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Development of 3D scaffolds based on the interaction of Elastin-Like Polymers and Hydroxyapatite for bone tissue Engineering

Tesis que para obtener el grado de:

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DEDICATORIA

A:

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Abstract

Bone disorders have trended steeply upward and it is expected to increase by double within the next five years in the worldwide population. Under these conditions, tissue engineering aims to induce new functional bone as a valid approach to the current therapies via the combination of biomaterials like hydroxyapatite (HA) and the ultimately so-called smart polymers namely, Elastin-Like Polymers (ELPs). It is well know that hydroxyapatite is a well-established bone graft for bone repair due to its biocompatibility, oseointegrity, and osseous inductivity, whereas ELPs have shown to be biocompatible, biodegradable, and to possess a non-immunogenic response. In this work, a new biomaterial was synthesised in the form of hydrogel based on Hydroxyapatite and ELPs. This new hydrogel has shown stability for a period of 10 days without additives such as cross-linking agents, furthermore, Scanning Electron Microscope (SEM) revealed different types of morphology under different concentrations of HA. Additionally, Fourier Transform Infrared Spectroscopy (FTIR) was used to analyse the composition and spectra of HA and ELPs before and after the synthesis of hydrogel. Difference on spectra bands was not observed before synthesis (peaks in the bands at 1028cm^{-1} and 1700cm^{-1}) for HA whereas ELPs showed bands at 1076cm^{-1} and 1647cm^{-1}, nonetheless decrease of the intensity in the bands was observed posterior synthesis. Finally, human mesenchymal stem cells were cultured for a period of 4 days in order to evaluate the biocompatibility of the material, showing cell adhesion revealed by SEM.

Keywords: Tissue engineering, Elastin like Polymers, Hydroxyapatite, Hydrogels, Human Mechenchimal Stem Cells.

RESUMEN

Las enfermedades óseas se han incrementado precipitadamente y se espera que aumenten al doble en los próximos 5 años dentro de la población mundial. Bajo estas condiciones, la ingeniería tisular tiene como objetivo crear nuevo tejido ósea funcional como un nuevo elemento en las terapias de recuperación actuales, mediante la combinación de biomateriales como la hidroxiapatita (HA) y los últimamente llamados "polímeros inteligentes" tales como los Polímeros similares a la Elastina (ELPs por sus siglas en inglés). Se sabe que la hidroxiapatita es un sustitutito óseo bien establecido debido a su biocompatibilidad, osteointegridad, e inductividad ósea, mientras que los Polímeros similares a la elastina han demostrado poseer biocompatibilidad, biodegradabilidad, además de no mostrar respuesta inmunológica. En este trabajo, un nuevo biomaterial se sintetizo en base a la hidroxiapatita y los ELPs. Este nuevo hidrogel ha mostrado una estabilidad por un periodo de 10 días sin aditivos tales como los agentes de entrecruzamiento, además, la microscopia de barrido de electrones revelo diferentes tipos de morfologías bajo diferentes concentraciones de hidroxiapatita. Adicionalmente, la espectroscopia infrarroja de transformada de Fourier se utilizó para analizar la composición y el infrarrojo del HA y los ELPs antes y después de la síntesis del hidrogel. No se observó diferencia en las bandas de espectro de los materiales antes de la síntesis (picos entre $1028cm^{-1}$ and $1700cm^{-1}$) para la hidroxiapatita, mientras que los ELPs mostraron picos en $1076cm^{-1}$ and $1647cm^{-1}$, sin embargo, se observó in decremento en la intensidad de las bandas posterior a la síntesis. Finalmente, se utilizaron células madre mesenquimales para cultivarlas por un periodo de 4 días, para así, evaluar la biocompatibilidad del material, el cual mostro adhesión celular que fue revelada por el microscopio de barrido de electrones.

Palabras clave: Ingeniería de tejidos, Polímeros similares a la elastina, Hidroxiapatita, Hidrogeles, Células madre mesenquimales.

ABREVIATIONS

CPD – Critical Point Dryer

ELP - Elastin Like Polymers

FTIR – Fourier Transform Infrared

HA - Hydroxyapatite

HAP – Recombinant statherin-containing protein polymer

HMSC – Human mesenchymal stem cells

ITT – Initial Transition Temperature

IK – Recombinant lysine-rich protein polymer

PBS – Phosphate buffered Saline

RGDS – Recombinant RGD-containing protein polymer

SBF – Simulated Body Fluid

SEM – Scanning Electron Microscope

ZP – Zeta Potential

GLOSARY

Stem Cells	An undifferentiated cell of a multicellular organism that is capable of giving rise to indefinitely more cells of the same type, and from which certain other kinds of cell arise by differentiation.
Elastin like Polymers	Polymers based on elastins that possess stimuli-responses either temperature, light of ph.
Mesenchymal Stem Cells	Multipotent stromal cells that can differentiate into a variety of cell types, including: osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells).
Hydrogels	Polymer networks extensively swollen with water.
Tissue	Group of connected cells in an animal or plant that are similar to each other, have the same purpose, and form the stated part of the animal or plant.
Organ	Group of tissues in a living organism that have been adapted to perform a specific function. In higher animals, organs are grouped into organ systems
Hydroxyapatite	A mineral, Ca 10 (PO 4) 6 OH 2, that is the principal storage form of calcium and phosphorus in bone.
Lysine	Lysine is an α-amino acid with the chemical formula $HO_2CCH(CH_2)_4NH_2$.
Tissue engineering	The study of the growth of new connective tissues, or organs, from cells and a collagenous scaffold to produce a fully functional organ for implantation back into the donor host.
Amide	A compound with the functional group $R_nE(O)_xNR'_2$ Most common are carboxamides.
Scaffold	A structure bade on the basis of a biomaterial capable of providing the structural support for cell attachment and subsequent tissue development.
Biomaterial	Synthetic or natural material suitable for use in constructing artificial organs and prostheses or to replace bone or tissue.

Extra Cellular Matrix	A collection of extracellular molecules secreted by cells that provides structural and biochemical support to the surrounding cells.

Chapter 1 Introduction

It is well recognised that musculoskeletal disorders such as osteoarthritis, rheumatoid arthritis, osteoporosis, and low back pain are prevalent and their impact is pervasive. The four major conditions are representing a major burden on individuals, health systems, and social care systems, with indirect costs being predominant. This burden has been recognised by the United Nations and WHO, by endorsing the Bone and Joint Decade 2000–2010 [1]. The prevalence of many of these conditions increases markedly with age, and many are affected by lifestyle factors, such as obesity and lack of physical activity.

Due to its prevalence worldwide, osteoporosis is considered a serious public health concern. Osteoporosis is a disease characterised by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures of the hip, spine, and wrist [2]. This is a global public health problem currently affecting more than 200 million people worldwide. Osteoporosis is estimated to affect 200 million women worldwide - approximately one-tenth of women aged 60, one-fifth of women aged 70, two-fifths of women aged 80 and two-thirds of women aged 90 [3] although it is estimated that one third of men are likely to suffer from this disorder at some point of their lives [4].

The biggest problem created by bone disease, especially osteoporosis, is fractures, which may be the first visible sign of this disease in patients. Every year an estimated of 1.7 million individuals suffer a fracture due to bone disease and 1.5 million of these fractures are attributed to osteoporosis including hip fractures [4]. Seventy per cent of those suffering from these fractures do not return to their pre-injury status. Worldwide, 1.66 million hip fractures were estimated to have occurred in 1990: about 1.19 million in women and 463 000 in men. Fracture rates vary in different countries, the highest rates are seen in North America and Europe, particularly Scandinavia [5,6,7]. The risk of osteoporotic fractures is lower in Africa and Asia, but worldwide projections suggest that it will increase markedly in the future [1].

Hip Fracture

Hip fracture is associated with serious disability and excess mortality. the incidence of hip fractures increases exponentially with age, with rates of 2 per100 000 person-years in women aged <35 years rising to 3032 per 100 000 person-years in women aged ≥85 years; respective rates in men are 4 and 1909 Women who have sustained a hip fracture have a 10-20% higher mortality than would be expected for their age[12]. The worldwide annual incidence of hip fracture is approximately 1.7 million [1,3].

Wrist Fracture

Wrist fractures are most likely to occur in women over 65 years old. An increase in age-adjusted incidence in white women between 45 and 60 years of age has been observed. Then the trend stabilises or slightly increases. Only 15% of wrist fractures occur in men and this rate does not increase much with age [3].

Vertebral Fracture

The incidence and prevalence of radiological findings increase with age. One in eight men and women aged >50 years in Europe have vertebral deformity [1-5], See fig. 1.

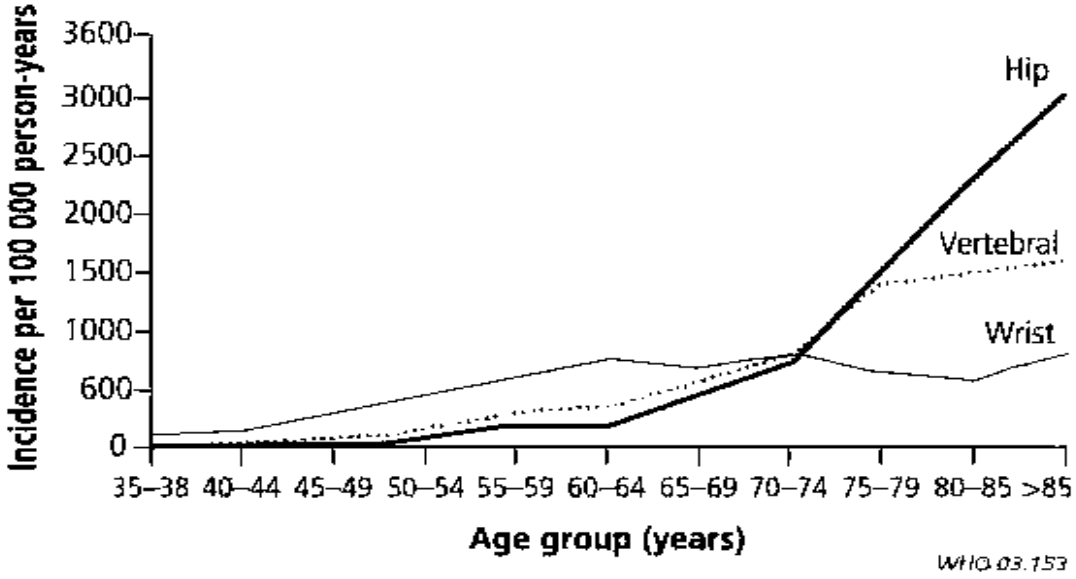

Fig. 1 Incidense of osteophorosis fractures in women, by age.

PROJECTIONS

With the dramatic growth of the elderly population, it is expected that bone diseases will rise by double within the next 25 years and much of these increase will occur when “baby boomers” reach their 70s and 80s.

With the increase in the elderly population, there has been a major incidence of fractures at earlier ages, which is why osteoporosis has become a major public health problem of epidemic proportions.

The worldwide incidence of hip fracture in men is projected to increase by 240% in women and 310% in men within the next 30 years (3,22). The estimated number of hip fractures worldwide will rise from 1.66 million in 1990 to 6.26 million in 2050, even if age-adjusted incidence rates remain stable [3,9], see fig. 2.

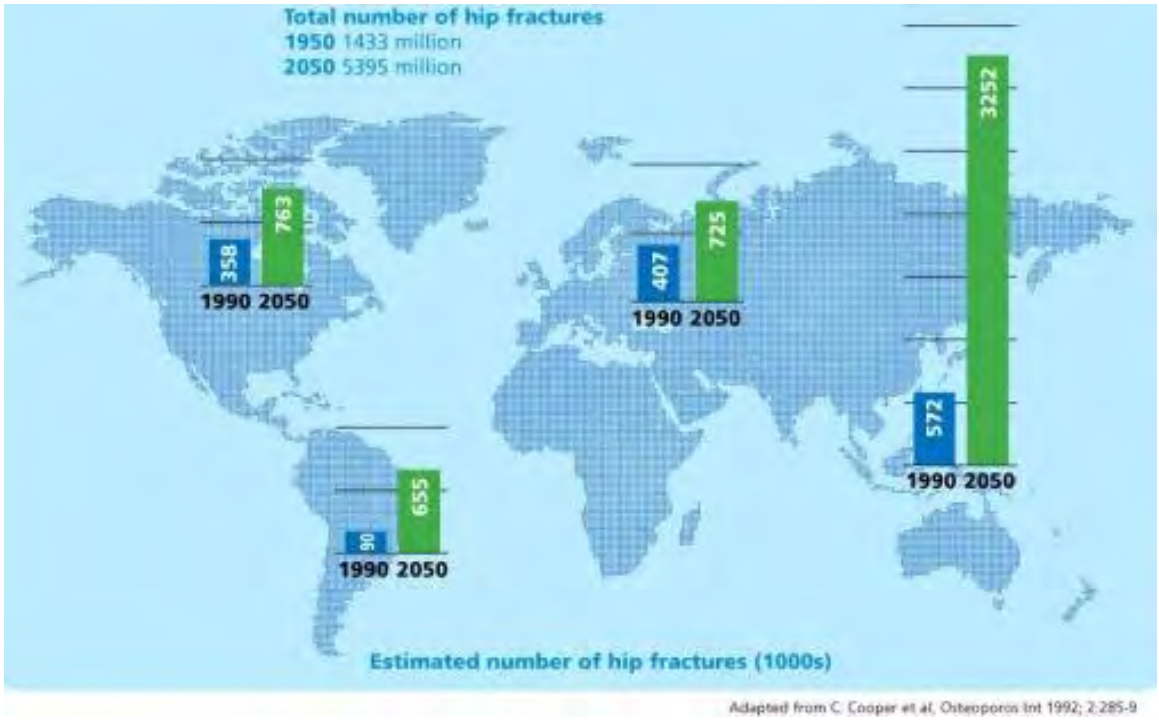

Fig. 2 Projected number of osteoporosis fractures worldwide [3].

Bone fractures

As previously described, one of the major consencuence of bone disorders is fractures. A fracture, also referred to as a bone fracture, is a medical condition where the continuity of the bone is broken. A significant percentage of bone fractures occur because of high force impact or stress fracture as well as a result of some medical conditions, which weaken the bones, (osteoporosis) some cancers or osteogenesis. A fracture caused by a medical condition is known as a *pathological fracture*.

There are basically eight types of bone fractures, which are be described as follows (see fig 3):

- Transverse fracture: A transverse temporal bone fracture is orientated perpendicular to the long axis of the petrous bone with the line of force running roughly anterior to posterior [10].
- Oblique fracture: An oblique fracture is a break which has a fracture line that runs diagonal to the bone shaft [11].
- Spiral fractures: spiral fractures are similar to oblique fractures because they also have a break line which is diagonal to the shaft, nevertheless, the oblique fracture tends to be a straight break while the spiral fracture has a pattern similar to a corkscrew [11].
- Comminuted fracture: A bone injury that results in more than 2 separate bone components [12].
- Avulsion Fracture: is an injury to the bone in a location where a tendon or ligament attaches to the bone [13].
- Impacted fracture: occurs when the broken ends of the bone are jammed together by the force of the injury [14].
- Fissure fracture: Cracks or fissure lines that occur when direct trauma is applied to any long or flat bone [15].
- Greenstick fracture: occurs when a bone bends and cracks, instead of breaking completely into separate pieces [16].

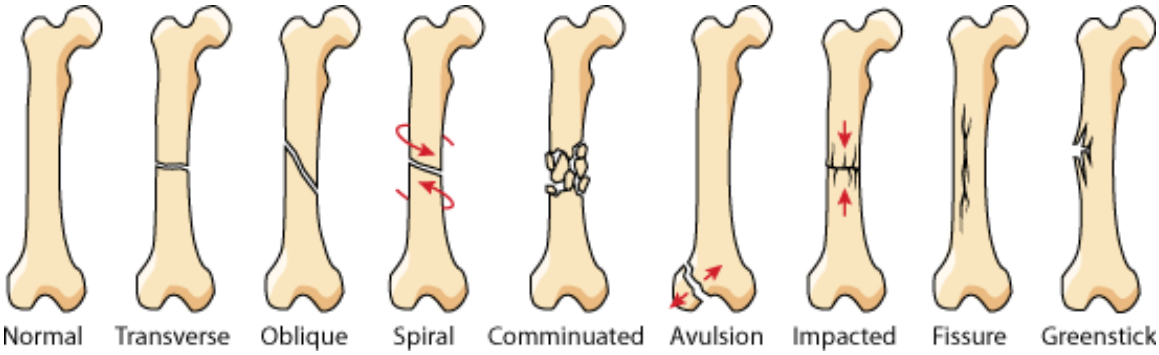

Fig. 3 Types of bone fractures.

Bone fractures healing

Normally, when bone fracture occurs, body possesses the capacity to heal itself, after immediately a bone fracture occurred; the fracture self- repair process starts the work of repairing. This is a spontaneous and natural process, which seeks no direction from us, but what is done during this time is of unrecognised importance.

Fracture healing involves a complex process of cell and tissue proliferation and differentiation. Many players are involved, including growth factors, inflammatory cytokines, antioxidants, bone breakdown (osteoclast) and bone- building (osteoblast) cells, hormones, amino acids, and uncounted nutrients [17].

For bone healing to happen, the bone needs adequate stability and blood supply. Good nutrition also plays a role in bone healing.

- **Stability.** All treatment of broken bones follows one basic rule: the broken pieces must be put back into position and prevented from moving out of place until they heal. Some fractures can be held in position with a cast. Some fractures require surgical fixation with devices like screws, plates, rods and frames.
- **Blood supply.** Blood delivers the components required for healing to the fracture site. These include oxygen, healing cells, and the body's own chemicals necessary

for healing (growth factors). The blood supply to the injured bone usually comes back on its own during the healing period.

Nonetheless, in some cases, spontaneous bone healing is not possible to be performed, in this case, non-unions happen when the bone lacks adequate stability, blood flow, or both. They also are more likely if the bone breaks from a high-energy injury, such as from a car wreck, because severe injuries often impair blood supply to the broken bone, which is why some external treatments are required for the total healing of bone. Such treatments may include surgical and non-surgical methods.

Non-surgical methods

In regard to non-surgical methods, some non-unions can be treated throughout this method. The most common nonsurgical treatment is a bone stimulator. This small device delivers ultrasonic or pulsed electromagnetic waves that stimulate healing. The patient places the stimulator on the skin over the non-union from 20 minutes to several hours daily. This treatment must be used every day to be effective [18].

Fig. 4 An external bone stimulator is applied to the skin overlying the non-union.

Surgical methods

As far as surgical methods, they are usually focused on three goals:

- *Establishing a healthy vascular area of bone and soft tissue around the fracture site*.
  This is accomplished by removal of any poorly dead bone or poorly vascularized tissue or scar from the fracture site. In some cases, the local tissues may be so badly damaged that plastic surgery in the way of rotational or micro vascular free muscle flaps may be necessary to bring new healthy tissue to the fracture site.
- *Establishing stability at the fracture site.*
  This usually involves use of a rod, plate or screws. This may also involve use of an "external fixator" -- external pins to hold the bones above and below the fracture.

Fig. 5 bone stabilisation throughout the distraction osteogenesis technique.

Nonetheless these methods contain some risks, which may include neurovascular injury, infection, bleeding, and stiffness [18,19], see fig. 5.

Bone Graft Healing

Nowadays, some other therapies such as bone grafts have been proposed for bone healing, in which said methods consists stimulating a new fracture healing response using grafts most often involve borrowing healthy chancellors or "spongy" bone from the pelvis, through a small incision at the level of the hip. This brings in many new bone forming cells and other supportive cells. In this case, a bone graft can be taken from the patient's own healthy bone (this is called an autograft). Or, it can be taken from frozen, donated bone (allograft). In some cases, a manmade (synthetic) bone substitute is used.

During surgery, the surgeon makes a cut over the bone defect. The bone graft is shaped and inserted into and around the area. The bone graft can be held in place with pins, plates, or screws.

Most of the times, due to the lack of patient's own healthy bone, it is compulsory the use of synthetic bone substitute grafts and here is where tissue engineering or currently so-called "Regenerative Medicine" [20].

Regenerative Medicine (Tissue Engineering)

Tissue engineering, or currently named "Regenerative Medicine" is a multidisciplinary area which comprises biology, medicine, engineering and chemistry with the potential to fully heal damaged tissues and organs, offering solutions and hope for people who have conditions that today are beyond repair [21].

Regenerative medicine also empowers scientists to grow tissues and organs in the laboratory and safely implant them when the body cannot heal itself. Importantly, regenerative medicine has the potential to solve the problem of the shortage of organs available through donation compared to the number of patients that require life-saving organ transplantation [22].

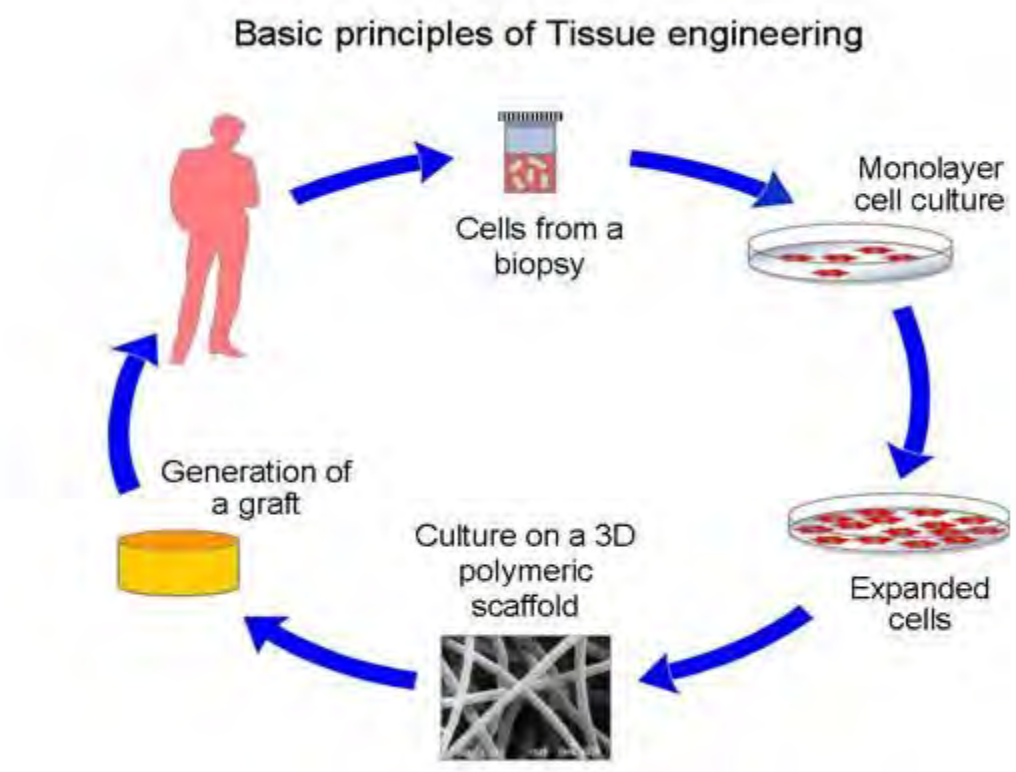

Fig. 6 Basic principles of tissue engineering where cells are extracted from patients' own tissue and then incubated in a monolayer cell culture for their expansion. Afterwards, cells are set and cultured on a 3D scaffolds, which is later implanted into patien.

- Rejuvenation. This means boosting the body's natural ability to heal itself. Though after a cut your skin heals within a few days, other organs don't repair themselves as readily. But cells in the body once thought to be no longer able to divide (terminally differentiated) — including the highly specialized cells constituting the heart, lungs and nerves — have been shown to be able to remodel and possess some ability to self-heal.

- Replacement. Replacement involves using healthy cells, tissues or organs from a living or deceased donor to replace damaged ones. Organ transplants, such as heart and liver transplants, are good examples.

- Regeneration. Regeneration involves delivering specific types of cells or cell products to diseased tissues or organs, where they will ultimately restore tissue and organ

function. This can be done through cell-based therapy or by using cell products, such as growth factors. Bone marrow transplants are an example [22].

To this end it is essential a support for the cultivation, differentiation as well as the cell growth for the development of new tissue and/or organs, which is why the use of scaffolds for providing said support have become widely used in regenerative medicine.

Scaffolds in tissue engineering

The scaffold or three-dimensional (3-D) construct provides the necessary support for cells to proliferate and maintain their differentiated function, and its architecture defines the ultimate shape of the new bone and cartilage [23], See figure 7.

Fig. 7 Scaffolds for bone repair syntehtised based on calcium phosphate.

Ideally, a scaffold should have the following characteristics [23].

- Three-dimensional and highly porous with an interconnected pore network for

cell growth and flow transport of nutrients and metabolic waste.

- Biocompatible and bio-resorbable with a controllable degradation and resorption rate to match cell/tissue growth in vitro and/or in vivo.
- Suitable surface chemistry for cell attachment, proliferation, and differentiation and (iv) mechanical properties to match those of the tissues at the site of implantation.

Numerous strategies currently used to engineer tissues depend on employing a material scaffold. These scaffolds serve as a synthetic extracellular matrix (ECM) to organise cells into a three-dimensional architecture and to present stimuli, which direct the growth and formation of a desired tissue. Depending on the tissue of interest and the specific application, the required scaffold material and its properties will be quite different [23, 24].

There are currently a number of biomaterials utilised in the synthesis of scaffolds for bone regeneration, from which calcium phosphate (hydroxyapatite) and the nowadays so-called smart polymers can be highlighted.

Hydroxyapatite

Bioceramics are a class of ceramics used for the repair and replacement of diseased and damaged parts of musculoskeletal systems. The most widely used bio-resorbable ceramics include calcium orthophosphates (CaPs). They are present in bones, teeth, deer antlers and the tendons of mammals, giving these organs hardness and stability. Non-ion-substituted calcium orthophosphates are in a range between 0.5 and 2.0 Ca/P molar ratio. The most widely used member of the family of CaPs is hydroxyapatite (HA) [26], see figure 8.

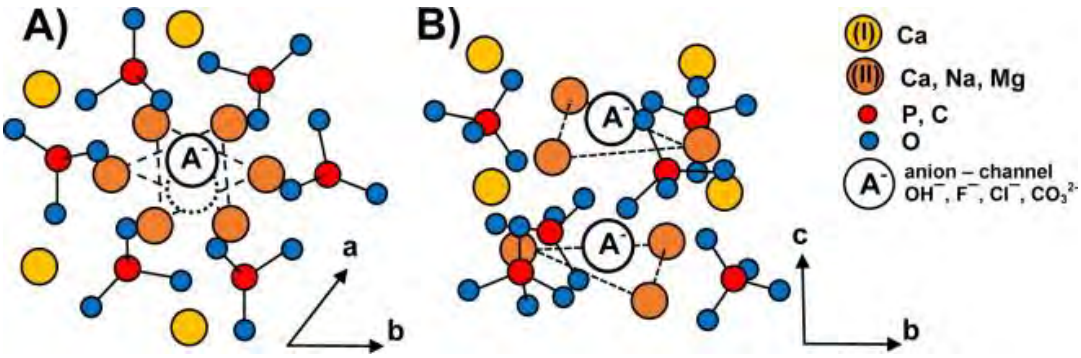

Fig. 8 View of the atomic structure of hydroxyapatite as a stand-in for bioapatite. Viewed (A) down the c-axis, and (B) perpendicular to the c-axis [26].

Hydroxyapatite is the major mineral constituent of vertebrate bones and teeth. It has been well documented that HA nanoparticles can significantly increase the biocompatibility and bioactivity of man-made biomaterials.

HA has been currently and commonly used a material for various biomedical applications such as the replacement for bony and periodontal defects [26,27], alveolar ridge [28], middle ear implants [29], tissue engineering systems [30,31], drug delivery [32], dental materials [33] and bioactive coating on metallic osseous implants [34,35].

Hydroxyapatite (HA) has been used for the repair of bone defects for over 30 years [36,38], mainly because of its similarity in crystallography to bone mineral [37,39]. One of the earliest events upon introduction to the physiological environment is protein adsorption (which occurs within fractions of a second) [40] and it is widely recognized that surface attributes, such as topography [41], surface charge [42] and surface energy or wettability strongly dictate and influence this biological reaction to implants [43,44], regulating protein conformation [45,46,], speciation [47] and stability [48].

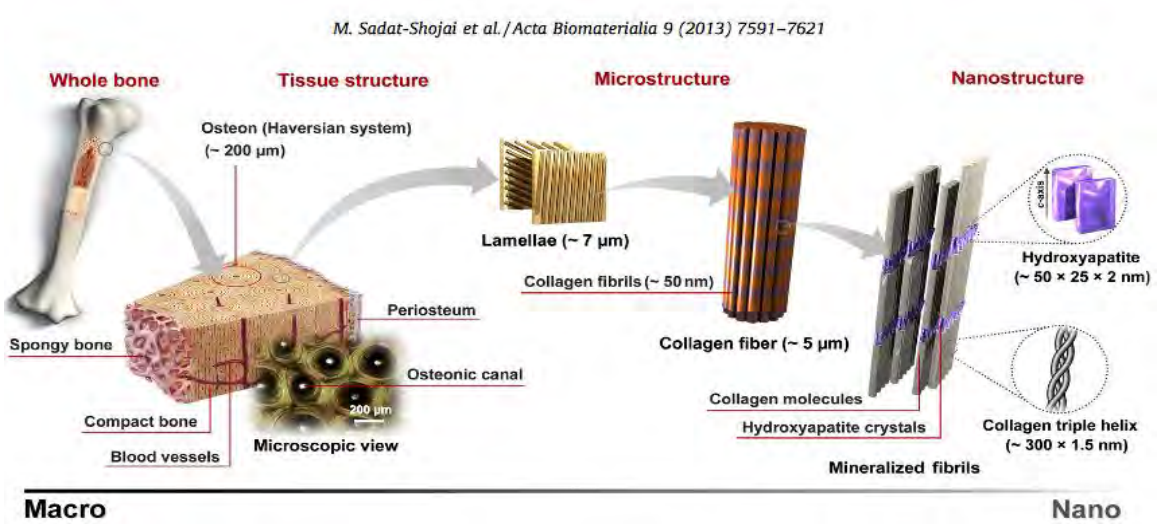

Fig. 9 The hierarchal structure of typical bone at various length scales. The microstructure of cortical or compact bone consists of harvesian systems (circles in the cross-section and microscopic view) with osteonic Canals and lamellae, and the Nano-scale, *the structural framework is collagen fibbers composed of bundles of mineralised collagen fibrils [ ].*

Substitution in hydroxyapatite

The number of ionic substitutions in bioapatites is smaller than for geological apatites, owing to the limited number of elements present in body fluids. Foreign ions known and/or reported in bone and tooth apatites are F^{-}, Cl^{-}, Na^{+}, K^{+}, Fe^{2+}, Zn^{2+}, Sr^{2+}, Mg^{2+}, citrate and carbonate. However, there are structural limits that determine how much of a given ion can be incorporated.

The major substituent in biological apatite consists of carbonates ($CO_3 2-$), which occur in bone mineral at levels typically of 5–8 wt. There is great number of elements utilised for the substitution of hydroxyapatite cations, such elements may include carbonates, fluorides, chlorides, sulphur and selenium oxyanions, magnesium, Lithium, potassium and sodium, zinc as well as silicate [37].

Levels of substitution of other biomaterials such as silicate have been found to have a significant effect on the biological response both in vitro and in vivo [49,50,51,52,53,54]. Currently, there is a growing body of evidence to suggest that there is an optimal level of 0.8 wt% Si in phase pure silicate substituted apatites which contributes for an optimal

response in both, the activity of bone formation and bone resorbing cells [50, 55, 56, and 57].

Elastin-Like Polymers

Artificial repetitive polypeptides have grown in popularity as a bioinspired alternative to synthetic polymers. The genetically encoded synthesis, monodispersity, potential lack of toxicity, and biocompatibility are attractive features of these biopolymers for biological applications. Elastin-like polypeptides (ELPs) are one such class of biopolymers that are of particular interest because of their ‘‘smart’’—stimuli responsive—properties [58], see figure 10.

Elastin-like Polymers are composites of repetitive artificial polypeptides with the sequence (V-P-G-X-G) V: valine, P: proline, G: glycine, where X could be any guest amino acid expect proline derived from recurred amino-acid sequences from a hydrophobic domain of tropoelastin, which is a soluble precursor of elastin [59,60].

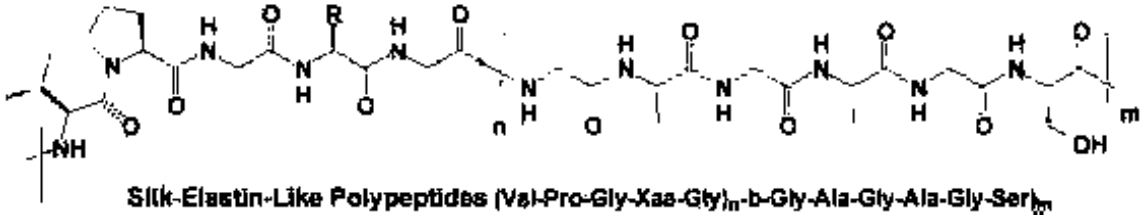

Fig. 10 Sequence of Elastin-like Polymers without any guest-amino acid inserted.

These protein polymers undergo a phase transition in response to changes in temperature. Herein, these polymers exhibit a thermodynamic inverse phase transition, which is completely reversible in aqueous solution at a specific temperature (ITT) Below this phase transition temperature ELPs are highly soluble, nonetheless above ITT, become insoluble and form a coacervate phase. These macromolecular polymers are considered smart materials capable of responding to thermal and optical stimuli [58,61,62], as illustrated in figure 11.

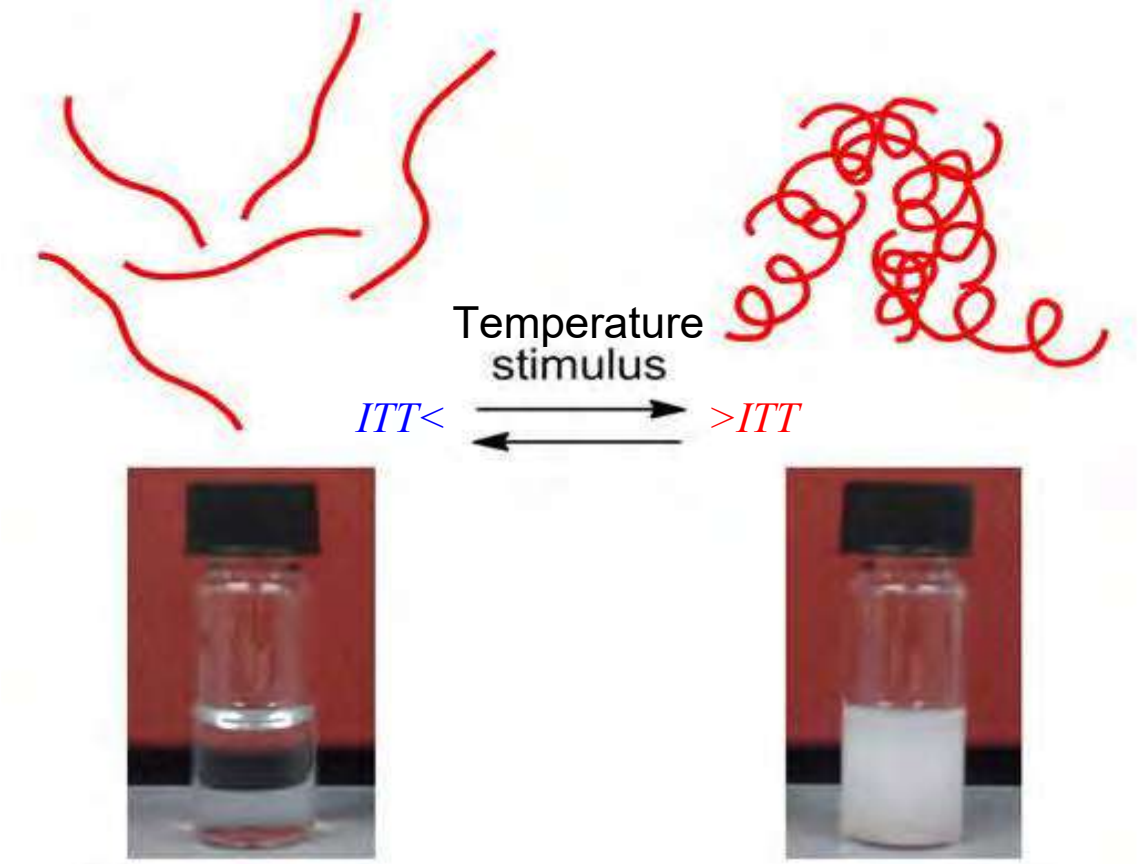

Fig. 11 ***Graphical scheme of Initial Transition Temperature of ELPs where bellow ITT, the ELPs free polymer chains remain disordered in the form of random coils that are fully hydrated, mainly by hydrophobic hydration. This hydration is characterised by ordered clathrate-like water structures surrounding the apolar moieties of the polymer, however, above ITT, the chain folds hydrophobically and assembles to form a phase-separated state where particles aggregates in micelle form.***

Because of their stimulus-response properties, ELP´s can be used as triggered molecular actuators for recombinant protein purification and drug delivery, as well as stimulus-response materials for tissue engineering [58,59,61].

ELP´s are largely used in the formation of scaffolds as they are derived from Extracellular Matrices (ECM), which can provide ECM-like environments for cell growth. Scaffolds for tissue engineering are created mainly via use of three methods [24-28]:

- Coacervation of Soluble ELP: heating the cell-ELP solution to create a viscous liquid. (No gel)
- Physical crosslinking: Physical crosslinking via chain entanglement and hydrophobic effects has been demonstrated with ELP triblocks composed of a central hydrophilic domain capped with hydro- phobic blocks at either end.103 Aggregation of the hydrophobic domains above their ITT creates a hydrogel that is stabilized by physical crosslinks between the hydrophobic domains of the triblock ELP. They posses lack of strength.
- Chemical cross-linking: ELPs with periodic lysine residues were cross-linked into hydrogels by McMillan and Conticello using an electrophilic cross-linker, bis(sulfosuccinimidyl) suberate. Cross-linkers are toxic.

As biologically inspired recombinant materials, ELPs have distinct properties that make them useful for applications in drug delivery. ELPs are biocompatible and are therefore suitable for local and systemic administration, as they induce minimal inflammatory and immune effects in animal models and can be administered to humans without eliciting an adverse immune response. Additionally, the genetically encoded design of ELPs permits exact control over the sequence of the ELP, which can be exploited to precisely specify the location at which a biological drug—peptide or protein—is fused to an ELP, or the location at which a reactive residue is placed for covalent conjugation of the ELP with small molecule drugs. Furthermore, their genetically encoded design leads to perfectly monodisperse polymers. As MW influences important biological parameters, like circulation clearance, this monodispersity allows improved prediction of the ELP behavior *in vivo*, as compared to alternative polydisperse materials [63].

The tunable physicochemical properties and good biocompatibility of ELPs have led to extensive biological applications as triggered molecular actuators for recombinant protein purification and drug delivery, and as stimuli-responsive materials for tissue engineering. Such applications include recombinant protein purification, drug delivery, systemic delivery of soluble carriers and local delivery [64].

Biomimetic hybrid materials based on a polymeric and an inorganic component such as calcium phosphate are potentially useful for bone repair, such materials include:

- Biomimetic Calcium Phosphate Mineralization with Multifunctional Elastin-Like Recombinamers (HAP)
- Recombinant RGD-containing protein polymer (RGDS)
- Recombinant lysine rich-protein polymer (IK)

Biomimetic Calcium Phosphate Mineralization with Multifunctional Elastin-Like Recombinamers (HAP)

Biomimetic hybrid materials based on a polymeric and an inorganic component such as calcium phosphate are potentially useful for bone repair, here, it is reported the use of a Recombinant Statherin-Containing Protein Polymer (HAP) which is the term of a triblock-structured recombinamer containing elements from elastin, (*an elastic structural protein*), and statherin, (*a salivary protein*) which have shown a very efficient control of the Calcium Phosphate formation, yielding spherical hydroxyapatite (HA) nanoparticles with diameters from 1 to 3 nm after 1 week in SBF at 37ºC.

The data thus suggest that ELRs containing statherin segments (HAP) and the selection of an appropriate polymer structure are key parameters to obtain functional materials for the development of intelligent systems for hard tissue engineering and subsequent in vivo applications [65,66].

Recombinant RGD-containing protein polymer (RGDS)

RGDS is a novel thermo-responsive Elastin-like Polypeptide containing cell-binding epitope (Arg-Gly-Asp-Ser sequence); arginine-glycine-aspartic acid-serine (RGDS). Is a sequence of amino acid that promotes cell adhesion and spreading. Also, in 1984, Pierschbacher and Ruoslahti found that RGDS sequence is the principal adhesive site of Fibronecting (FN), which is a predominant Extra Cellular Matrix (ECM) protein that

mediates the adhesion, and spreading of many cell types. It was also proved that the RGDS sequence specifically binds to integrin receptors that are present on cell surface [67].

Recombinant lysine rich-protein polymer (IK)

The monomer unit contains two different functional blocks in order to achieve an adequate balance of mechanical and bioactive response. The VPGIG sequence confers the mechanical properties (similar to the natural elastin), the biocompatibility and the stimuli-responsive nature. The second building block VPGKG is a modification of the first, containing lysine, so that the lysine ε- amino groups can be used for crosslinking purposes and other chemical modifications [68].

ELR	ELR Sequence (Bioactive sequence)	Bioactivity
HAP	**[[(VPGIG)$_2$(VPGKG)(VPGIG)$_2$)$_2$ DDDEEKFLRRIGRFG [(VPGIG)$_2$(VPGKG)(VPGIG)$_2$]$_2$]$_6$**	**Mineralization**
RGDS	**[[(VPGIG)$_2$(VPGKG)(VPGIG)$_2$)$_2$AVTGRGDSPASS[(VPGIG)$_2$(VPGKG)(VPGIG)$_2$]$_2$]$_6$**	**Cell Adhesion**
IK	**(VPGIG VPGIG VPGKG VPGIG VPGIG)$_{24}$**	**Control**

Table 1. Sequence and functionality (bioactivity) of ELP, modified from [68].

1.1. Problem statement

The search for alternative therapies to improve bone regeneration continues to be a major challenge for the medical community, an important challenge in tissue engineering and regenerative medicine is the design of novel scaffolds for cell adhesion, spreading and proliferation that mimic the natural extra-cellular matrix (ECM). As previously described, the current therapies for bone regeneration continue to be a non-stimuli and well-established materials for bone repair, which is why it is important to develop bone grafts capable to work as enhancers of cell growth and adhesion, as well as possessing biocompatibility and behave as stimulators of bone repair.

Initially, a literature search was performed to assess the technological aspects and research covering the materials previously mentioned. No publications were found regarding the development of hydrogels combining HA with ELPs, furthermore, no publication was found mentioning the use of HA for the development of a hydrogel. However, the preparation of hydrogels using Elastin like recombinants combined with other substances were analysed, and their methods of preparation were used as a guide for the developing of a hydrogel based on HA and ELPs.

1.2. Hypothesis

Once analysed the biological response of these two biomaterials (ELPs and HA), each one with their respective advantages and disadvantages, it might be possible to create a new biomaterial for the development of a 3D scaffold, combining the advantages that both HA and ELPs possess, whether possible in the form of hydrogel, capable of providing an organic and or inorganic environment for bone regeneration therapies.

1.3. Objectives and expected contributions

1.3.1. General Objective

To create a new biomaterial in the form of 3D scaffold, specifically a hydrogel, based on HA and ELP, capable of providing an organic/inorganic environment for bone regeneration therapies.

1.3.2. Specific objectives

- To standardise the synthesis for the hydrogel creation with the most optimum properties

- To visualise the morphology of hydrogel under Scanning Electron Microscope
- To obtain the Zeta potential of Elastin like polymers and Hydroxyapatite to analyse the mechanism attraction of these two biomaterials
- To make an FTIR characterisation to confirm the presence of ELPs and HA after hydrogel synthesis.
- To make a cell culture of human mesenchymal stem cells for the analysis of biocompatibility of hydrogels.

Chapter 2 Development of hydrogel based on Elastin like polymers and hydroxyapatite

2.1. Introduction

2.1.1. Silicate substituted hydroxyapatite 0.8%wt

To this end, Karin et al performed some experiments where HA and SA porous discs were created to identify whether protein absorption layer is abolished in the presence of porous, giving a result that porosity encourage the development of this layer in a faster way within SA discs than HA [50]. Furthermore, porous HA and SA scaffolds with no significant variation in porosity level (as-sintered total porosities 72.971.4 and 73.474.3%; strut porosities 22.272.3 and 23.974.9%) were produced using a novel slip foaming technique [24] and were implanted in rabbits demonstrating that those who contain Si substituted with 0.8 wt% showed a higher bone growth regeneration.

2.1.2. ELPs in tissue engineering

As previously described, ELP's are becoming a well class of biomaterial for bone regeneration. Works performed Here we report on the enhanced mineralization, osteogenesis, and in vivo bone regeneration properties of a bioactive elastin-like recombinamer (ELR) membrane. To this end, three bioactive ELRs exhibiting epitopes designed to promote mesenchymal stem cell adhesion with the use of ELP (RGDS), for mineralization ELP (HAP), and both cell adhesion and mineralization ELP (RGDS-HAP). These ELP's were synthesized using standard recombinant protein techniques. The ELPs materials were then used to fabricate membranes comprising either a smooth surface (smooth) or channel microtopographies (channels). Mineralization and osteo-blastic differentiation of primary rat mesenchymal stem cells (rMSCs) were analysed in both static and dynamic conditions (uniaxial strain of 8% at 1 Hz frequency). Smooth mineralization membranes in static condition exhibited the highest quantity of calcium phosphate (Ca/P of 1.78) deposition with and without the presence of cells, the highest

Young's modulus, and the highest production of alkaline phosphatase on day 10 in the presence of cells growing in non-osteogenic differentiation medium. These membranes were tested in a 5 mm-diameter critical-size rat calvarial defect model and analyzed for bone formation on day 36 after implantation. Animals treated with the mineralization membranes exhibited the highest bone volume within the defect as measured by micro-computed tomography and histology with no significant increase in inflammation. This study demonstrates the possibility of using bioactive ELR membranes for bone regeneration applications [69-71].

Having performed the search in the literature besides getting the opportunity to work with this biomaterial, it can be concluded that 0.8wt% is the optimal level of silicate substitution of HA, allowing us to use this apatite for the creation of a new biomaterial based on hydroxyapatite and Elastin like polymers, using the ELP more specifically termed as HAP.

2.2. Materials and methods

For the creation of gels the following materials were used:

Hydroxyapatite (HA)

- Silicate substitute apatite (0.8 wt% Si).

Elastin like polypeptides (ELPs)

- Recombinant statherin-containing protein polymer (HAP).
- Recombinant lysine rich protein polymer (IK) as a control.
- Recombinant RGD containing protein polymer (RGDS).

2.3. Synthesis of hydrogel

An assay, varying the temperatures and stirring intervals to mix these two substances (HA and ELP's) within the aqueous medium such as Ultrapure MiliQ-Water and Phosphate buffered Saline (PBS) and Cell Culture Media was performed. The objective of this was to see whether certain reactions would be observed. After numerous attempts performed, at a certain concentration of HA (50mg/ml) and ELP (100mg/ml), temperature (37ºC for 3 minutes) and method of stirring (200 RPM for 1 minute) and pH of 7.5, the creation a new hydrogel was achievable. This method was named "preliminary gel process"

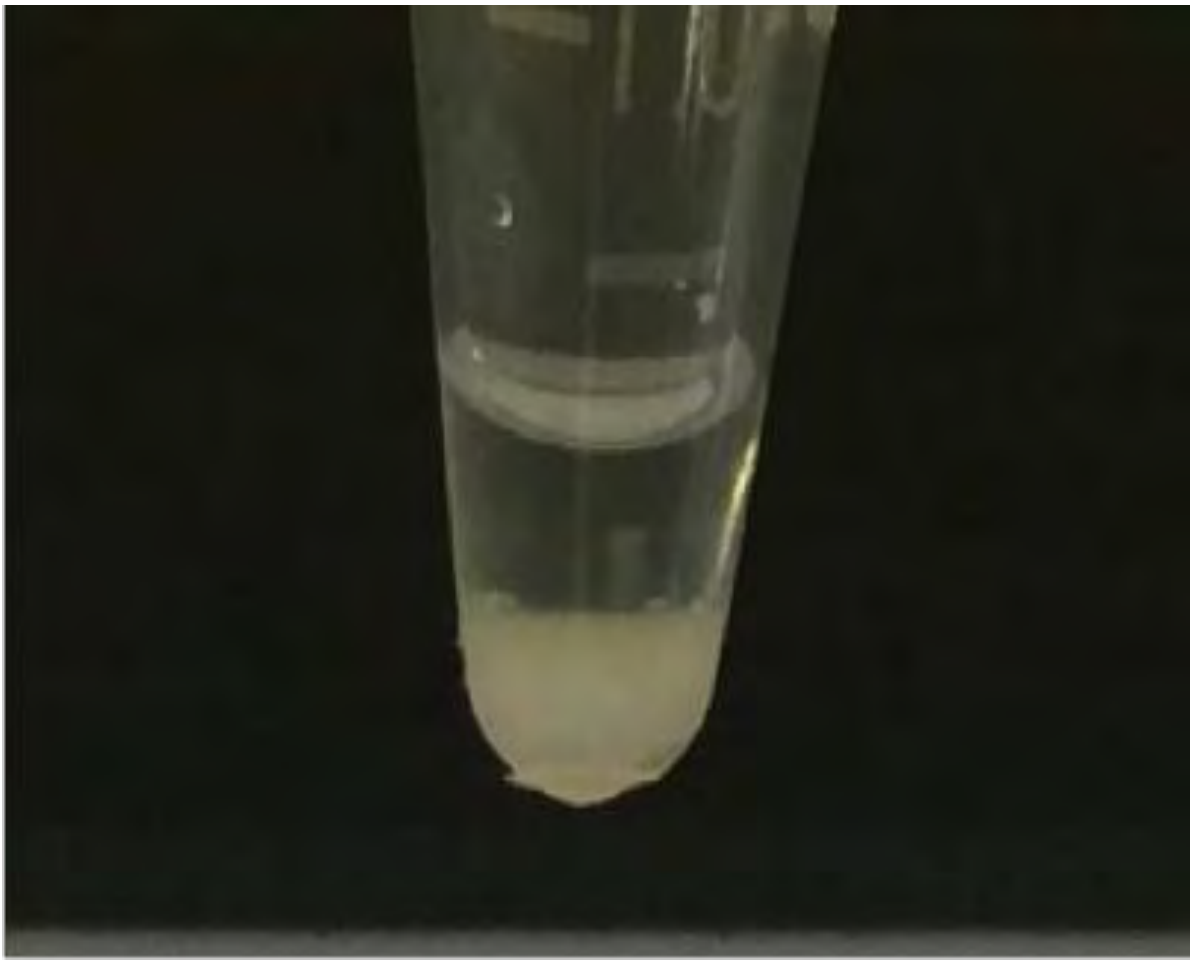

Fig. 12 Hydrogel (white) created on the basis of Hydroxyapatite and Elastin like polymers.

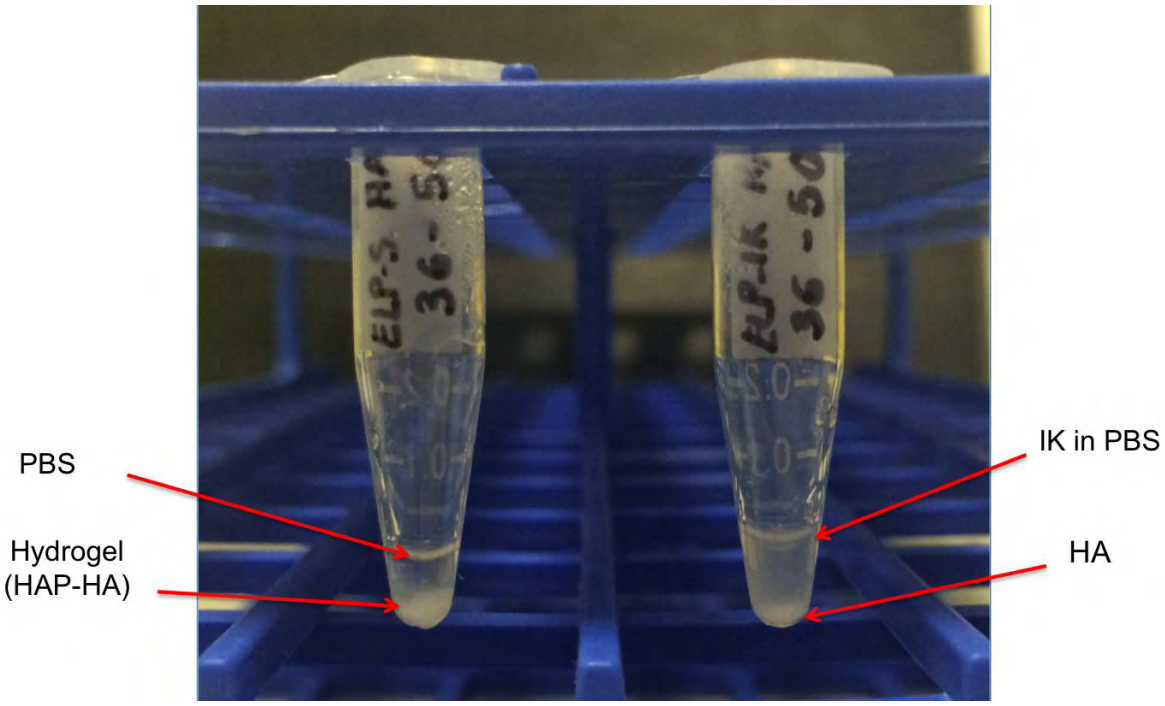

Fig. 13 A hydrogel (left) created with ELP-S and HA. As a control, ELP-IK with HA was used (right). In the control, hydrogel was not possible to be created.

The following chart represent the concentrations used for the synthesis of hydrogels:

HA / ELP-S	0	15	25	50	75	100	200
0							
25							
50							
100							

Table 2. Solutions based on hydroxyapatite and Elastin like polymers using different concentrations of HA as well as ELP. Hydrogels formation is represented in green and non-reaction solutions (no hydrogels) are represented in red. Concentrations are in mg/ml.

2.4. Standardisation for hydrogel synthesis process

An essay for the standardization of the process for the preparation of hydrogel was performed. Taking the "preliminary gel process as a starting point" all variables present during the synthesis of hydrogel was considered to improve the stiffness, quality and to analyse where it was possible or not to create a larger amount of gel with less material. Said variables considered within this process are represented in the following diagram: a) Mix of materials, b)temperature, c)stirring, d)Incubation for stabilization, e)Time of stirring, f) Time of incubation, g)Time of incubation for stabilization, as shown in figure 14.

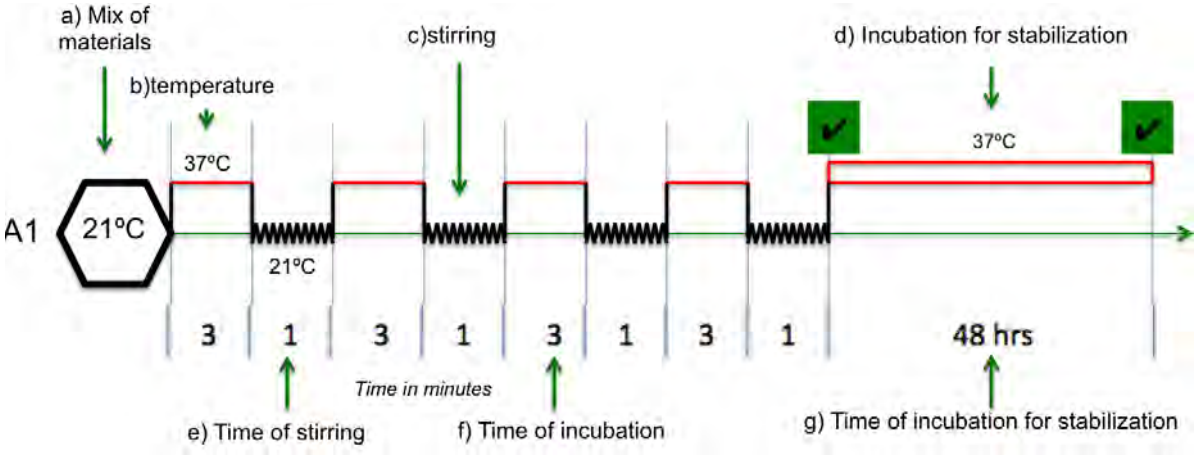

Fig. 14 Diagram for hydrogel synthesis process, with the different variables. A)mix of materials, b) temperature (above ITT red, bellow ITT black), c)stirring, d) incubation for stabilisation, e) time of stirring, f) time of incubation and g) time of incubation

Essay was performed and divided in "static conditions" as well as dynamic conditions". In regard to "static conditions", stirring and temperature of incubation are worked separately as far as in "dynamic conditions" were worked at the same time. Variables such as time of stirring, time of incubation and time of incubation for stabilization remained constantly as well as the concentrations of ELP and HA, (100mg/ml and 75mg/ml respectively) whereas temperature, stirring and the temperature of materials mixing were not worked constantly. The essay was performed at room temperature (21ºC) unless otherwise stated:

Static conditions

A1) ELP and HA were mixed in PBS, being stirred for 3 minutes, the solution was then incubated at 37ºC for 1 minute and stirred again at room temperature. The process was repeated five times for a better interaction between ELP and HA. Gel was observed at the end of the process of stirring and incubating (green tick). Finally, the gel was then placed at 37ºC for 48hrs for stabilization. Gel formation was not affected after incubation for stabilization (green tick).

A2) ELP and HA were mixed in PBS being stirred for 3 minutes, the solution was then incubated at room temperature for 1 minute and stirred again at the same temperature. The process was repeated five times for a better interaction between ELP and HA. Gel was not observed at the end of the process of stirring and incubation. Gel formation was not observed at the end of stirring and incubation. Finally, the solution was then placed at 37ºC for 48 hours for stabilization. Gel formation was observed after 48 hours of incubation (green tick), nonetheless it was not stable.

A3) ELP and HA were mixed in PBS at 37ºC, being stirred for 3 minutes, the solution was then incubated at the same temperature (37ºC) for 1 minute and stirred again at 37ºC. The process was repeated five times for a better interaction between ELP and HA. Gel formation was not observed at the end of the process of stirring and incubating. At the end, the solution was then placed at 37ºC for 48hrs for stabilization. Gel formation was observed after 48 hours of incubation (green tick), nonetheless it was not stable.

A4)) ELP and HA were mixed in PBS at 37ºC, being stirred for 3 minutes, the solution was then incubated at room temperature for 1 minute and stirred again at 37ºC. The process was repeated five times for a better interaction between ELP and HA. Gel formation was observed at the end of the process of stirring and incubating (green tick). At the end, the solution was then placed at 37ºC for 48hrs for stabilization. Gel formation was affected after 48 hours of incubation resulting in a non-stable gel, as shown in figure 15.

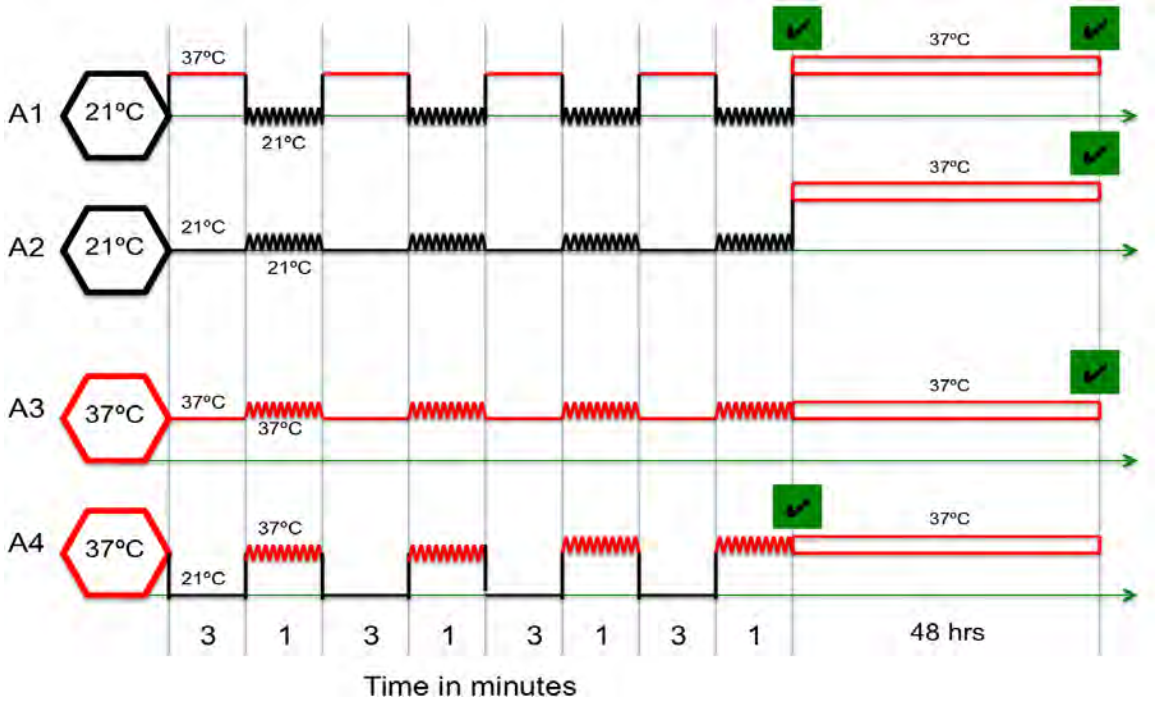

Fig. 15 ***Diagrams of hydrogel synthesis under "Static Conditions" where temperature of stirring is bellow ITT whereas temperature of incubation is above (A1), temperature of stirring and incubation are bellow ITT (A2), Temperature of stirring and incubation above ITT (A3) and lastly, temperature of incubation remained bellow ITT whereas temperature of stirring was worked above ITT (A4).***

Dynamic Conditions

Previously, stirring and incubation variables were worked separately. In this case, for dynamic conditions, said variables were worked simultaneously as follows:

B1) ELP and HA were mixed in PBS at room temperature. The solution was then stirred and incubated at 37ºC at the same time for four minutes. Gel was observed at the end of the process of stirring and incubating (green tick). Finally, the gel was then placed at 37ºC for 48hrs for stabilization. Gel formation was not affected after incubation for stabilization (green tick).

B2) ELP and HA were mixed in PBS at room temperature. The solution was then stirred and incubated at 21ºC at the same time for four minutes. Gel was not observed at the end of the process of stirring and incubating. Finally, the gel was then placed at 37ºC for

48hrs for stabilization. Gel formation was observed after 48 hours of incubation resulting in a non-stable gel.

B3) ELP and HA were mixed in PBS at 37ºC. The solution was then stirred and incubated at 37ºC at the same time for four minutes. Gel formation was not observed at the end of the process of stirring and incubating. The solution remained at 37ºC for 48hrs for stabilization. Gel formation was observed after 48 hours of incubation resulting in a non-stable gel.

B3) ELP and HA were mixed in PBS at room temperature. The solution was then stirred and incubated at the same temperature at the same time for four minutes. Gel formation was observed at the end of the process of stirring and incubating. The solution was then incubated at 37ºC for 48hrs for stabilization. Gel formation was affected after 48 hours of incubation resulting in a non-stable gel.

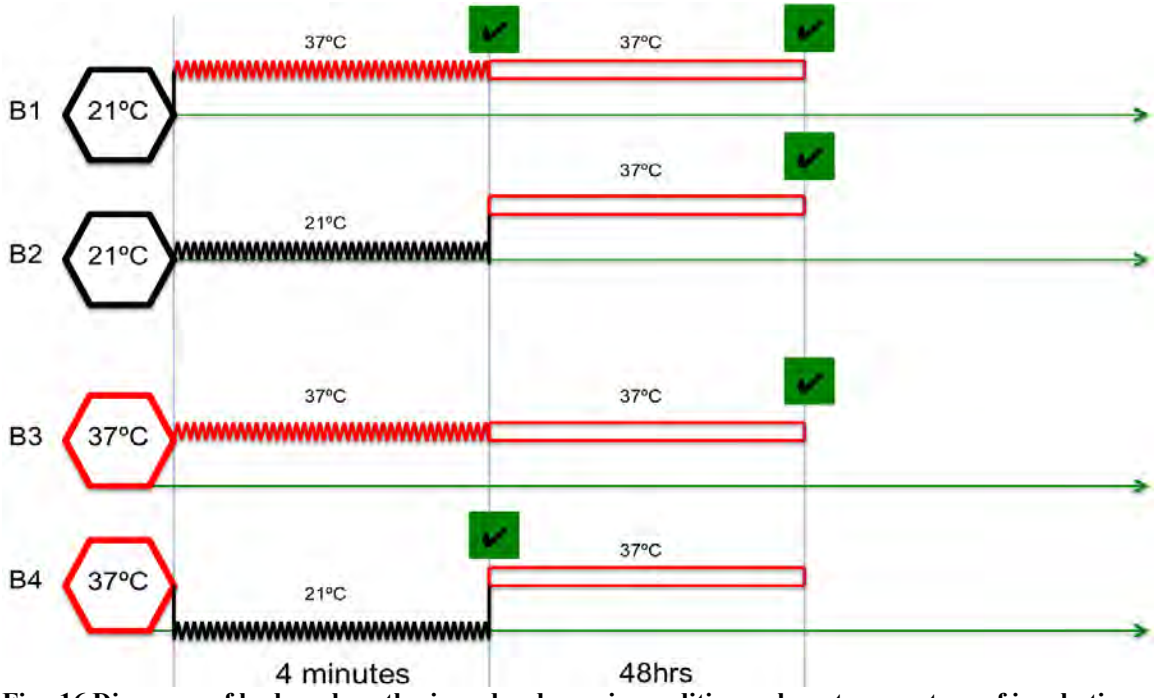

Fig. 16 Diagrams of hydrogel synthesis under dynamic conditions where temperature of incubation and stirring are above the ITT (B1) and (B3) with mixing temperature of materials bellow and above respectively, whereas (B2) and (B4) remained a temperature of stirring and incubation bellow ITT.

According to the experiments previously performed, the best processes for hydrogels characterisation tuned out to be procedures A1 and B1, nonetheless, B1 represent a

technician drawback (difficulty to stir and incubate at the same time) compared to A1, therefore, A1 remained as the most optimal process for hydrogel synthesis, where final steps are represented in the following diagram:

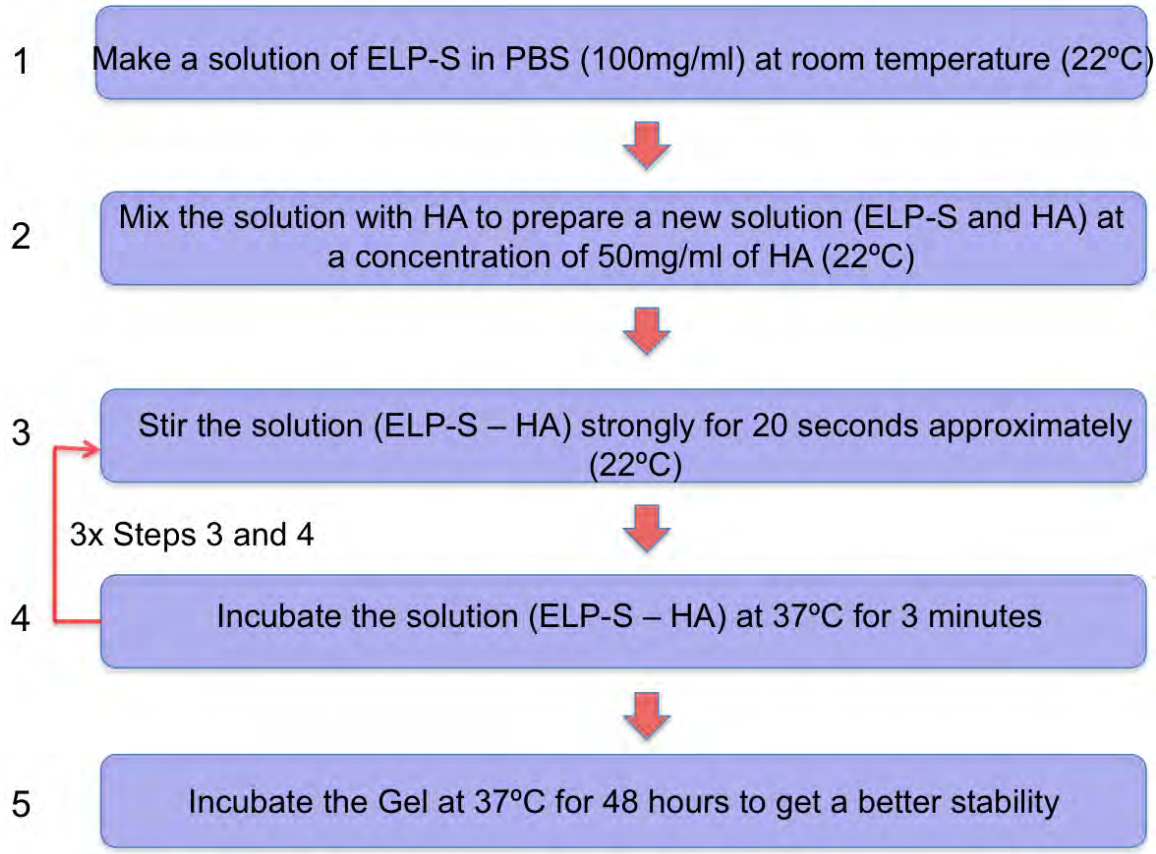

Fig. 17 Diagram representing the most optimal steps for the hydrogels synthesis.

Chapter 3 Characterization of hydrogel

3.1. Visualisation of hydrogel morphology under Scanning Electron Microscope (SEM)

The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of

materials making up the sample. In most applications, data are collected over a selected area of the surface of the sample, and a 2-dimensional image is generated that displays spatial variations in these properties. Areas ranging from approximately 1 cm to 5 microns in width can be imaged in a scanning mode using conventional SEM techniques (magnification ranging from 20X to approximately 30,000X, spatial resolution of 50 to 100 nm). The SEM is also capable of performing analyses of selected point locations on the sample; this approach is especially useful in qualitatively or semi-quantitatively determining chemical compositions (using EDS), crystalline structure, and crystal orientations (using EBSD). The design and function of the SEM is very similar to the EPMA and considerable overlap in capabilities exists between the two instruments.

Morphology of hydrogels and controls was studied using FEI Inspect F-50 Scanning Electron Microscope (London, U.K). Hydrogels were fixed in 2.5% glutaraldehyde in 0.1 M PB (pH 7.4) at room temperature for 2 h with shaking, followed by a 10 min wash in 0.1 M PB, at room temperature for 30 min. Samples were then dehydrated in 50, 70, 90, 95 and 100% ethanol for 30 minutes each. Finally, hydrogels were critical point-dried (CPD-030, EMS 850, London, UK), mounted on SEM-holders and gold sputtered (EMITECH, SC7620, London, UK). Hydrogels were then observed using an FEG scanning electron microscope at 10kV. Images were recorded using a secondary electron (SE)-detector with the voltage of the collector grid biased to +300 V in order to improve the signal-to-noise ratio and to reveal optimal topographical contrast.

Scanning electron microscopic analysis of ELP-HA hydrogels showed a different morphology at different concentrations of HA. Hydrogels were prepared on the basis of three types of elastin like recombinants (HAP, RGDS and IK) maintaining a concentration of 100mg/ml of ELPs. Hydroxyapatite varied mainly at 4 different concentrations: 25, 50, 75 and 100mg/ml.

Four samples of hydrogels based on HAP were prepared maintaining the concentrations of ELP and HA as previously described (Figure 18, 1a,1b,1,c,1d). SEM showed that HAP-HA hydrogels at 25mg/ml HA presented a plate or step-like feature with null

porosity (1a), the presence of porosity can be observed at concentrations of 50 and 75mg/ml showing a mayor dense of porosity at 75mg/ml (figure 18, 1b and 1c) whereas a spherical or slightly ellipsoidal morphology can be observed at a concentration of 100mg/ml of HA, 1:1 ELP-HA (figure 18, 1d).

Hydrogels based on RGDS presented both, spherical and micro-fibrous morphology. Spherical and micro-aggregates can be observed at 25mg/ml concentration (figure 18, 2a), nonetheless low-density spherical morphology can be observed at 50mg/ml (figure 18, 2b) whereas nanofibrous patterns are observed at 75mg/ml (figure 18, 2c), lastly, spherical or slightly ellipsoidal morphology can be observed at a concentration of 100mg/ml of HA, 1:1 RGDS-HA (figure18, 2d).

In the case of controls based on IK and HA, the formation of gels could not be observed. However it was possible to observe a plate or step-like feature with null porosity at 25mg/ml (figure 18, 3a), a low density porosity with flat morphology could be observed at 50mg/ml (figure 3b) whereas spherical patterns (possibly HA particles) attached to a smooth- flat morphology (figure 18, 3c), as for the samples 1:1 IK-HA, a granulated shape is observed with wrinkled aspect, no porosity nor fibrous structure could not be observed (figure18, 3d).

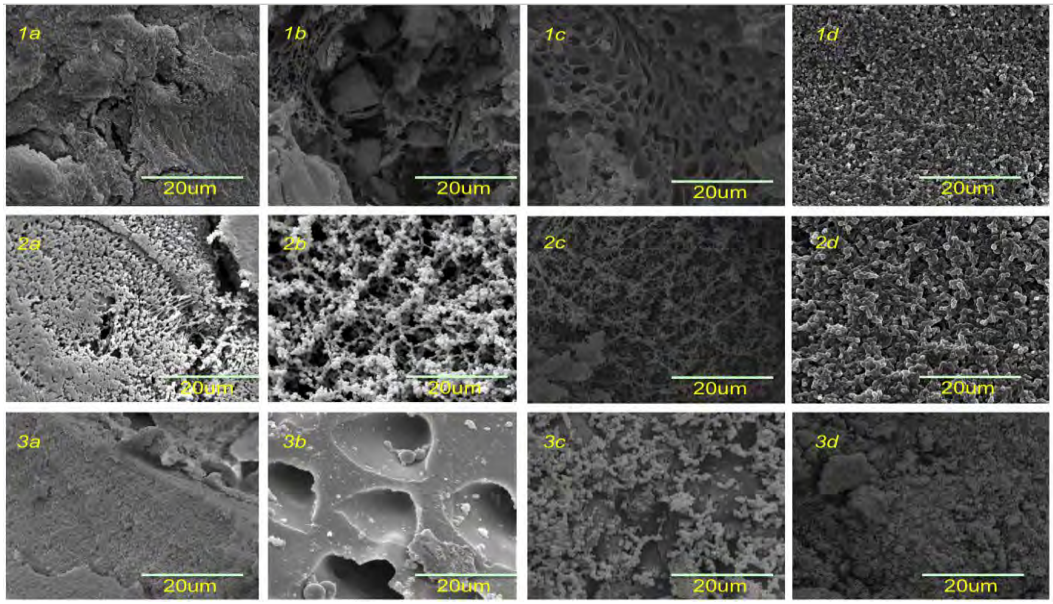

Fig. 18 Electron micrograph of hydrogels, (1) HAP-HA, (2) RGDS-HA, (3) IK-HA with HA concentrations at 25, 50, 75, and 100mg/ml (a,b,c,d) respectively.

It is well know that porosity helps to the development of Extracellular matrix as well as the attachment of cells, due to this, besides the high porosity density showed within HAP-HA at HA concentrations of 75mg/ml, it was decided to work in the synthesis of hydrogels at this concentration of hydroxyapatite in the hope of improve not just the mechanical properties oh hydrogel, but also its biocompatibility.

3.2. Zeta Potential of Elastin like polymers and Hydroxiapatite.

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles, and is one of the fundamental parameters known to affect stability. Its measurement brings detailed insight into the causes of dispersion, aggregation or flocculation, and can be applied to improve the formulation of dispersions, emulsions and suspensions [72].

To evaluate the surface charge of ELP nanoparticles, zeta potential of ELPs was measured using a Malvern Zetasizer 2000 (London, UK). ELPs were dissolved in water at pH 7.5 at 0.1% (W/W) concentrations and passed through a 20nm membrane filters to be introduced into a disposable folder capillary cell DTS1070. Measurements were

carried out at temperatures of 37ªC and 10ªC to obtain the charge above and below the ITT. The experiment was performed in triplicate for reproducibility and results presented as mean in +/- SD, (see fig. 19).

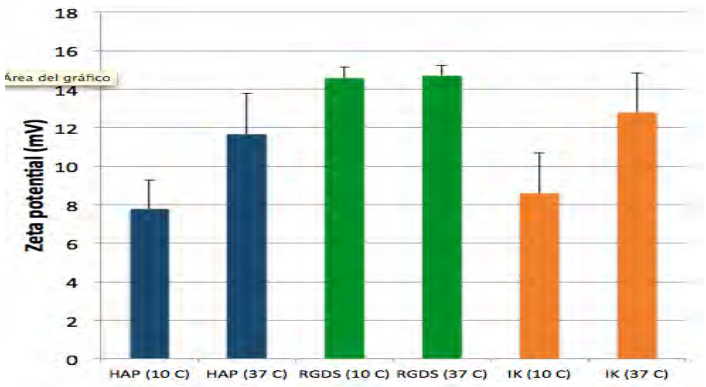

Fig. 19 ZP measurements of ELPs (HAP, RGDS, IK) at 0.1% concentration in PBS at pH 7.5 and temperatures of 10 C and 37 C. Data reported as mean +/- SD, n=3.

It is well know that Ionic attraction is involved in the synthesis of hydrogels, to this end, Elastin-Like Recombinants and Hydroxyapatite ought to contain either a positive and negative charge respect to the material in which is to be combined, based on this, zeta potential measurements showed that ELPs were positively charged at hydrogel creation conditions. Charge present in all ELPs were significantly greater above the ITT, more specifically in HAP and IK, (HAP-11.68mV, IK-12.79mV) compared to bellow ITT, (HAP-7.781mV, IK-8.587mV) whereas in RGDS there is a slightly difference of 0.1178 in charges above and below the ITT, showing a result of 14.6868mV and 14.569mV respectively.

Surface charge of HA

According to K.A. Hing [73], "Titrimetric analysis enables direct investigation of the buffering capacity of a surface (i.e. the ability of a surface to soak up H+ and/or OH− ions from solution) at a range of pHs, and has previously been used to determine the Point of Zero Charge (PZC) (the pH at which the simultaneous surface adsorption/desorption of potential determining cations and anions is in equilibrium) of stoichiometric HA as a method to characterise surface charge (and its pH dependency) at

the electrolyte/surface interface and to yield information on the accessibility of chemical groups at the surface of interest (Harding et al., 2005)".

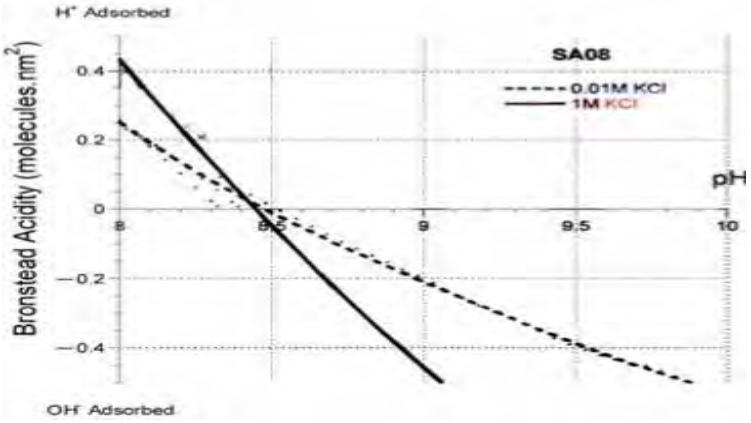

Fig. 20 Bronstead acidity data at the crossing points of phase pure 0.8 wt% of Silicate substituted Hydroxyapatite [73].

In regard to the previous table reference, values bellow PZC which corresponds to a pH=8.4, absorb OH^-, obtaining an HA surface charged negatively at pH of 7.5, the pH conditions in which hydrogels were synthesised.

Herein it is concluded that superficial charges are not responsible for union of ELPs and HA, having as result of positive charges within both surfaces.

3.3. Fourier Transform Infrared Spectroscopy (FTIR)

FT-IR stands for Fourier Transform InfraRed, the preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum. This makes infrared spectroscopy useful for several types of analysis.

Infrared spectroscopy has been a workhorse technique for materials analysis in the laboratory for over seventy years. An infrared spectrum represents a fingerprint of a sample with absorption peaks, which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, infrared is an excellent tool for quantitative analysis.

Thus, the Fourier Transform Infrared (FT-IR) technique has brought significant practical advantages to infrared spectroscopy. It has made possible the development of many new sampling techniques, which were designed to tackle challenging problems, which were impossible, by older technology. It has made the use of infrared analysis virtually limitless.

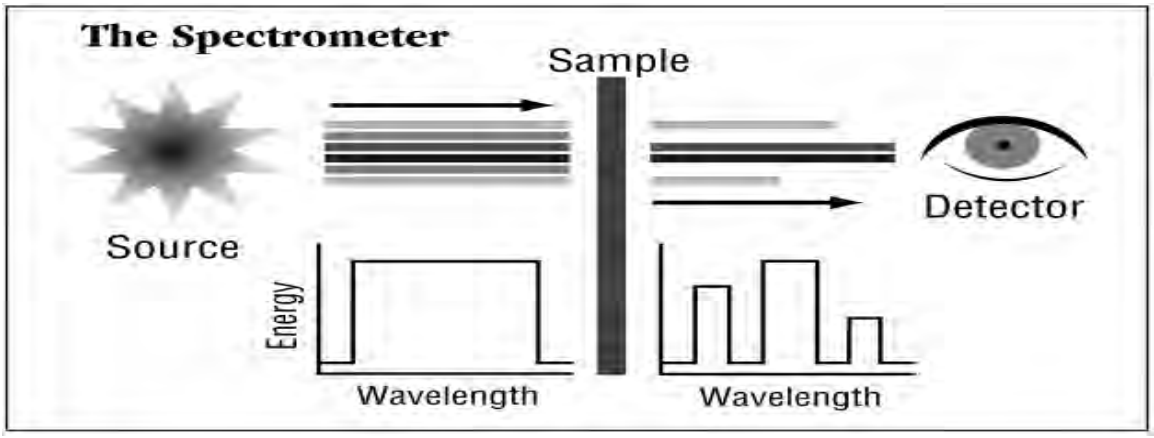

Fig. 21 A simple spectrometer layout.

FTIR was carried out to confirm the presence of ELPs and HA into the gel. For this purpose, Perkin Elmer spectrometer (London, UK) was used. A scanning of background was performed before each experiment and samples were then collected on the aluminium mirror surface and analysed in reflectance mode at room temperature. All spectra were acquired at a resolution of 4 wave number operating between the spectral range of 4000-650cm^{-1}.

As a manner of control, IR spectra of each hydrogel component (ELPs, HA and PBS) were recorded individually and data was then compared from literatures. For the case of ELPs and HA, the analysis was performed in “Solid form” and “Liquid form” (with and without the presence of PBS).

To analyse ELP and HA in solid form, 1mg of ELP fibbers were collected and placed onto the spectrometer as well as 1mg of HA powder.

In the case of liquid form analysis, ELP and HA were dissolved separately in PBS at a concentration of 100mg/ml and 75mg/ml respectively. One millilitre of each solution was then dropped onto the spectrometer for their analysis.

In order to obtain the IR spectra of hydrogels, one millilitre of gel (HAP-HA, RGDS-HA and Control IK-HA) was synthesised and ground the mirror surface for their individual analysis.

Figure 22a shows the IR spectra of controls in solid form. FTIR showed the characteristic broad bands. The characteristic FTIR broad bands of ELR are indicated between the spectra range of 3500 and 2500cm^{-1}. a peak in 3334cm^{-1} can be observed which corresponds to the combination bands of OH and a peak in 3121cm^{-1} corresponding to the stretching modes NH whereas the peak at 2997-2899 and 1457 can be assigned to C-H stretching vibrations. Peaks between the ranges of 1640-1240 showed the characteristic bands for Amid I, Amid II and Amid III. The band at 1640 can be attributed to amid I whereas the peaks at 1533 and 1248 are due to Amid II and Amid III respectively [76]. The IR spectra of HA showed the characteristic bands of OH^- at peaks of 3600-3228cm^{-1} whereas the bands at 3000-2850cm^{-1} can be assigned to absorbed water. The broad band between the spectra range of 1100-960cm^{-1} is due to the PO_4^- ion, characteristic of hydroxyapatite [75].

As for the controls in liquid form, all samples presented an increase in the OH stretch vibration at the broad band between the range of 3500-3120cm^{-1}. nonetheless, the relative ratios of the bands heights associated to the ELPs and HA decreased with combining in water present in PBS (figure 22b),

The FTIR spectra of hydrogels (HAP-HA, RGDS-HA and control IK-HA) are shown in figure c. The IR analysis demonstrated the presence of ELPs. Amid I, Amid II and Amid III are associated as previously described, to the narrow peaks at 1600, 1533 and 1248cm^{-1} respectively. These amide groups were present for gels HAP-HA and RGDS-IK followed synthesis. However, no peaks from these amide groups were observed within the control IK-HA. The FTIR also yielded information regarding PO_4^- ion commonly assigned to hydroxyapatite. This can be easily attributed to the common peak in the 1100-960cm^{-1} region. Nevertheless a bit stronger PO_4^- ion peak at 1051cm^{-1} was observed in Gel HAP-HA, this might be due to the mineralisation property of HAP. Lastly, IR spectra showed a broad band in the peak at 3600-3220cm^{-1} regions, which corresponds to the OH^- stretch vibration [74-76].

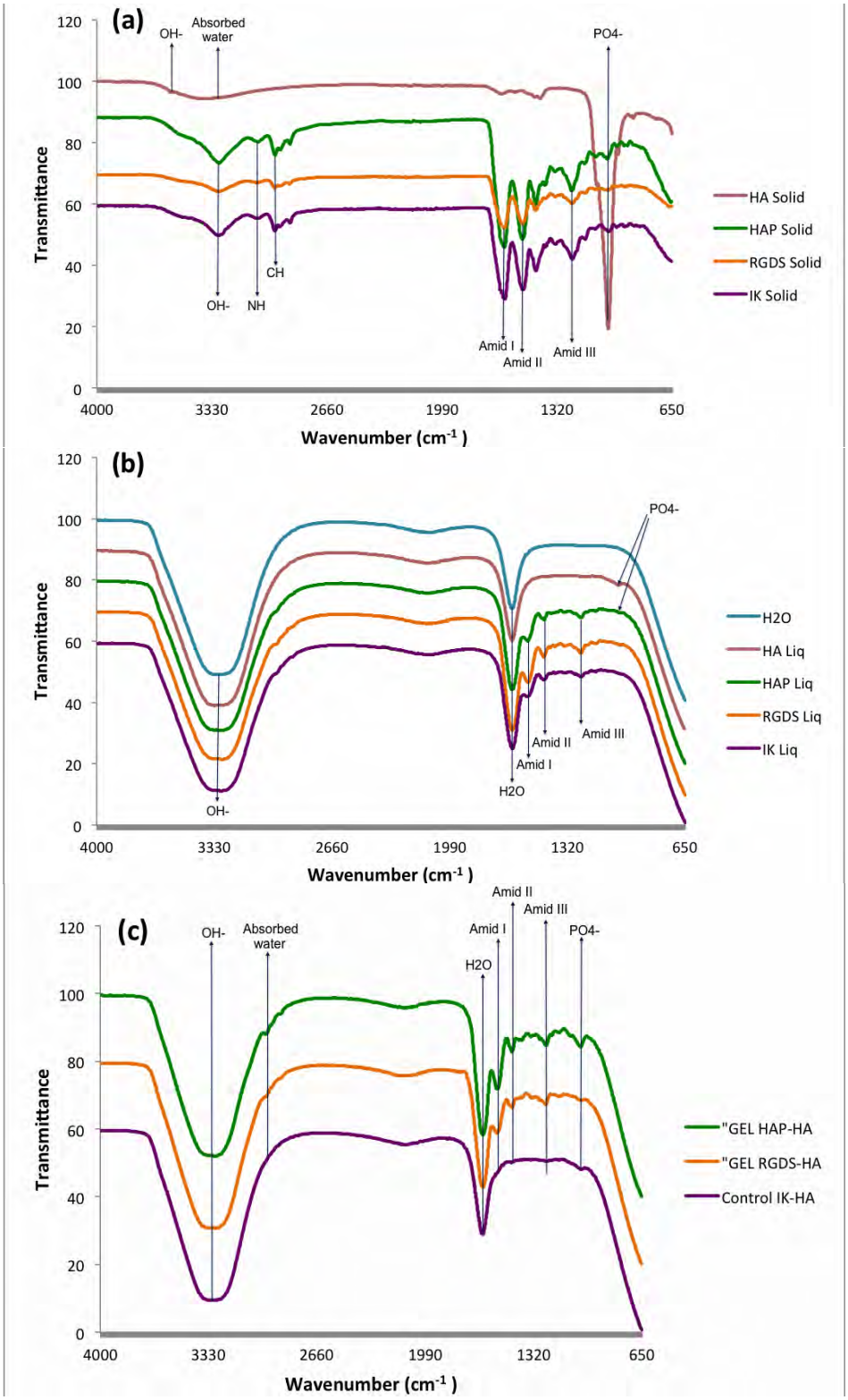

Fig. 22 ***FTIR spectroscopy of hydrogels, control biomaterials in solid state (a), control biomaterials dissolved in PBS (b) and hydrogels (c).***

Chapter 4 Cell culture

4.1. Introduction

Stem cells have the remarkable potential to develop into many different cell different specialized cells in the body. Serving as a sort of repair system for the body, they can theoretically divide without limit to repletion other cells as long as the person or animal is still alive When a stem cell divides, each new cell has the potential to either endure a stem cell or become another type of cell with a more specialized function, such as a muscle cell, or a red blood cell, or a brain cell.

Stem cells have an extensive capacity to proliferate, differentiate & self-renewal, enabling them to repopulate recipients after transplantation.

4.1.1. Human Mesenchymal Stem Cells (hMSC)

The mesenchymal stem cells (MSCs) in the adult bone marrow are necessary for the body to generate tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and bone marrow. Reproducible differentiation of human embryonic stem cells (hESCs) into MSCs does not require the use of any feeder layer. MSC stem cells can be grown for many generations in the laboratory and still retain a stable morphology and normal chromosome complement.

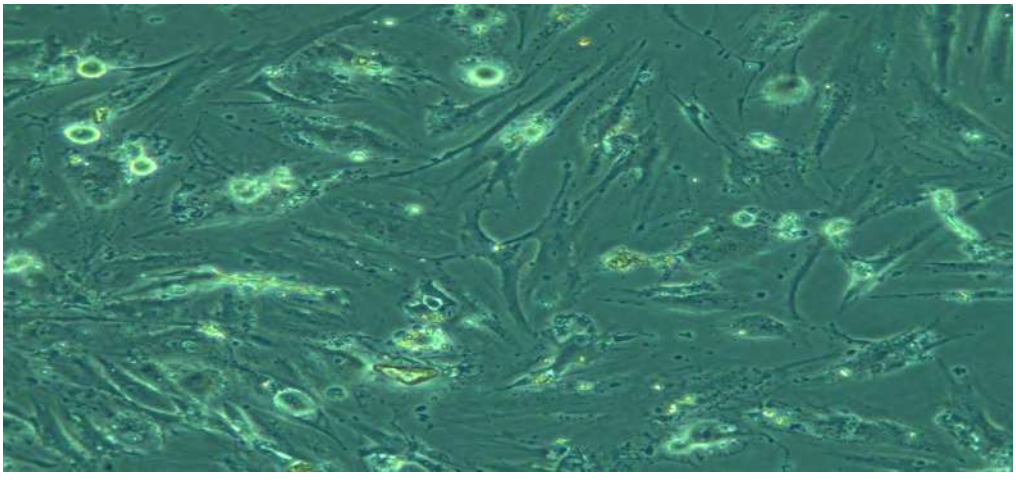

Fig. 23 Photograph oh hMSC taken from culture control of hydrogels.

MSCs are non-haematopoeitic stromal cells that are capable of differentiating into and contribute to the regeneration of mesenchymal tissues like bone, cartilage, muscle, ligament, tendon, and adipose. MSCs are very less in bone marrow, representing 1 in 10,000 nucleated cells. And there is no immortal, they have the ability to expand many fold in culture while retaining their growth and multilineage potential.

Mesenchymal stem cells (MSC) are specialised cells present in bone-marrow stroma and the stroma of various organs with the capacity for mesoderm-like cell differentiation into, osteoblasts, adipocytes, and chondrocytes. MSC are being introduced in the clinic for the treatment of a variety of clinical conditions.

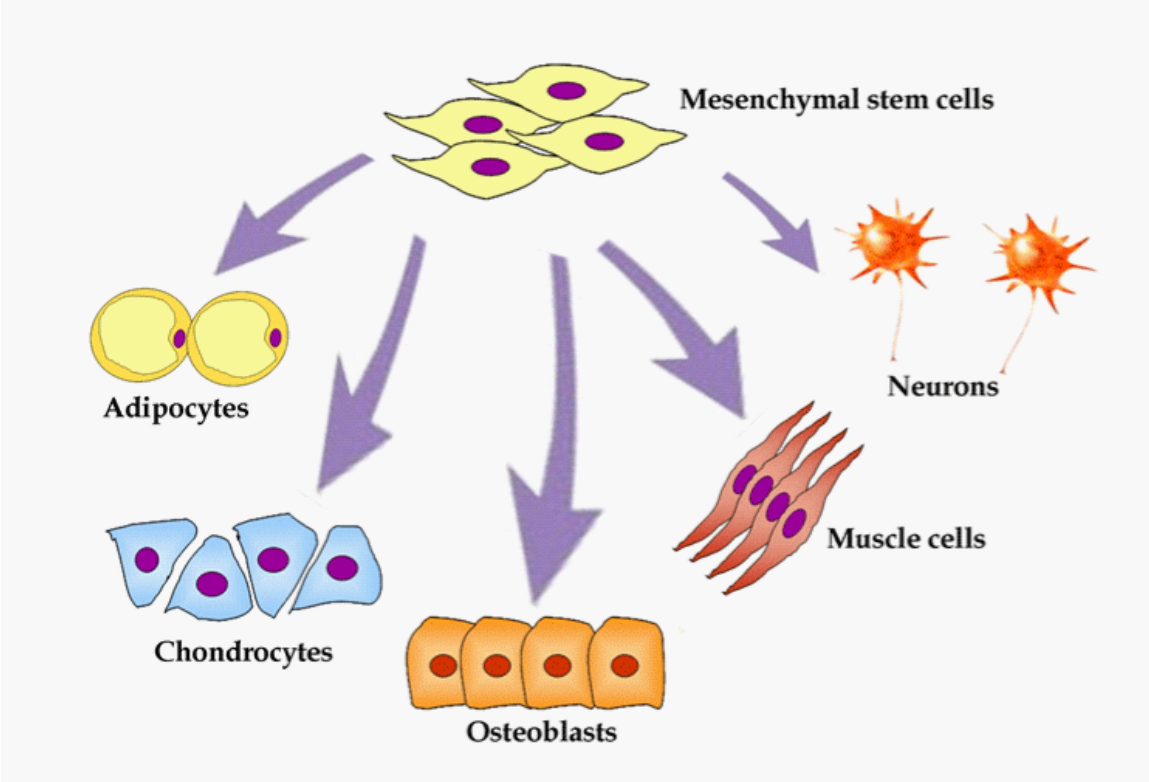

Fig. 24 Scheme of hMSC differentiation, these types of cells are capable to differentiate into adipocytes, chondrocytes, osteoblasts, muscle cells and neurons.

Bone marrow-derived mesenchymal stem cells (BMSCs) are a mostly researched adult stem cell population capable to differentiation into various lineages. Because many promising applications of tissue engineering wants cell expansion following harvest and

involve the treatment of diseases and conditions of old age population, the effect of donor age and ex vivo should be understood to develop clinical techniques and therapeutics based on these cell.

To this end, stem cells as well as any other type of cell require an environment capable of providing stability and nutrients for their growth. Scaffolds made based on biomaterials may provide such environment in order for cells to grow and differentiate adequately. Such scaffolds are required to possess biocompatibility, which is a word that is extensively used within biomaterials science and can also be defined as the ability of a material to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimising the clinically relevant performance of that therapy" (Williams, 2008).

To assess a part of the biocompatibility of hydrogels, gels were created within 4 96mm diameter eppendorf caps. Briefly, 9mg of ELP-HAP and 6.6mg of HA were dissolved in 90ul of PBS to create a gel at a concentration of 100mg/ml of ELP and 75mg/ of HA using the standardized process. Human Mesenchymal Stem Cells (hMSC) were cultivated in the Tissue Laboratory, Queen Mary University of London in Alpha-minimum essential medium (DMEM no FBS + amphotlricin, penicillin and FGF). Cells were seeded at a concentration of $2x10^4$ cells/cm^2 on top of gel. As a control, cells were seeded in a well plate without gel. Caps with the gel and cells on top were set in a 24 well plate. Immediately, plates were left incubated at 37 °C for 4 h in a fully humidified atmosphere at 5% CO_2 in air. After 4 hours, dead cells were removed and caps were covered with 600um of Cell Culture Media and left incubated for 3 days.

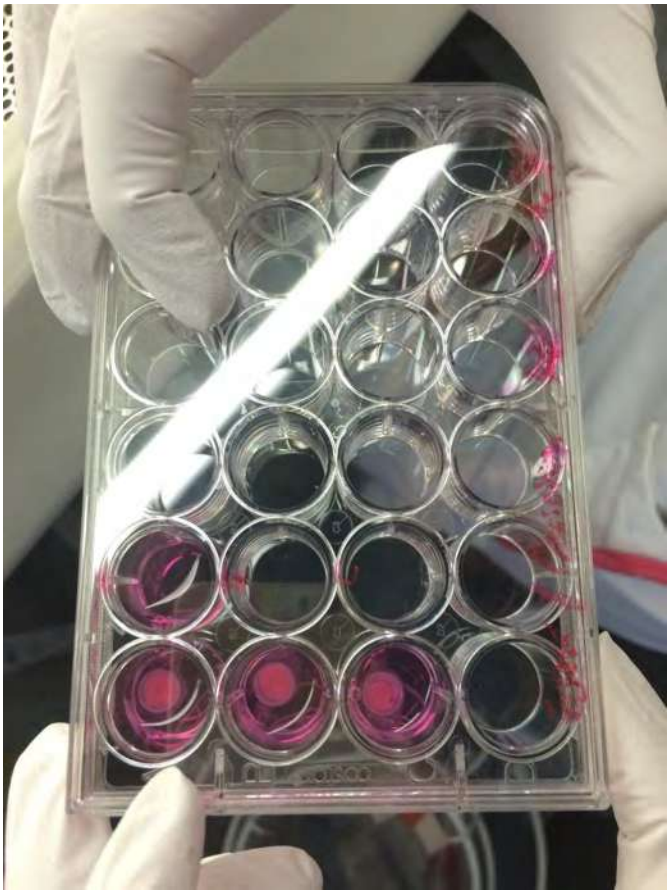

Fig. 25 Photograph of well plates containing hydrogels submerged in Cell-Culture Media for hMCS growth.

4.1.2. Staining, SEM and Optical microscope analysis

For SEM analysis, cells were fixed with glutaraldehyde and washed in Phosphate Buffered (PB) according to the standard protocol. For Optical microscope analysis, cells were washed with PBS warmed at 37ºC and PFA 4% was added in PBS for 30 minutes. Afterwards, cells were washed twice with PBS and Triton X-10 in PBS was added for 5 minutes. Cells were washed three times with PBS to remove Triton and phalloidin FTIC (or Rhodamain) was added for 30 minutes. [1um – 250um for phalloidin or 1ul in 500ul for Rhodamain]. Cells were washed again three times during 5 minutes.

Observations by electron microscopy of hydrogels and human mesenchymal stem cells samples from various points of the material showed the presence of stem cells attached to

the gel throughout the development of filopodes, which is a good signal that cells are establishing contact to the material as well as to other cells surrounding to each other. It can also be observed the development of extracellular matrix (ECM) throughout the cells and material. hydrogels compared in cell-free culture gels. hMCS measured between 7 and µm and 10 µm (see figure 26).

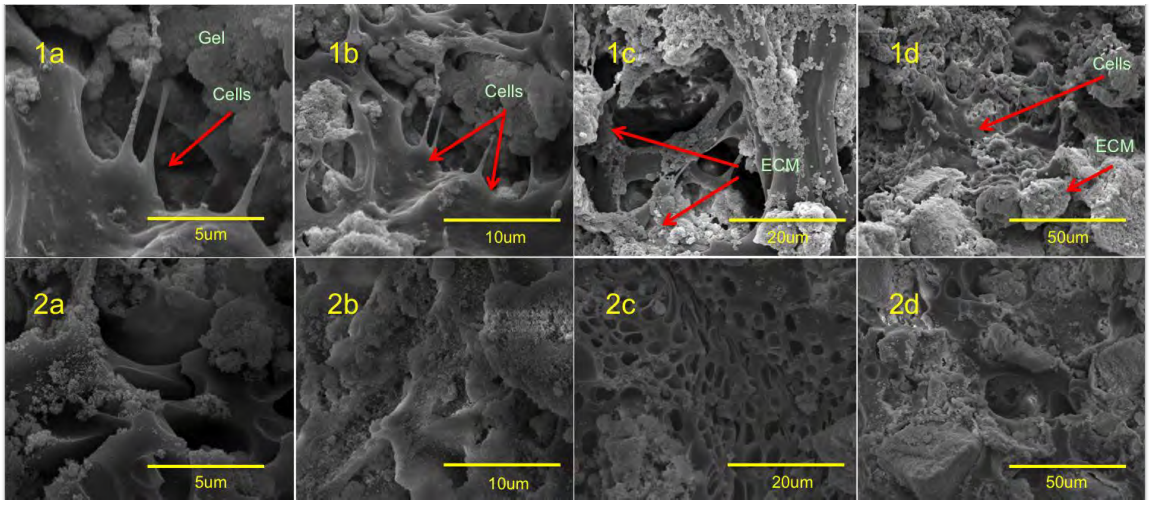

Fig. 26 Electron micrograph of (1) hMSC culture within hydrogels at different resolutions (a,b,c,d) compared to free hMSC culture hydrogels (2) at the same resolutions after 48 hrs of incubation.

Conclusion and Discussion

As previously mentioned, the creation of this hydrogel based on HA and ELPS has been a great achievement not just for the challenge of combining both materials but also because of the combining of their properties. Said properties may include a well-establishment graft for bone regeneration and cell attachment for the case of HA as well as its capability for bone ingrowth and bone coverage in the presence of Silica. In regard to ELP, such properties like temperature stimulus response, mineralization and enhancement of Extracellular Matrix provide a good environment capable of providing cell ingrowth or other possible applications as a mean of transportation of substances for drug delivery therapies. One significant point to highlight is the great stability of the gel presented without the need of cross-linking agents, making it suitable for bone regeneration and biomedical applications without the use of toxic chemicals to improve its stability.

It is clear that much additional work is required to be performed before a complete understanding of the properties of this biomaterial as well as its possible applications; however it is hoped that this study will stimulate further investigations in this work.

To conclude, the development of this new material might be promising for the stimulation of bone regeneration therapies; in the meantime, new methods are being tested in an attempt to create a stronger gel. Furthermore, within the following months further studies will be performed such as characterisation of the new biomaterial as well as some biological tests. It is expected to publish a paper at the end of this work.

Conclusión y Discusión

Como se mencionó previamente, la creación de este hidrogel a base de hidroxiapatita y Polímeros similares a la elastina fue un logro llevado a cabo no solo por el reto de mezclar ambos materiales, sino por la combinación de sus propiedades de estimulación, regeneración y capacidad de crecimiento de tejido que ya están establecidas como en el caso de la hidroxiapatita que se sabe, ya es un material bien establecido para la regeneración ósea, adhesión celular al igual que su capacidad para crecimiento de tejido óseo en presencia de silica. En cuanto a los Polímeros similares a la elastina, se puede destacar sus propiedades de estimulación a la temperatura, mineralización y un mejoramiento de la matriz extracelular, capaz de proveer crecimiento celular u otras aplicaciones como medio de transporte para la liberación de fármacos. Es importante destacar también la gran estabilidad del gel sin la presencia de agentes de entrecruzamiento (químicos tóxicos para mejorar la estabilidad de un material tales como el glutaldeido), haciendo adecuado para la regeneración ósea entre otras aplicaciones biomédicas.

Es sabido que mucho trabajo se requiere llevar a cabo antes de un completo entendimiento de las propiedades de este material al igual que sus posibles aplicaciones, sin embargo se espera que este estudio estimule futuras investigaciones.

Para concluir, el desarrollo de este nuevo material seria prometedor para las terapias de regeneración sin embargo, por el momento, nuevos métodos están siendo desarrollados para crear un gel más fuerte, además, en el corto plazo, se realizaran más estudios tantos de caracterización como de cultivo celular para examinar sus propiedades. Se espera publicar un artículo al final de este trabajo escrito.

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