would have been filled correctly.

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2.4 Concluding remarks

The development of biodegradable thermoplastic bone cement has been successful. Our product is able to be injected at 50ºC and remains mechanically stable when the temperature reaches 37ºC preserving its initial properties since injection is homogeneous.

Also, the fractured vertebral body can be easily treated by the surgeon because the setting time only takes a few minutes; and there is no need for patient immobilization after the surgical treatment or the use of corsets as working time only takes several minutes too.

The vertebroplasty procedure is entirely conducted under fluoroscopic control and our bone cement mixed with Zirconia is radiopaque and have the same rheological properties of the final product, needed to support the load of the vertebrae. In fact, our paste has an excellent compression resistance and after the compression test, it does not present fractures and it is able to fill the entire gap.

At this point, we can conclude that the new formulation may find potential application in vertebroplasty. So it is selected for its adequate rheological and mechanical properties before and after the intervention, and for its correct manipulation during surgical intervention in vertebroplasty too.

So, the next step is to evaluate its citotoxicity and its biological activity in vitro and in vivo. The reason why bone cement is wanted to be biologically active is because its main function is to regenerate the bone. Therefore, the product has to be osteoinductive and osteoconductive, that is, able to differentiate bone stem cells to osteoblasts and osteoblasts to osteocytes, that is, bone matrix. If it is not able to fulfill this function it should be discarded for this function.

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2.5 References

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Chapter III. Bone cement bioactivity study

“Gracias por soportarme”

-Eduardo Bardají Ais

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3.1 Introduction

The bone cement studied until now has exhibited the ability to fill the vertebral defects generated by a compression fracture. Thus, thanks to its good injectability without presenting heterogeneity in its composition after the injection and thanks to its high viscosity, our bone cement has shown to be a good support for the fractured vertebra, easily manipulated in the technique of vertebroplasty and have optimal mechanical compression properties. Also, pour paste presents a correct manipulation during surgical intervention in vertebroplasty as evidenced in the ex vivo experiment.

At this point, the objective is to evaluate if the product, besides being able to fill the gap created by the fracture, is also capable of regenerate the bone, that is, to activate it at a biological level. This study will be carried out by evaluating the biocompatibility of the product through cell viability assays and its bioactivity by cellular differentiation experiments. Finally, its behavior in vivo will be studied too.

Although bone is a tissue with a great capacity to regenerate bone defects that can be generated by a fracture, when a fracture is caused by several traumas like bone cancer or osteoporosis, then it is difficult to regenerate it or heal it spontaneously (Langer and Vacanti 1993).

Nowadays, there are several solutions to this problem. One example is an allograft but it is not the most successful. Although they present positive characteristics, they also carry a lot of problems as a long recovery process, the risk of transfer of pathogens from one patient to another, additional surgical time, lack of supply, among others (Amini, Laurencin et al. 2012).

The bone cement that has been worked on throughout this thesis does not present these problems because its compounds are synthetic and they are not coming from another human being. In addition, there is no problem of lack of supply since it can be synthesized as much as necessary.

Another option is the regenerative medicine. In this case, biomaterials are inserted in the fracture, and they are able to regulate and direct cell behavior and function (Scheller, Krebsbach et al. 2009). Biomaterials are very versatile; they can be biodegradable or

permanent, synthetic or naturally derived. In addition, some of them bear the mechanical load of the bone and they are osteoconductive, osteoinductive, porous and biocompatible (Kretlow, Young et al. 2009).

A lot of studies have been done, but only a few of them have shown efficacy in vivo. However, the materials synthesized with hydroxyapatite have quite approached what is sought because of its excellent biocompatibility and bioactivity (Dorozhkin 2011). For this reason, our bone cement has been combined with HAp, for its effectiveness.

However, it is important to know that the blood-biomaterial interactions just begin after implantation of the biomaterial, with the formation of the provisional matrix in the bone, with the development of granulation tissue and the development of the fibrous capsule. Therefore, they can lead to inflammation and foreign body reaction (Anderson, Rodriguez et al. 2008).

So, the implantation of biomaterials or tissue engineered can trigger a body's reaction to constructs. But, during this process a number of proteins are generated, which are in the same provisional matrix, which modulates the healing process and the foreign body reaction (Sheikh, Brooks et al. 2015).

There is a wide variety of cellular responses to the implantation of biomaterial; one of them is chronic inflammation. This type of inflammation is recognized by the presence of mononuclear cells such as lymphocytes and plasma cells. This inflammation is usually confined to the implant zone(Anderson, Rodriguez et al. 2008).

Another type of chronic inflammation is detected by the presence of monocytes, macrophages and/or foreign body giant cells (FBG cells), which are present at the tissue biomaterials interface. This type of inflammation is identified by the formation of new granulation tissue formed which is recognized by macrophages. Often, the granulation tissue is separated from biomaterial by cellular components of the foreign body reaction and ends up like several layers of monocytes, macrophages or FBGC. The FBGC are much more effective reacting against the implant than individual macrophages. Then, the material degrades much more efficiently (Sheikh, Brooks et al. 2015).

After surgery, the number of macrophages normally peaks in about a week; however, they may persist at the site of the injury for months. Their presence is critical for tissue repair and regeneration.

Also, the inflammatory response can be significantly enhanced as a result of foreign body reaction induced. Device interactions or other tissue lead to protein deposition in the biomaterial forming a provisional matrix which affects subsequent interactions. Therefore, chemistry and topography of the implant surface may be primarily responsible for the intensity of the reactions caused by the infiltration of immune cells (Ghrebi 2009).

In addition, matrix derived from cells can contain biological impurities or allogeneic signals resulting in increased inflammation in the implant site (Boehler, Graham et al. 2011).

However, the bone cement studied in this thesis is able of taking bone fracture shape because it is an injectable paste, and therefore it will not present the problems described with the topography of the implant. Also, the sterility of the bone cement will be checked, and if it is not sterile, it will be sterilized to avoid biological impurities.

Another question that needs to be considered is the process that occurs in bone remodeling. This process is carried out by two types of cells: osteoblasts and osteoclasts. Osteoblasts are responsible for synthesizing the calcified bone matrix by depositing, so that, they are in charge of maintenance, growing and reparation of the bone. In addition, they form a cellular layer at sites of bone formation. Osteoclasts are dealing with the degradation and absorption of the bone. As the osteoblast, the osteoclast is involved in the natural bone remodeling (Cao 2011).

Therefore, the injected product in the vertebra should enhance the activity of osteoblasts especially. In this bone repair process, where there is a lot of damaged tissue that needs to be regenerated, there is no point in enhancing bone degradation and absorption (osteoclasts).

It is also important to know that osteoblasts and osteoclasts are morphologically similar to fibroblasts when cultured in vitro (Ducy, Schinke et al. 2000).So, it is worthwhile to carry out the first cell viability tests with fibroblasts.

Finally, the goal is to enhance the activity of osteoblasts to differentiate them until they become osteocytes, which are formed from differentiated osteoblasts, which are located in

the bone surface. When osteoblasts are surrounded by extracellular matrix materials, they become osteocytes. These cells are unable to divide and they have the ability to segregate or resorb bone matrix that surrounds them. In fact, these cells are like trapped in their own secretion substance. In spite of the distance between osteocytes because of the extracellular matrix, they remain in contact through small channels, which are along the bone. Osteocytes communication is important to control the amount of formed and deteriorate bone (Bonewald 2011).

So, osteoblastic differentiation from human bone marrow mesenchymal stem cells to osteoblasts is an important step of bone formation and regeneration. The maturation of hBMSCs into osteoblasts is essential in bone growth, fracture healing and the osseointegration of bone-anchored implants, as well as the general bone turnover process, governed by the interactions between osteocytes, osteoblasts and osteoclasts.

The differentiation process towards osteoblasts is regulated by a number of key factors and signaling pathways. Some of the factors involved are commonly used as markers (Granéli, Thorfve et al. 2014). These markers are very important to evaluate if the bone cement is regenerating or not the vertebrae fracture.

One of the most important markers is the effector protein, alkaline phosphatase (ALP), which is responsible for the mineralization of the extracellular matrix (ECM). In fact, the use of ALP enzyme activity assays are the most common to follow the mineralization process(Granéli, Thorfve et al. 2014) because if a bone cement regenerates the fractured bone, then it is able to differentiate the hBMSCs until they mineralize.

Respect of the ECM, its main constituent of the organic part is collagen type I. However, there are two non-collagenous bone ECM proteins too; osteopontin (OPN) and osteocalcin (OCN).They are commonly used as early and late markers of osteogenic differentiation, respectively.

On the one hand, OPN is implicated in bone formation and remodeling. On the other hand, OCN is expressed at later stages of osteoblast differentiation, indicating a mature osteoblast phenotype (Mckee and Nanci 1996). So, osteopontin and osteocalcin are two adequate differentiation markers to evaluate if the cement promotes or not the bone differentiation.

In the process of bone remodeling, the transcriptional factors play an essential role too. Specifically, BMP-2 has unique biologic activities in vitro that lead to cells playing a critical role in bone formation (Hiraki, Inoue et al. 1991).

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) family that play essential roles in osteogenesis. BMPs play an essential role in the commitment and differentiation of osteoblastic lineage cells. BMP-2, a prototype of BMPs, promotes osteoblast maturation by increasing the expression of the transcription factor Runx2, and the expression of osteoblast marker genes (Haÿ, Lemonnier et al. 2001).

Therefore, BMP-2 is an adequate positive control respect to the studied bone cement to observe the increase in maturation of the stem cells hBMSCs. For this reason, it will be used as a positive control in cell differentiation assays that will be carried out with our paste. To get a more general idea of the complete mechanism of coupling between bone resorption and formation, and the transcriptional factors involved a scheme is displayed below:

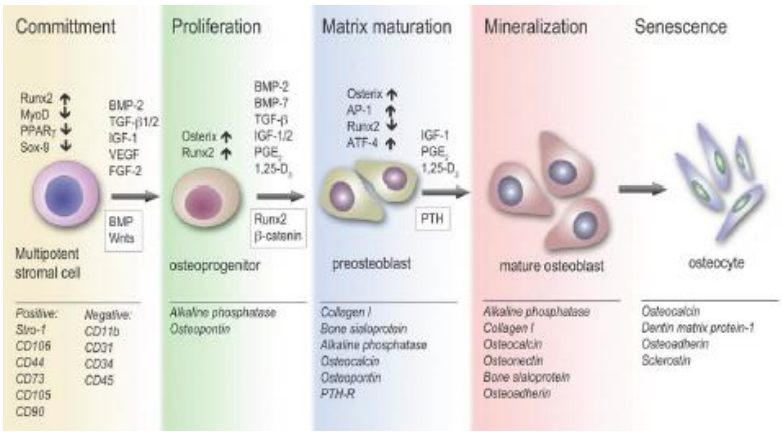

Figure III - 1: Transcriptional factors involved in osteoblasts differentiation and corresponding cell markers expressed at different stages. (Up arrow is activation, down arrow is inhibition) (Tour 2012)

Growth factors (GFs) are expressed during different phases of tissue healing and they are a key element in promoting tissue regeneration. In fact, GFs carried on orthopedic devices have

been reported to enhance osteoblastic activity and implant integration. Therefore, it is important to consider adding them in the bone cement to promote the bone healing.

However, obtaining specific growth factors is very expensive. An easy and inexpensive way to obtain growth factors in physiologic proportions that might stimulate the regenerative process is Platelet-rich plasma (PRP) (Roffi, Filardo et al. 2013).

Platelet-rich plasma (PRP) is a new approach to tissue regeneration and it is becoming a valuable complement to promote healing in many procedures in dental and oral surgery, especially in aging patients. PRP derives from the centrifugation of the patient's own blood and it contains growth factors that influence wound healing, thereby playing an important role in tissue repairing mechanisms. The use of PRP in surgical practice have beneficial outcomes, reducing bleeding and enhancing soft tissue healing and bone regeneration. Studies conducted on humans have yielded promising results regarding the application of PRP to many dental and oral surgical procedures (i.e. tooth extractions, periodontal surgery, and implant surgery)(Albanese, Licata et al. 2013).

The use of PRP with bone graft significantly improves the quality of bone healing. However, the use of PRP without bone substitute does not provide adequate repair tissue because PRP needs a mechanical support to be distributed. It provides less benefit when it is used independently(Kanthan, Kavitha et al. 2011).

Therefore, PRP will be added in our product to own the many advantages that this growth factors combo has in the bone regeneration. In fact, it is already used in other surgical applications in bone similar tissues but what fails in the other cases is the mechanical support.

To sum up, in this chapter, our product will be combined with PRP to prove its regenerative capacity. This study will be carried out through the evaluation of the biologically induced osteogenic differentiation of human bone marrow mesenchymal stem cells to bone and the implantation studies in critical-size calvarial bone defect healing. In this way, it will be verify that bone cement is really able to regenerate the bone, activating the bone biologically while it fills the gap generated in the fractured vertebra and supporting the mechanical load of sick vertebra.

3.2 Materials and Methods

3.2.1 PRP preparation

A protocol for extracting Rich Platelet Fibrin from our own blood samples was established. The procedure tested allowed extracting and isolated the PRP, from our own extracted blood samples after two sequential centrifugation processes. The requisites of blood extraction and the PRP conservation were developed. In figure 2 a scheme of the procedure was presented.

To collect the blood our laboratory contacted to a hospital center which gave us the bag where the blood donations were kept. Later, the donor went to another center to extract 450ml of blood because the bag contained 50mL of anticoagulant. The donor's name is Joan Gilabert and he is a 27 years old man.

First of all, the fresh blood was mixed with ACD-A Anticoagulant Citrate Dextrose Solution (ACD-A Anticoagulant Citrate Dextrose Solution, Solution A, Citralabs, FL9350-ACD-A) in volum ratio of 10:1 respectively.

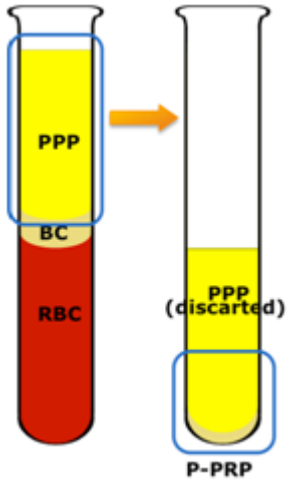

Figure III - 2: Scheme of the protocol for extracting Platelet Rich Plasma from our own blood samples

The mixture was added in 50ml falcon and was centrifuged 5 minutes at 1200G. After centrifugation, three phases were observed; red blood cells (RBCs), buffy coat (BC) layer and platelet-poor plasma or acellular plasma (PPP).

BC is typically of whitish color and contains the major proportion of the platelets and leucocytes. For production of pure PRP, PPP and superficial BC were transferred to another tube. This mixture was centrifuged at 1200G during 10 minutes. The top of the PPP layer was discarded.

The final PRP consisted of an undetermined fraction of BC (containing a large number of platelets) suspended in some fibrin-rich plasma. Most leucocytes were not collected. The product was frozen and freeze-drying. With this procedure, the platelets were broken and the growth factors were free (Dohan Ehrenfest, Rasmusson, and Albrektsson 2009).The PRP was stable store in the freezer at -20ºC.

This method gots a high concentration of platelets with high concentration of growth factors, and then, the addition of PRP in the paste improved healing. In addition, the RBC phase was discarded; so, the body's immune response was avoided.

The incorporation of the Platelet Rich Plasma in our product was a suspension with HAp and PRP mixed during 2 hours in an orbital mixer. After that, the powder was mixed with polymer in a ratio of 0.5:1 in a silicon bath at 50ºC during 2 minutes. Samples were stored in a fridge at -20ºC.
Three combinations of these product were made; 100% of polymer + 50% of HAp + 5% of PRP, 100% of polymer + 50% of HAp + 1,2% of PRP and 100% of polymer + 50% of HAp + 0,7% of PRP.

3.2.2 In vitro. Cytotoxicity in COS-7 and fibroblasts cell line

3.2.2.1 Cell line and culture conditions

To determine the cytotoxicity of the final product, a cell viability assay was performed with fibroblasts in collaboration with Karolinska Institutet. They used rat dermal fibroblasts and two products were tested: paste *+ 30%ZrO_2 +* 0,7% PRP (P2Z+0,7) and paste *+ 30%ZrO_2 +* 1,2% PRP (P2Z+1,2).

The selected cells were fibroblasts because they are morphologically similar to osteoblasts when cultured in vitro (Ducy, Zhang et al. 1997). They were cultured at 37°C in presence of 5% CO_2 and a relative humidity of 70-80% with DMEM, Dulbecco's Modified Eagle Medium with L-glucose (4500 mg / ml) (Sigma, D5671), supplemented with 10% FBS (Lonza, DE14-801F), 1% L-glutamine (PAA, M11-004) and 1% penicillin / streptomycin (PAA, P11-010).

3.2.2.2 MTS test

MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corporation, USA L0615) was used to determine the toxic effect of the final product on cell viability.

The MTS assay is a colorimetric test which measures the number of viable cells in cytotoxicity assays. This test is performed with a tetrazolium compound, 3 - (4, 5-dimethylthiazol-2-yl) -5 - (3-carboxymethoxyphenyl) - 2- (4-sulfophenyl) -2H-tetrazolium, and a coupling reagent electrons, like phenazinemethosulfate (PMS).

MTS is bioreduced to formazan by metabolically active cells. Formazan is soluble in tissue culture medium. The reaction is shown below:

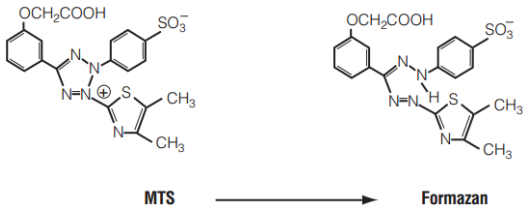

Figure III - 3: Reduction of MTS to formazan

The formazan can be quantified by colorimetric methods, because it is able to absorbe light at 490nm. And there is a correlation between the absorbance value obtained and the number of living cells in culture.

To perform the MTS assay with fibroblasts, 40.000 cells/well were seeded in 12 well plates (Labclinics, PLC30012). After 24 hours of culture, when the cell confluence was 80%, 2mg of the P2Z+0.7 and 2mg of P2Z+1.2 were incubated with 2mL of media (direct contact). These

two products were suspended in 500 μl of DMEM separately and they were added in an insert (Labclinics, PLC30012). Also, 1500 μl of medium were added in every well (indirect contact).

Three days later, before adding the MTS reagent, the cells were washed with PBS 1x. Then, 600μl of DMEM were added per well for 12 well plates with 20% (120μl) of MTS reagent (v/v) and the cells were incubated in 5% atmosphere of CO_2 at 37°C. Absorbance was measured every 30 minutes using a plate reader (Biotek ELx808 Instruments Ltd, USA) until the adequate absorbance values were obtained.

Cell viability was calculated using the following equation:

$$\%\, Cellviability = \frac{Absorbanceoftreatedcells}{Absorbanceofuntreatedcells} \cdot 100$$

Equation III - 1: Cell viability

3.2.3 Differentiation of the Human Bone Marrow Mesenchymal Stem Cells

3.2.3.1 Cell line and culture conditions

To determine the cellular differentiation that promotes the final product in the cells, Human Bone Marrow Mesenchymal Stem Cells were cultured. These primary cells were acquired through Tebu bio and cultured at 37°C in presence of 5% CO_2 and a relative humidity of 70-80%.

Also, HBMSC cells were expanded and seeded in α-MEM, Minimum Essential Medium Eagle Alpha Modification with sodium bicarbonate (M4526), supplemented with 10% FBS (Lonza, DE14-801F), 1% L-glutamine (PAA, M11-004) and 1% penicillin/streptomycin (PAA, P11-010).

To induce the differentiation in the Human Bone Mesenchymal Stem Cells (Bone marrow MSC) of passage 6, they were plated at 24-well culture plate. 40.000 cells/well were seeded with α-MEM, and when the cultured cells reached approximately 80% confluence and they were attached to the plate, the osteoinductive medium was introduced for 28 days. Two different

experiments were performed; one with inserts and another one without them, and the cells were analyzed at day 14, 21 and 28.

The osteoinductive media was α-MEM but supplemented with 10mM of glycerol-2-phosphate (Sigma, 50020), 50 μg/ml of ascorbic acid (Sigma, A4544), and 10^{-8}M of dexamethasone (Sigma, D4902).

3.2.3.2 Alkaline phosphatase activity

After the cells were seeded and the confluence was 80%, they were put in contact with BMP-2, PRP, P2Z+1, P2Z+5 and control. On days 7 and 14, they were washed with DPBS, lysed with 1mL of 0.2% Triton X-100 (Sigma Aldrich) per well and sonicated to destroy cell membranes.

100 μl of supernatant were added to 100 μl of *p*-nitrophenylphosphatesubstrate buffer (Sigma-Aldrich) in a 96-well plate and incubated at 37ºC for 30 min. Reaction was blocked with 50ml of NaOH 0.1 M and the absorbance was measured at 405nm in a Spectra Max M2^{e} microplate reader (Tour, Wendel et al. 2012).

To normalize ALP activity, total protein content was measured using Pierce^{TM} BCA Protein Assay Kit, according to the manufacturer's instructions (Thermo Scientific). BSA standards were freshly prepared in RIPA 1X buffer.

3.2.3.3 Western blot to measure osteopontin and osteocalcin

Cell lysis and protein solubilization

Western blot analysis was performed on HBMSCs protein extracts from monoculture on four different conditions. In addition, monocultures of HMSC cells were also analyzed as positive control. Cells were washed twice with cold PBS and then lysed in RIPA 1X buffer (Merck Millipore) containing protease inhibitor cocktail (Calbiochem) for 20 minutes on ice (Helms, Waagepetersen et al. 2010). Next, cell suspension was sonicated for 5 min to complete cell disruption, and centrifuged (14000 rpm, 5 min).

*Total protein concentration in each sample was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Absorbance was measured at 562 nm in a Spectra Max M2^{e} microplate reader. BSA standards were freshly prepared in RIPA 1X buffer.

Gel electrophoresis

Proteins were separated on 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Chen, D O'Neill et al. 2002) at 140V during 1-2 hours. Each lane was charged with 10 µg of total protein extracts. In order to determine the molecular weight of the proteins of interest, a protein marker (Precision PlusProtein™ Dual Color Standards, Bio-Rad) was used.

Electrophoresis buffer was prepared with Tris-base (1.5 % w/v), glycine (7.2 % w/v), SDS (0.5 % w/v) and Milli-Q water. Samples were heated at 95°C before being charged during 10 minutes.

Transfer

Proteins were transferred onto PVDF membranes (Immobilon-P, Merck Millipore) at 300 mA during 2 hours at 4°C. PVDF membranes were previously activated with methanol, and then washed with Milli-Q water and washing buffer (PBST, phosphate buffered saline solution with 0.1% Tween 20). Transfer buffer was prepared with Tris-base (0.3 % w/v), glycine (1.4 % w/v), methanol (20% v/v) and Milli-Q water (Ye, Skates et al. 2006).

Blocking and detection

Blocking of non-specific binding in the membrane was achieved by placing the membrane in a blocking solution, 5% (w/v) non-fat powered milk in PBST (phosphate buffered saline solution with 0.1% Tween 20) overnight at 4ºC in an orbital shaker.

Specific immunoreactivity for osteopontin was detected with anti-osteopontin antibody (AB10910, Merk Millipore) diluted 1:1000 and visualized by chemiluminescence. Another membrane was also immunoblotted using anti-osteocalcin antibody (AB10911, Merck Millipore) diluted 1:1000. Both antibodies were diluted with blocking buffer.

The membrane 1 was incubated with anti-osteopontin antibody (AB10910, MerkMillipore) and the membrane 2 was incubated with anti-osteocalcin antibody (AB10911, Merck Millipore).

Both membranes were incubated for 2 hours in an orbital shaker at room temperature. After that, primary antibodies were removed by washing the membrane with blocking buffer with 0.1% Triton X-100 three times for 10 minutes each wash.
Then, blots were incubated with the secondary antibody, goat anti-rabbit IgG antibody, HRP-conjugate (12.348, MerckMilipore). The secondary antibody was diluted 1:1000 in blocking buffer and incubated for 2 hours in an orbital shaker.

As the secondary antibody was conjugated to a fluorophore, all the steps must be performed in the dark, covering the membrane with aluminum foil. Afterwards, the secondary antibody was removed by washing the membrane with washing buffer (PBST) three times for 10 minutes each wash. HRP conjugated secondary antibody was detected by chemiluminescence method.

Briefly, the membrane was incubated with the Luminata^{TM} Forte Western HRP Substrate (WBLUF0100, Merck Millipore) for 3-4 minutes. Then the membrane was revealed in the luminescent image analyzer, ImageQuant LAS 4000 mini (GE, Healthcare). The total exposure time of osteocalcin and osteopontin was 300 seconds. The molecular weight of the bands was determined using *ImageQuant TL software*, and the predicted molecular weights were 20kDa and 60Kda for osteocalcin and osteopontin, respectively.

3.2.3.4 Staining with alizarin red

HBMSC were seeded into 12-well plates (Labclinics, PLC30012) and cultured in standard osteogenic media (α-MEM, Minimum Essential Medium Eagle Alpha Modification with sodium bicarbonate (M4526), supplemented with 10% FBS (Lonza, DE14-801F), 1% L-glutamine (PAA, M11-004) 1% penicillin / streptomycin (PAA, P11-010),10mM of glycerol-2-phosphate (Sigma, 50020), 50 μg/ml of ascorbic acid (Sigma, A4544) and 10^{-8}M of dexamethasone (Sigma, D4902)) for 28 days. The cell monolayer was washed with DPBS, stained for 5 min with a 2% (w/v) solution of Alizarin Red S (Merk, Germany) adjusted to pH 4.2 with ammonium hydroxide and washed with water and ethanol (Tour, Wendel et al. 2012).

3.2.4 In vivo experimental design

The in vivo experiments were carried out at the Department of Dental Medicine, Karolinska Institutet, Stockholm, Sweden. The experiment was approved by the Research Ethics Committee of Karolinska University Huddinge Hospital in accordance with the policy on human care and use of laboratory animals.

Six adult Sprague–Dawley male rats (~350 g) were used. The rats were kept under uniform conditions for a period of at least 1 week before commencement of the experiment. Free access to water and standard pelleted food was provided throughout the experiment.

The critical-sized calvarial defect model was used (Tour et al. 2014). An 8mm full-thickness circular defect was created on the left parietal region using a trephine drill under sterile saline irrigation. The defect area was evenly covered using a periosteum elevator and forceps with 100 µl of our bone cement containing PRP and preheated to 50ºC right before application. The incisions were closed with single sutures in two layers. The rats were followed for 2 (n = 3) and 12 weeks (n = 3) after surgery.

3.2.4.1 Tissue preparation and histological analysis

The rats were euthanized by CO_2 inhalation. The calvarial bones were dissected and fixed in 4% neutral-buffered formaldehyde overnight at +4ºC, then decalcified in 12.5% EDTA and later embedded in paraffin; 5 mm serial tissue sections were prepared parallel to the sagittal line, and stained with haematoxylin and eosin for the assessment of general morphology and new bone formation or Wright-Giemsa for the assessment of local inflammation (Tour, Wendel et al. 2014).

3.2.5 Paste biodegradability test

To perform the biodegradability test, one plate of 6 wells (Labclinics PLC30006) was used and 6 inserts (Millipore, 6 - Well Millicell, 1mm) were get to put the polymeric paste. 50 mg of polymer were added in each insert, in 3mL of DMEM Dulbecco's Modified Eagle Medium, with L-glucose (4500 mg / ml) (Sigma, D5671), supplemented with 10% of FBS (Lonza, DE14-801F),

1% L-Glutamine (PAA, M11-004) and 1% of Penicillin / Streptomycin (PAA, P11-010). 5mL of DMEM were added into the wells.

The paste was added like a lentil in three inserts, and like scattered dust in the other three inserts to try to mimic the conditions in which the paste will be in the organism.
The two plates were left for 17 days in an oven at 37 degrees. On day 17, the DMEM was extracted from the wells, frozen and lyophilized. Finally, it was analyzed by GPC using tetrahydrofuran as eluent and with a concentration of 1mg / mL of sample.

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3.3 Results and discussion

3.3.1 Bone cement biocompatibility by fibroblasts cell viability test

Bone healing is an intricate cascade of processes involving cellular migration, adhesion, differentiation, proliferation, and synthesis of the ECM(Tour, Wendel et al. 2010). In this first test, the proliferation that the new formulation and PRP make in the cells is explored.

As commented, in tissue engineering for bone, appropriate artificial bioresorbable and biocompatible scaffold materials may be combined growth factors for the stimulation of new bone (Buma, Schreurs, and Verdonschot 2004).For this reason, the challenge we face is combined the bone cement with PRP and to study if it improves its bioactivity.

The first test before to evaluate if the biomaterial has an impact on the osteoblastic differentiation of Human Bone Marrow Mesenchymal Stem Cells in vitro and in vivo is to check its biocompatibility but with primary cells. This first cellular viability screening with primary cells is realized in collaboration with Karolinksa Institutet.

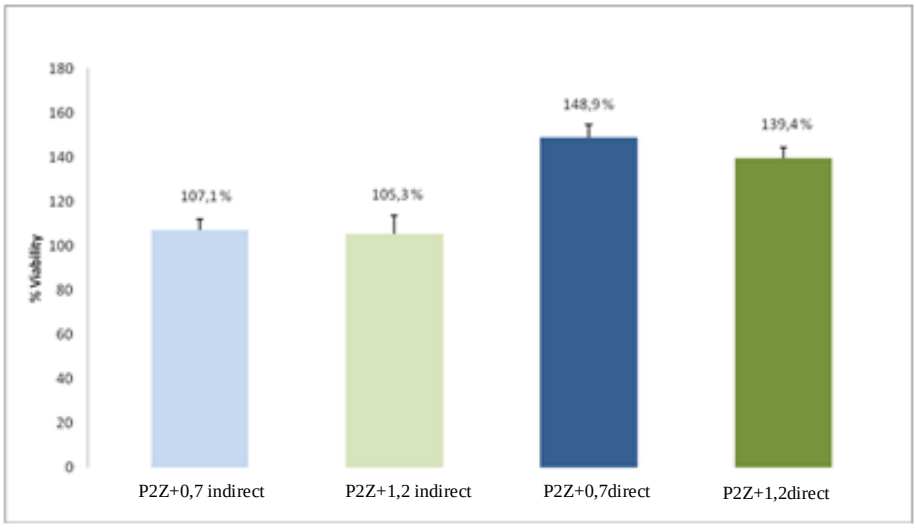


Figure III - 4: Fibroblasts viability in contact with 2mg of P2Z+0,7% and P2Z+1,2% after 72h of incubation

The new formulation is non-toxic in direct contact with fibroblasts and in indirect contact with them. The addition of a different percentage of PRP does not cause a significant difference in cell viability. As noted, none of the products is toxic.

Also, all cell viability values are greater than 100. Therefore, there is an increased in cell proliferation in the presence of bone cement combined with PRP.

So, the next step is to realize a study of the degree of cell differentiation promoted by the new product to observe the biological activity that promotes in a cellular tissue to know if it will be able to regenerate the bone tissue.

3.3.2 Human Bone Marrow Mesenchymal Stem Cells differentiation

3.3.2.1 ALP activity determination and mineralization. Positive control with BMP-2 and PRP

The importance of the measurement of alkaline phosphatase (Orthophosphoric monoester phosphohydrolase) activity in serum has not decreased during the half century that has elapsed since its introduction into diagnostic enzymology(Berstine, Hooper et al. 1973).

Alkaline phosphatase determinations were first applied to the investigation of bone diseases on a firm theoretical basis established by Robinson and others (Robinson and Pierce 1964), through their demonstration of the association between an increased alkaline phosphatase activity in serum and increased osteoblastic activity in bone; an association which still forms the basis of interpretation of serum alkaline phosphatase activities in bone (Fishman).

First of all, the levels of alkaline phosphatase that BMP-2 and PRP generates in the cells are analyzed because the intention is to check if BMP-2 and PRP activate the cells biologically. So, they could be the positive controls in future experiments.

In fact, they are the controls in terms of cellular differentiation because new formulation contains PRP and PRP contains BMP-2; one of the growth factors that most affects the maturation of osteoblasts. So, Bone Morphogenic Protein 2 (BMP2) is chosen as PRP alone positive control and PRP alone is used as a positive control of the bone cement. Two end points are chosen: 7 and 14 days.

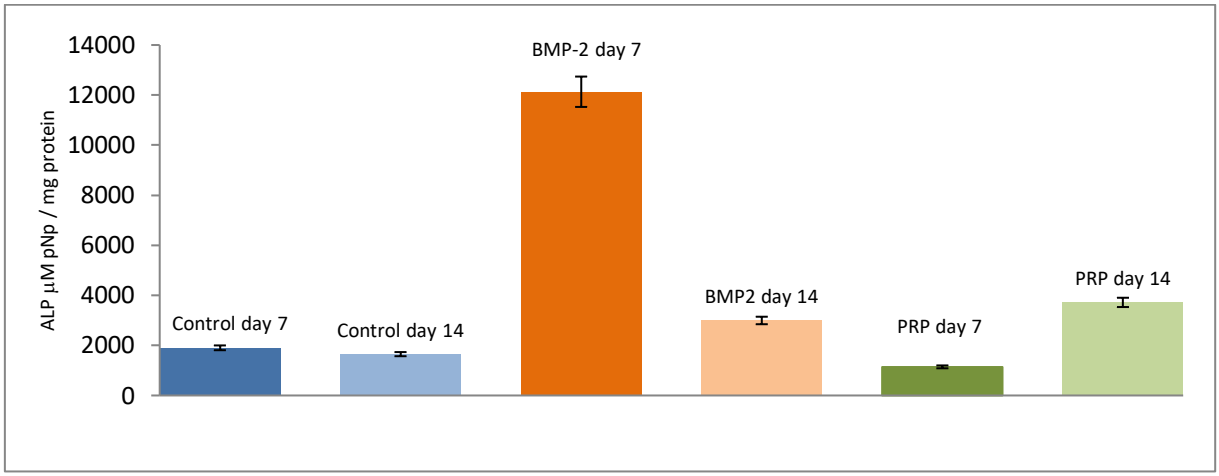


Figure III - 5: Determination of ALP. PRP: 0,0015g per well and BMP-2: 0,09ng per well

A peak of Alkaline phosphatase is observed at day 7 in BMP-2 and in control slightly. However, PRP has a peak at day 14; and this profile is the typical profile of differentiation in bone marrow mesenchymal stem cells (Tour, Wendel et al. 2012). Therefore, something has happened in the measure of ALP respect to control and BMP2.

Even though ALP levels are not as expected, the mineralization of these samples will be analyzed because BMP-2 and PRP are cell differentiation promoting agents. On the one hand, BMP-2 plays an essential role in the commitment and differentiation of cells of osteoblastic lineage. In fact, promotes osteoblast maturation (Haÿ, Lemonnier et al. 2001). On the other hand, PRP is a cocktail of growth factors in physiologic proportions and play an important role in tissue repairing mechanisms (Roffi, Filardo et al. 2013, Albanese, Licata et al. 2013). The use of PRP with bone graft significantly improves the quality of bone healing (Kanthan, Kavitha et al. 2011). So, it is worth working with them and expects the most definitive results of mineralization.

Osteoblasts are specialized fibroblasts that secrete and mineralize the bone matrix. They develop from mesenchymal stem cells, which are used to perform this experiment. The mineralized extracellular matrix is mainly composed of collagen type I and small quantities of osteocalcin (OC), matrix gla protein, osteopontin (OPN), bone sialoprotein (BSP), BMPs, TGF-β, and the inorganic mineral hydroxyapatite (Stein and Lian 1993). For this reason, OPN and OCN will be determined later too.

Osteoblast differentiation in vitro and in vivo can be described in three stages; Cell proliferation; which is analyzed with cell viability test of fibroblasts in our case, Matrixmaturation; which it is checked with the differentiation markers identification of the bone human mesenchymal stem cells (Osteopontin, Osteocalcin and Alkaline phosphatase activity) and Matrix mineralization (Kasperk, Wergedal et al. 1995).

Therefore, the in vitro human bone formation can be described as a mineralization of human osteoblast cultures originating from bone human mesenchymal stem cells, and it is associated with the expression of mature bone specific markers such as osteopontin, osteocalcin and alkaline phosphatase. In fact, in the absence of mineralization, OP and OC are not induced at high level(Kostenuik, Harris et al. 1999).

This human bone marrow cell culture model provided an ideal means of obtaining a homogeneous population of mature osteoblasts after progressive subcultures, and somehow we get closer to what happens in a real bone. These human differentiated cells are not modified by any stimulating treatment with the exception of BMP-2 and PRP.

Next, the images of the stained immobilized cells with Alizarin Red at day 21 and 28 are shown.

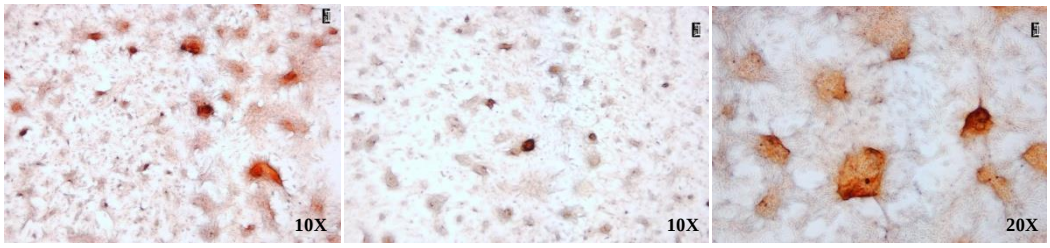
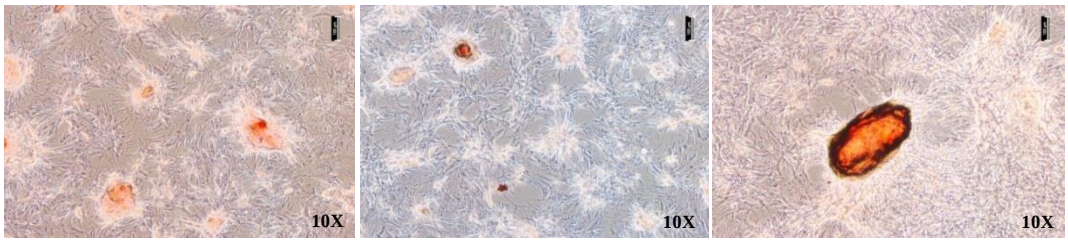


Figure III - 6: Alizarin Red at day 21. Control, BMP-2: 0,09ng per well, PRP: 1,5mg per well



Control BMP-2 PRP 5

Figure III - 7: Alizarin Red at day 28. Control, BMP-2: 0,09ng per well, PRP : 1,5mg per well

The mineralization is present in all the samples except in the control at day 21 because it is too early. The mineralization is usually given from day 28 unless the cells are in contact with agents that accelerate the cellular differentiation. Clearly, the greatest cell differentiation enhancer is PRP at day 21 and day 28.

3.3.2.2 ALP activity determination and mineralization of bone cement

In the previous experiment, it has been observed that BMP2 and PRP are clear cell differentiation inducing agents. However, the typical profile of differentiation in bone marrow mesenchymal stem cells has only been observed in the cells in contact with PRP.
In the next experiment, the concentration of BMP2 is increased to get a typical cell differentiation profile in HBMSCs. In addition, P2Z+5 is tested like a cellular differentiation agent too.

It has been a very important step to verify the cellular differentiation generated by PRP. However, in practice, the PRP will be mixed with the bone cement at 50 ° C. Therefore, it is imperative to see if the mixing temperature between components has an effect of deactivating the growth factors contained in the PRP.

For this reason, the cell differentiation generated by the new formulation is tested with 5% of PRP. 5% of PRP is added instead of 1.2% or 0.7% like in cell viability experiments, because of the differentiation power of the PRP which has been observed on the cells in the previous study.

So the quantity of PRP is increased from 1.2% to 5% PRP in the new formulation. Adding a higher amount of PRP in the bone cement may lead to greater mineralization.

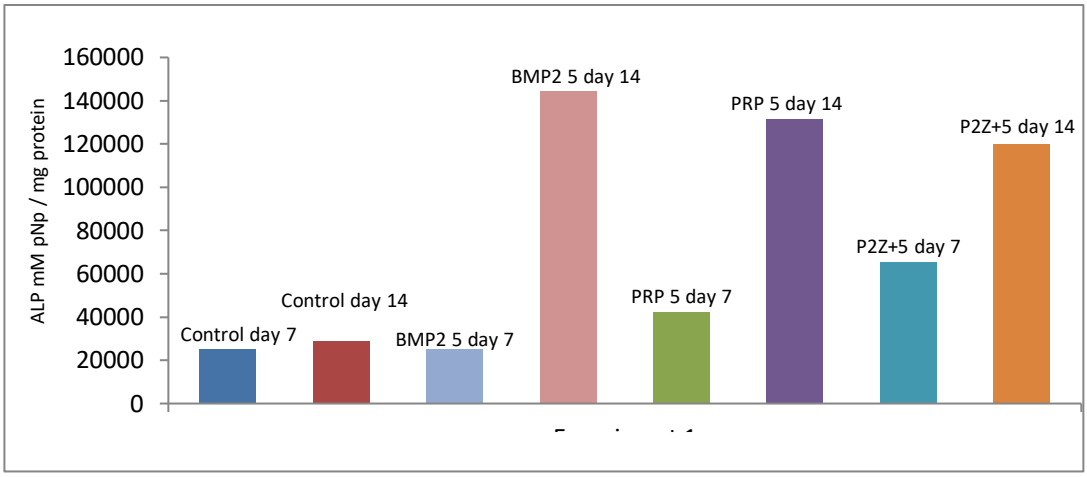


Figure III - 8: Determination of ALP. BMP-2: 0,15ng per well, PRP: 0,125mg per well and P2Z: 2,5mg per well

A peak of alkaline phosphatase is noted in all the products at day 14 respect to day 7, especiallyin cells in contact with BMP-2 5, PRP 5 and P2Z+5. This form is the typical profile of differentiation in bone marrow mesenchymal stem cells (Tour, Wendel et al. 2012). Then, the results suggest that HBMSCs are differentiated in presence of BMP-2 5, PRP 5 and P2Z+5.

So, the inactivation of the growth factors due to the sample preparation is tested too with this experiment. It has been demonstrated that the growth factors remain active even after the bone cement is heating at 50ºC for the mixing process and injection. The device maintains the integrity of the different ingredients of the biomaterial (specially the platelet rich fibrin active components).

In vitro matrix maturation and mineralization are usually enhanced by growing the cells to complete confluence and by adding specific osteogenic factors like BMP-2, PRP and P2Z+5 like in our case. The matrix maturation phase is characterized by maximal expression of alkaline phosphatase. Also, at the beginning of matrix mineralization, osteocalcin and osteopontin are expressed and once mineralization is completed, calcium deposition can be visualized using adequate staining methods.

The mineralization process of osteoblasts in vitro culture is usually used as a model for testing bone formation (Kostenuik, Halloran et al. 1997). The calcium deposits generated in the cells in contact with BMP-2, PRP and P2Z+5 are shown:

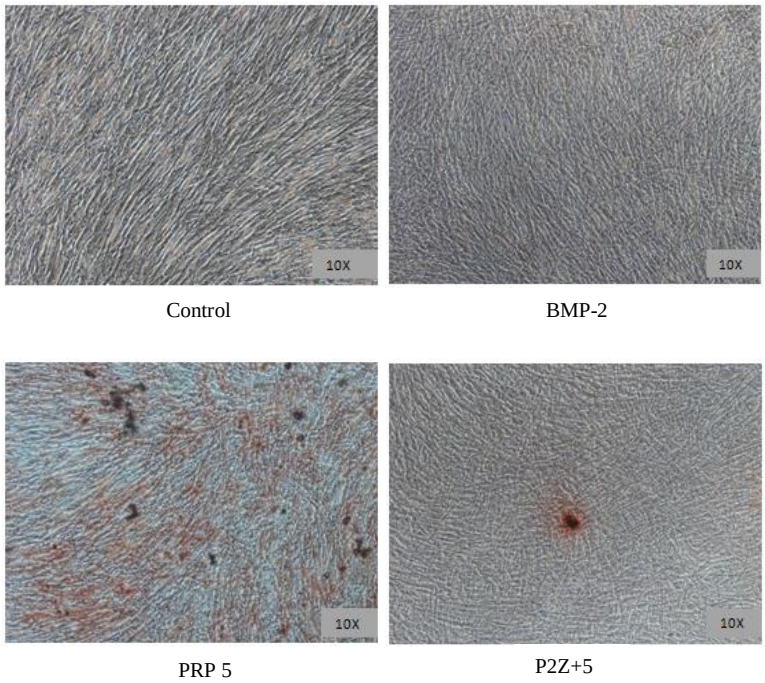


Figure III - 9: Alizarin Red at day 21.Control 10X, BMP-2 10X: 0,15ng per well, PRP 10X: 0,125mg per welland P2Z 10X: 2,5mg per well

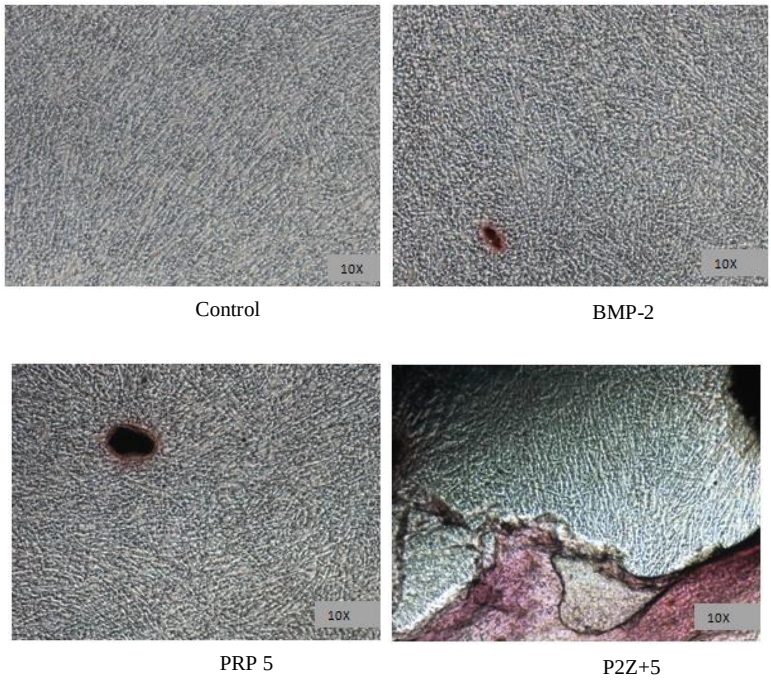


Figure III - 10: Alizarin Red at day 28. Control 10X, BMP-2 10X: 0,15ng per well, PRP 10X: 0,125mg per well and P2Z 10X: 2,5mg per well

Mineralization is observed specially in the wells with PRP 5 and P2Z+5. But in control and in BMP2 5, mineralization is not observed until day 28. Although in all cases a leap of ALP is

observed at day 14, not all of them have the same mineralization rate at the same day. That observation makes sense because PRP is a combination of growth factors, which cause very quick cell differentiation (Dolder, Mooren et al. 2006).

So, the most differentiation products are clearly PRP 5 and P2Z+5.The last one is the bone cement that meets all the desired conditions and tested in bone cement.

3.3.2.3 Differentiation effect of the paste

Due to the strong calcium deposits that cells show in the presence of PRP and P2Z+5, another experiment is carried out. The objective is to observe if the paste alone is able to generate the same mineralization rather than paste with PRP. Also, inserts are used to avoid direct contact of the product with the cells because it is very difficult to change the media or to take measurements of alkaline phosphatase and not to take away part of the scaffold that PRP forms with the cells when it is in contact with them. It is like a layer that bonds the cells with the product and it is not easy to separate the product from the cells without damaging or losing them.

In addition, the products are added in greater quantity because insert could restrain the complete process of differentiation and mineralization by the little contact of the product with the cells.

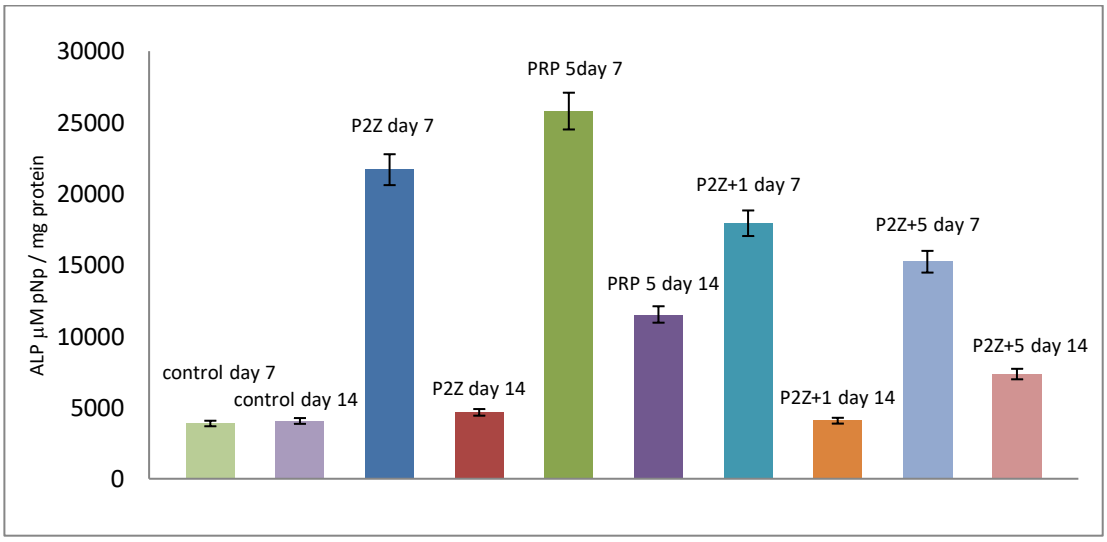


Figure III - 11: Determination of ALP. P2Z,P2Z+1,P2Z+5; 0,075g per well, PRP 5: 3,75mg per well

In this experiment, the peak is observed at day 7 in all the cases. Probably, this result is consequence of the high quantity of added product at the insert.

Mineralization appears before than in the other tests. This experiment is done with more quantity of product that the other two to promote the differentiation in the cells despite of the presence of the insert.

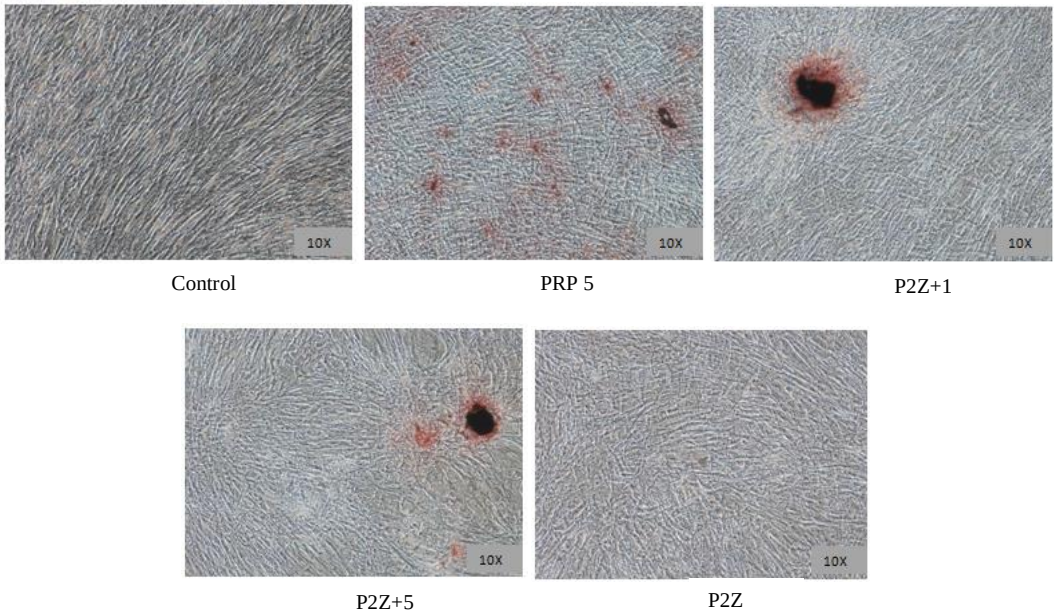
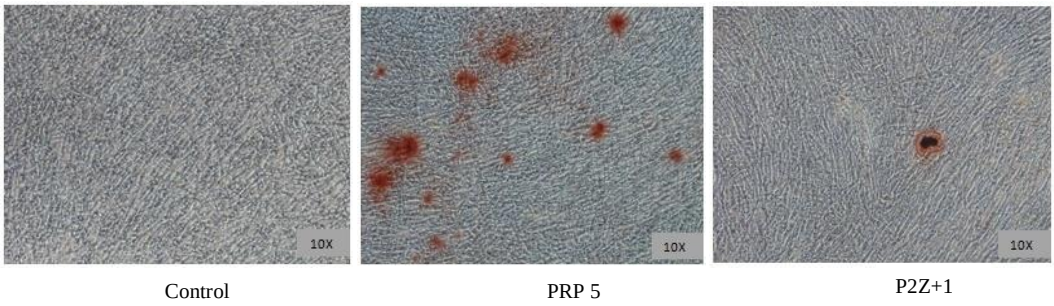
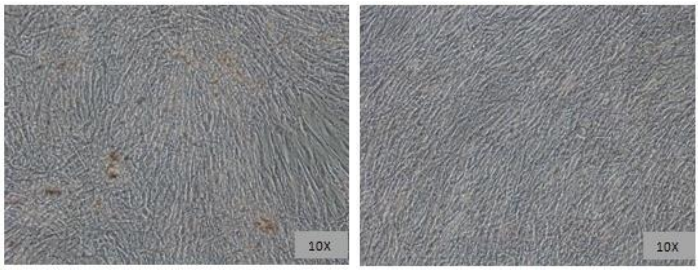


Figure III - 12: Alizarin Red at day 21.Control 10X; P2Z 10X, P2Z+1 10X, P2Z+5 10X: 0,075g per well; PRP 5 10X: 3,75mg per well





P2Z+5 P2Z

Figure III - 13: Alizarin Red at day 28.Control 10X; P2Z 10X, P2Z+1 10X, P2Z+5 10X: 0,075g per well; PRP 5 10X: 3,75mg per well

Mineralization is present in the wells with PRP 5, P2Z+1 and P2Z+5. Nevertheless, in the control and in the paste alone (P2Z), mineralization is not observed. Although in this experiment a leap of ALP is observed at day 7, not all of them have the same speed of mineralization. In fact, in this last differentiation experiment, P2Z+5 and P2Z+1 are tested at the same time to study if the change of percentage of PRP in the bone cement is significant in terms of cell differentiation.

Comparing the wells with P2Z+5 and P2Z+1 in this assay, more mineralization is observed in the wells with P2+5.Therefore, the percentage of PRP has a great influence on cell differentiation and mineralization of cells.

3.3.2.4 Differentiation markers. Osteopontin and osteocalcin

Considering the importance of alkaline phosphatase in bone growth and the final mineralization of the HBMSCs, the information obtained about the differentiation markersiscomplementary to all the experiments carried out until now.

Osteopontin (OPN) is measured because is a multifunctional matricellular protein that plays a key role in wound healing and cellular response to mechanical stress. In addition, it contributes to diverse physiological and pathological processes including immune responses, inflammation, tumor growth and metastasis, bone formation and remodeling. Itsexpression is tissue-specific and subject to regulation by many transcription factors(Zhao, Wang et al. 2015).

In addition, this matricellular protein is routinely present in mineralized tissues, which is believed to play an integral role in cellular responses to mechanical stimuli. In fact, OPN

appears to be necessary for mechano-stimulation of osteogenesis in the developing cranial suture. Conversely, mechanical stimuli are known to induce and increase the expression of OPN in osteoblasts and osteocytes (Perrien, Brown et al. 2002).

Due to the intimate relationship between osteopontin and tissue mineralization, and because of OPN is a Human Bone Mesenchymal Stem Cells differentiation indicator, osteopontin is analyzed as a differentiation marker.

Next, the gel is shown with the proteins separated by weight. The bands correspond to osteopontin at day 14 and 21.

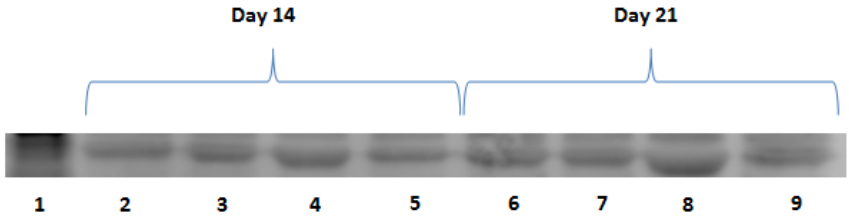


Figure III - 14: Gel with the proteins separated by weight. The bands correspond to osteopontin at day 14 and 21. Marker: 1; Control: 2, 6; BMP2 (1): 3, 7; PRP (1): 4, 8; P2Z: 5, 9

Osteopontin is present in all the samples at 60KDa, so there is a cellular differentiation in all media conditions. However, the most marked band is the number eight. So apparently, PRP at day 21 is the most differentiating cell material. Then, the differentiation in bone marrow mesenchymal stem cells is increasing, especially in PRP conditions. In addition, the level of secreted OPN becomes more intense as time progresses (from day 14 to day 21).

In fact, this study notes a progressive increase in OPN detection in the membranous bone matrix that accompanies maturation of the new bone. In fact, these results are not unique; but also, other studies have noted a biphasic pattern of OPN expression corresponding to proliferation and differentiation of osteoblastic cells during bone formation(Perrien, Brown et al. 2002).

So, this qualitative assay corroborates the results obtained in ALP determination and mineralization test.

Osteocalcin (OC) is also measured. It is a bone-specific protein that circulates in blood. Many studies have shown over the last 10 years that the serum OC level is a sensitive marker of bone turnover that reflects specifically bone formation (Szulc, Chapuy et al. 1993). In addition, OC is the most abundant non-collagenous protein (NCP) of the bone extracellular matrix (ECM) and is synthesized only by osteoblasts. So, OC plays a pivotal role during bone growth and mineralization (Lian, Stewart et al. 1989, Ducy and Karsenty 1995)**.**Because of the specificity of this differentiation marker, OC is qualitatively determined.

Next, the gel is shown with the proteins separated by weight. The bands correspond to osteocalcin at day 14 and 21.

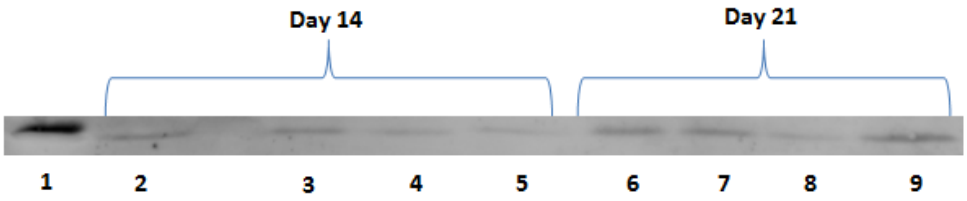


Figure III - 15: Gel with the proteins separated by weight. The bands correspond to osteocalcin at day 14 and 21. Marker: 1; Control: 2, 6; BMP2 (1): 3, 7; PRP (1): 4, 8; P2Z: 5, 9

Osteocalcin is expressed in four conditions at day 14 and day 21 (20KDa) but the intensity of the bands is much higher in the case of osteopontin.

These data indicate that osteocalcin expression increases slightly from day 14 today 21, so, the cellular differentiation in bone marrow mesenchymal stem cells increases a little bit as days go by, especially in PRP conditions (5vs9). Then, the level of secreted OC does not remain clearly unchanged and shows the same expression. In fact, 20 kDa bands become a bit more intense as time progresses. So, again, the influence of PRP on cell differentiation is confirmed throughout the days.

3.3.3 Sterility of bone cement

The bone cement + Zirconia + 5%PRP is determined like the bone cement that most promotes cell differentiation at all. Bioburden test is performed with the new formulation to proceed to the in vivo experiment.

Bioburden testing is required by regulatory authorities for investigational new drug (IND) submission and is especially critical in process development for biologicals and devices. Bioburden testing will provide the quantity of viable microorganisms on a therapeutic medical device or raw material before sterilization (Bioreliance, 2017).

Parámetro Método-Técnica	**Resultado** [1]
Determinación de la población de microorganismos Bioburden (Filtración)	
Bacterias aerobias (estrictas y facultativas) FP0024-FIL	<1 UFC/g
Mohos FP0024-FIL	<1 UFC/g
Levaduras FP0024-FIL	<1 UFC/g
Bacterias anaerobias (estrictas y facultativas) FP0024-FIL	<1 UFC/g

[1] El Laboratorio tiene a disposición del cliente la incertidumbre de medida de los ensayos cuantitativos acreditados.

Figure III - 16: Bioburden

No colony-forming units (CFU) are observed on our product. So, it is not necessary to sterilize it before the in vivo test.

Because of all the results obtained so far, it is decided to carry out the in vivo test with the new formulation mixed with PRP. It is performed using the bone critical size defect model.

3.3.4 Biodegradability of bone cement

One of the most important characteristics of the product is biodegradability. The paste must be biodegradable, because as the healing process progresses, the material reduces its stiffness as part of the load is supported by the regenerated tissue. For this reason, a biodegradability paste test was carried out at 17 days and analysed by GPC.

The paste was added like a lentil in three inserts, and like scattered dust in the other three inserts to try to mimic the conditions in which the paste will be in the organism. The polymer GPC at day 0 is compared with the GPC of the same polymer at day 17. The degradation products are observed by GPC at day 17.

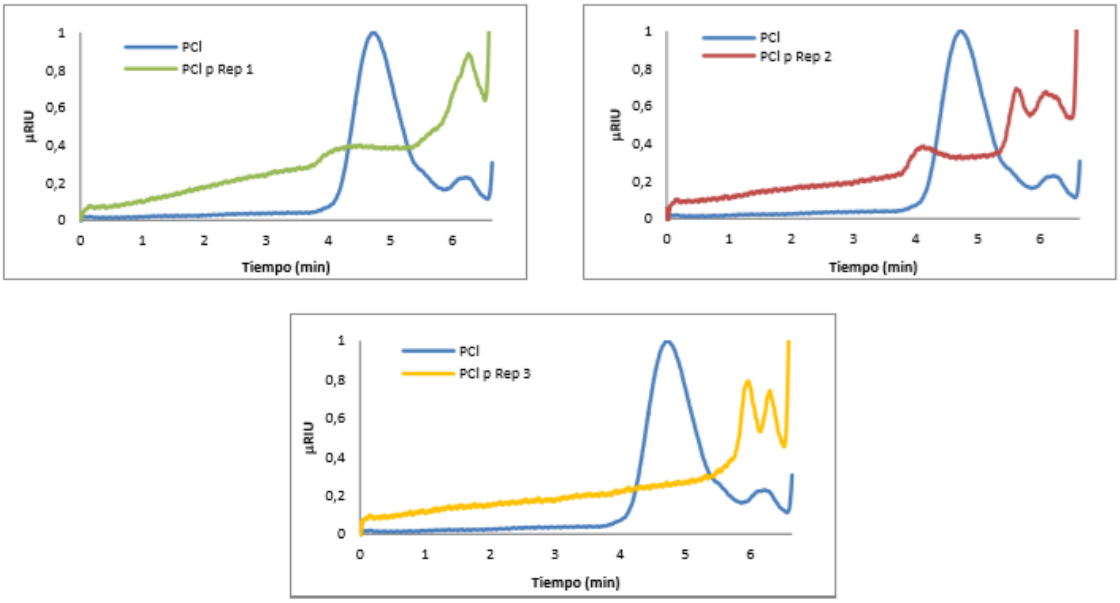


Figure III – 17: Comparison of the polymer degradation products at day 17 and scattered dust polymer at day 0 by GPC

In replicate 1 (PCl p Rep 1) a peak is observed in the minute 6.1 that may correspond to a paste polymer chain that has been degraded. In replicates 2 and 3 two closely spaced peaks are observed which probably correspond to two degradation products with different chain lengths. In replica 2 they appear in minutes 5.5 and 5.9, and in replica 3 in minutes 5.9 and 6.24. Next, the values of Mn, Mw and PDI of the degradation products are shown in comparison with the values of the polymer without degrading:

	PCl p Rep 1	PCl p Rep 2	PCl p Rep 3	PCl
Mn	1602,9	1332,2	820,4	11400,2
Mw	1673,4	1857,8	1092,2	18019,2
PDI	1,04	1,39	1,33	1,58

Figure III – 18: Comparision of Mn, Mw and PDI between polymer and scattered dush degradation products

In Table 1 it is observed how the molecular weight of the degradation products is much lower than the molecular weight of the polymer without degrading.

Next, the GPC show the degradation products for each well for the paste in lentin form. PC is paste at day 0, and PCl p Rep1, PCl p Rep2 and PCl p Rep3 are paste at day 17. There are three replicates of the same experiment.

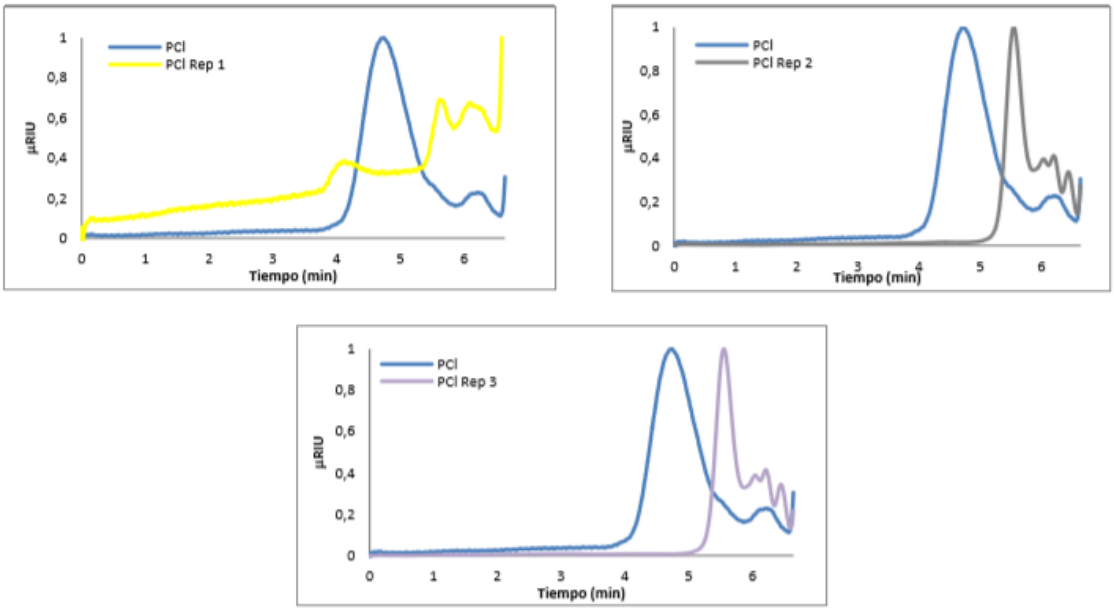


Figure III – 19: Comparison of the polymer degradation products at day 17 and lentil polymer at day 0 by GPC

In replicate 1, 2 and 3, diferent peaks are observed and all have a lower molecular weight than polymer at day 0.

Next, the values of Mn, Mw and PDI of the degradation products are shown in comparison with the values of the polymer without degrading:

	PCl Rep 1	PCl Rep 2	PCl Rep 3	PCl
Mn	908,8	2244,2	2234,5	11400,2
Mw	1222,7	2523,7	2452,0	18019,2
PDI	1,35	1,12	1,10	1,58

Figure III – 20: Comparision of Mn, Mw and PDI between polymer and lentil degradation products

In Table 2 it is observed how the molecular weight of the degradation products is much lower than the molecular weight of the polymer without degrading.

So, we can conclude that paste has already begun to biodegrade at day 17. The estimated time for a complete paste resorption in vivo have to be 6 months, but we would really need to check it with an in vivo test.

3.3.5 In vivo: Bone critical size defect model

By definition, the critical size bone defect (CSD) is the smallest size defect that will not heal spontaneously and completely cover the natural lifetime of an animal. Therefore that type of in vivo models allows testing materials or strategies capable to create new bone at a site where bone would not be present otherwise(Spicer et al. 2012).

That model is highly reproducible and allows testing for non-load-bearing orthotopic applications .The surgical procedure itself takes approximately 30 minutes to complete with approximately 2 hours of perioperative care.

3.3.5.1 Local tissue reaction

It is well known that the wound type and the type of targeted tissue determine the course and intensity of the local inflammatory reaction. 'Foreign body reaction' is the term used to describe the local chronic inflammation triggered by biomaterials implantation. The physical properties and chemical structure of the biomaterial are the key factors that guide the foreign body reaction and the eventual course of wound healing (Tour et al. 2014).

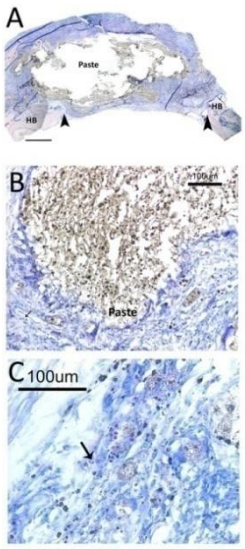


Figure III – 2117: Calvarial defects treated with the polymeric paste containing PRP, 2 weeks after implantation. (A) -overview image; Bar = 1 mm. (B) and (C) – enlargement views of selected areas from image (A).HB – host bone; defect margins indicated by arrow heads; arrows in (B) and (C) pointing to the eosinophils (cells with red-pink cytoplasm); Wright-Giemsa staining.

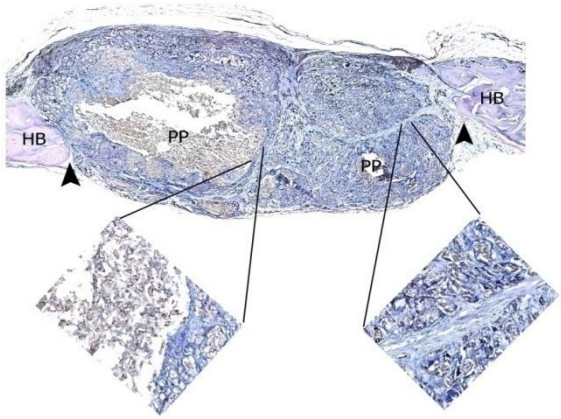


Figure III – 22: Calvarial defect treated with the polymeric paste containing PRP, 12 weeks after implantation. HB – host bone; arrow heads – bone defect margins; PP – polymeric paste; Wright-Giemsa staining

The bone cement was tested in a calvarial model with a critical size defect and the calvarial defects did not reveal new bone formation at 2 and at 12 weeks postoperatively. The calvarial

bone defects were filled with the new product by syringe spread among fibrous connective tissue comprising inflammatory cells, fibroblasts and blood vessels.

All rats survived the surgery and the post-operative follow-up. Large amount of the new bone cement was present at the site of implantation both at 2 weeks and 12 weeks after surgery. The paste was fragmented, penetrated by connective fibrous tissue, and infiltrated with a very high number of macrophages, inflammatory giant cells, and eosinophils demonstrating a strong local inflammatory reaction towards the paste. No new bone was formed.

Although all in vitro experiments gave hopeful results, this is not the case in vivo, where new bone has not been formed.

However, this in vivo model is not the most suitable to test a vertebra prosthesis. Rats are very small animals, and therefore, the result that bone cement does in them is not entirely determinant. For these reason, the inflammatory response is so high. Our new bone cement should be tested in sheep vertebrae, because they are very similar to human vertebrae. Unfortunately, this in vivo has a cost that a doctoral thesis cannot assume.

3.4 Concluding remarks

First of all, different combinations of paste with different percentages of PRP have been prepared and send to Karolinska Institutet for evaluation. The results confirmed that our bone cement is not toxic and that the presence of PRP promotes fibroblasts viability.

Moreover the incorporation of PRP to the paste confers osteoinductive properties to the material that would promote bone regeneration while the bone cement is biodegraded in the vertebra.

Finally, bone cement has been developed and selected for yielding the most promising results. The new formulation promotes cell proliferation and the presence of the differentiation markers of the bone human mesenchymal stem cells; Osteopontin, Osteocalcin and Alkaline phosphatase activity.

Osteopontin and Osteocalcin play a pivotal role during bone growth and mineralization. These matricellular proteins are routinely present in mineralized tissues and they have an intimate relationship with tissue mineralization. Also, Alkaline Phosphatase activity in serum is related to increased osteoblastic activity in bone. In fact, the new bone cement promotes the typical ALP profile of differentiation in bone marrow mesenchymal stem cells (Tour, Wendel et al. 2012).

In addition, our product generates clear matrix maturation, because it is able to mature the HBMSCs into osteoblasts, and these last ones in osteocytes. They interact between them and generate calcium deposits. So, the product accomplish the three stages of osteoblast differentiation until mineralization(Kasperk, Wergedal et al. 1995).

In conclusion, this human bone marrow cell culture model have provided an ideal means of obtaining a population of mature osteoblasts which later mineralize and generate calcium deposits. That process has happened after progressive treatment our bone cement, and somehow we get closer to what happens in a real bone.

Because of the successful results obtained, an implantation studies in critical-size calvarial bone defect healing are done to check if the bone cement is equally effective in vitro to in vivo.

However, the new product does not enhance the calvarial bone repair. Also, the cement creates a high foreign body reaction with macrophage accumulation. So, no new bone formation is observed, and the major amount of the implanted product remained in the same place. The cellular profiles of the inflammatory infiltrates at the defect sites are analyzed.The connective tissue surrounding the implanted paste is infiltrated with a large number of inflammatory cells, including eosinophils, therefore demonstrating strong local inflammatory reactions towards the paste.

But, as commented, this in vivo model is not the most suitable to test a vertebra prosthesis because rats are very small animals and the inflammatory response is too high. The new bone cement should be tested in sheep vertebrae, because sheep vertebrae are very similar to human vertebrae, but this in vivo has a cost that a doctoral thesis cannot assume.

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Conclusions

“El médico divino lo tiene todo controlado.”

-Eduardo Bardají Ais

Conclusions

1. New bone cement has been developed to apply in vertebroplasty. For achieving that a family of polyesters has been developed and their viscosity has been controlled by the length of each chain and by the reactivity of their monomers.

2. These polymers have been mixed with Hydroxyapatite to form a paste which has been complete characterized in terms of rheological characteristics and hardness to observe its thermoplastic behavior and its higher viscosity.

3. The selected bone cement assessment has been successful. The product is able to be injected at 50ºC and remains mechanically stable when the temperature reaches 37ºC preserving its initial properties because injection is homogeneous and avoiding cement extravasations into the surrounding tissues.

4. Also, the fractured vertebral body can be easily treated by the surgeon because the setting time only takes a few minutes; and there is no need for patient immobilization after the surgical treatment or the use of corsets as working time only takes several minutes too.

5. The cement bone has an excellent compression resistance too. And after the compression test, it does not present fractures and it is able to fill the entire gap. So, have the strength and stiffness to hold up the loads that support a healthy vertebral body and then augment and stabilize it.

6. In addition, our product generates clear matrix maturation, because it is able to mature the HBMSCs into osteoblasts, and these last ones in osteocytes. They interact between them and generate calcium deposits. So, the bone cement is osteoconductive and osteoinductive.

7. Finally, the biocompatibility and biodegradability of the bone cement has been verified by rat calvaria in vivo model. But the restoration of the bone has not been observed due to the limitations presented by this model.The cementhas created a high foreign body reaction with macrophage accumulation. So, no new bone formation has been observed.

8. Therefore, the use of a sheep vertebra in vivo model is proposed, which is more representative of what could happen in a real patient.