Purification of Konjac Glucomannan for Tissue Engineering Applications

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*"Success is not the key to happiness.
Happiness is the key to success. If you love
what you are doing, you will be successful."*

Albert Schweitzer

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Abstract

The growing need to develop novel therapies aiming to improve or replace biological functions for the repair of damaged or diseased tissues and organs led to the development of Tissue engineering during the last decades. In recent years, particular attention was dedicated to the development and optimization of the use of natural polymers as a scaffold because of their similarity to extracellular matrix, biodegradability profile, biocompatibility and non-toxicity.

Natural polymers present contaminants, such as foreign proteins, polyphenols and endotoxins among others (metals, ashes). The first step must always be the validation of biomaterials in terms of safety to the host organism to avoid an acute immune reaction and consequently a rejection of the biomaterial by the human body. The work developed under the scope of this thesis is within the framework of an ongoing project that aims to develop multifunctional scaffolds to treat bone cancer. The strategy is the use of Konjac Glucomannan as an injectable carrier of polydopamine nanoparticles to induce hyperthermia at bone tumour sites in future approaches. The main goal of this work is to develop and characterize an effective, reproducible and cost-effective purification protocol for Konjac Glucomannan. Different combinations of filtration, activated charcoal washes dialysis and ultra-centrifugations were tested.Our results showed that all purification methods reduced the contaminants content such as polyphenols and proteins while preserving the structural properties of Konjac Glucomannan. However, the purification protocol including filtrations combined with washings of activated charcoal and ultracentrifugation is the most efficient and cost-effective method for purification of Konjac Glucomannan, while it preserves structural properties and shear-thinning behaviour of the polymer.

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Abbreviation List

3D - Three-Dimensional

ATR-FTIR - Attenuated total reflectance-Fourier-transform infrared spectroscopy

ALP- Alkaline phosphatase

bFGF - Basic fibroblast growth factors

BMPs - Bone morphogenic proteins

BSA - Bovine Serum Albumin

BTE - Bone Tissue Engineering

DA - Dopamine

ECM - Extracellular matrix

GMP – Good manufacturing practice

HA - Hydroxyapatite

^{1}H NMR - Proton Nuclear Magnetic Resonance

IGF - Insulin-like growth factors

KGM - Konjac glucomannan

L-DOPA - 3, 4-dihydroxyphenylalanine

MIH - Magnetically induced hyperthermia

MW – Microwave

NIR - Near-infrared light

OC - Osteocalcin

ON - Osteonectin

OPN – Osteopontin

PDA - Polydopamine

PDGF - Platelet-derived growth factors

PDLLA - Poly-(D,L-lactic acid)

PEG - Poly-ethylene-glycol

PGA - Polyglycolide or poly glycol acid

PLA - Polylactide

PLGA - Poly-lactide-co-glycolide

RF - radiofrequency

TE – Tissue engineering

TGF-β - Transforming growth factor-beta

VEGFR - Receptor for vascular endothelial growth factor

US - Ultrasounds

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Introduction

1. Tissue Engineering

Tissue Engineering (TE) is a growing field of research that has emerged due to the limitations of traditional treatments, such as organ/tissue transplantation and surgery [1]. Nowadays, tissue engineering has been defined as a multidisciplinary field that encompasses engineering principles and the biological sciences. The main goal of TE is the manufacture of biological substitutes to restore, replace or regenerate the natural tissue form and function. The main components of tissue engineering are biomaterials, cells and bioactive molecules (Figure 1) [2].
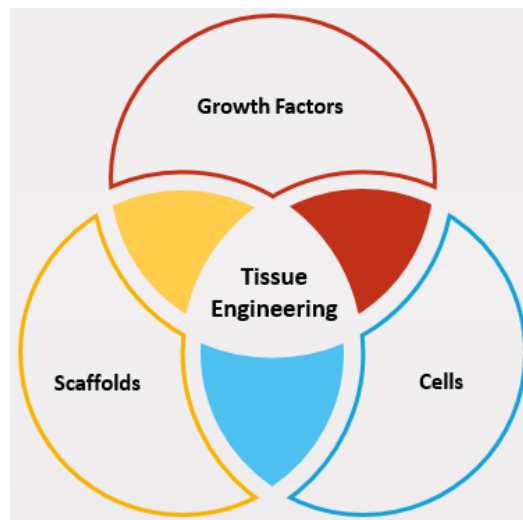
Figure 1- Basic Tissue Engineering strategy. Main components of Tissue Engineering: growth factors, scaffolds and cells. Adapted from [3].

TE strategies can be divided in two main approaches: scaffold-free and scaffold-based approaches [4]. Scaffold-free tissue engineering approaches aims to efficiently exploit the inherent capacity of cells to generate extracellular matrices and assemble into organized and functional 3D tissues. The basic strategy arose from the works showing that cells isolated from a tissue could stratify to reform an anatomically appropriate replica of the original tissue [5].

Scaffolds are devices for TE with perfect adjusted chemistry and architecture which will help to develop the target cell or tissue. Scaffold based methods include the use of scaffolds as back-bone support on which the cells are grown. They are usually made of biomaterials providing elasticity and mechanical strength to the tissue. The biomaterial will eventually degrade and the cells colonize the scaffold grown on the surface, produce extracellular matrix (ECM) [5].

Depending on the composition and architecture of scaffolds, cell behaviour and the interaction cell-scaffold can be influenced [6]. In addition, cell behaviour is also influenced by the micro or nanoscale

topography of scaffolds due to the modification of cytoskeleton arrangements [7]. The interaction cell-scaffold depends on the type of material used as a scaffold and the cell type used [8, 9]. The major issues with scaffolds are degradation, adjustment to the environment, immunological response, toxicity of the material used to the surrounding tissues [10]. Various types of cells can be used successfully in TE. Primary cells are isolated from the patient, however, this process has limitations because the process to collect these cells is invasive and there is the possibility that cells can be in a diseased state. Stem cells are promising to use in tissue engineered constructs, involving embryonic stem cells, bone marrow mesenchymal stem cells umbilical cord-derived mesenchymal stem cells and human neural stem cells. Differentiated cells from stem cells have also been used such as fibroblasts, osteogenic cells and human vascular endothelial cell [6].

Scaffolds must be fine-tuned to perform according to the following criteria: biocompatibility, biodegradability, mechanical properties and architecture [11].

First criterion to take in consideration is biocompatibility as cells need to be able to adhere to the scaffold, perform normal cell functions such as the ability to migrate on the surface, to proliferate and also to produce ECM. In addition, when implanted in the patient, the biomaterial should not cause an immune response, which could cause a rejection of the scaffold by the patient's body [10, 11]. Degradation products resultant from biomaterial degradation should be non-toxic and eliminated from the body without harming others tissues. Mechanical integrity upon handling is also an important pre-requisite since must be able to withstand manipulation during surgical implantation [11].

Regarding scaffold architecture, some requirements must be present to elicit an efficient TE strategy. A porous interconnected structure and high porosity are beneficial to allow cell penetration and diffusion of nutrients to cells into scaffold but also diffusion of waste of products and the degradation products. The mean pore size is also important because cells interacts with the scaffold through their ligands, and the density of ligands will depend on the available surface where the cells will adhere [11]. The scaffold or construct needs to be cost effective; to be scale-up and its manufacturing process needs to be develop based on good manufacturing practices (GMP) [11, 12].

1.1. Biomaterials

Biomaterial is defined as a material that has been designed to be used in any therapeutic or diagnostic procedure to regulate the interactions of single or multiple components of living systems when applied alone or as part of a complex device [13]. Biomaterials range from natural and synthetic polymers to inorganic biomaterials and organic-inorganic hybrid biomaterials [14, 15].

Synthetic biodegradable polymers with well-defined structure and without immunological concerns associated with naturally derived polymers are widely used in TE. Polyesters, polyanhydrides, polyphosphazenes, polyurethane, and poly (glycerol sebacate) display important advantages over natural

polymers such as processing features in terms of their molecular weight, degradation, mechanical proprieties and property profiles for specific applications [16]. Some of the synthetic polymers widely used in TE are polyglycolide (or poly glycol acid (PGA)), polylactide (PLA), poly-lactide-co-glycolide (PLGA), poly-(D,L-lactic acid) (PDLLA) and poly-ethylene-glycol (PEG) [17].

Natural and synthetic inorganic biomaterials (metallic and ceramic) have characteristics such as long-term reproducibility, microstructures and specific defined compositions [15]. Ceramics (e.g. alumina,zirconia, calcium phosphates, calcium phosphates cements and silicates) are highly biocompatible, and display osteoconductive and osteogenic ability, whereas, metallic biomaterials (e.g. titanium) have high strength, low density and low modulus [18, 19].

Inorganic biomaterials are classified as bioinert, bioactive or bioresorbable. An inorganic biomaterial is classified as bioinert when there is no interaction between tissue and the implant (alumina, zirconia and titanium) [18, 19]. The material is considered bioactive when the material links directly with native tissue. Examples of these materials are bioglasses and glass-ceramics. The material is bioresorbable when, a gradually absorption of the material occurs at same time that the replacement of the new tissue is undergoing [15]. Natural inorganic biomaterials are obtained from marine shells, corals, sponges, nacres and animal, once they are rich in calcium compounds such as calcium carbonate and calcium phosphate [20]. Calcium phosphates-based materials obtained from corals have been applied in bone tissue engineering due to their mechanical features and microstructure [15]. The synthetic inorganic materials result from diverse methods such as aqueous precipitation, hydrolysis, sol-gel synthesis, hydrothermal synthesis, mechanochemical synthesis, microwave processing and spray drying [21, 22]. These methods lead to materials with highly morphology and crystal size [23]. There are many synthetic inorganic materials used in tissue engineering such as alumina, zirconia, bioactive glasses, glass-ceramics [24, 25].

Organic-Inorganic Hybrid biomaterials are obtained from a combination of organic and inorganic compounds. They have some characteristics such as mechanical, thermal, and structural stability [26]. Within the hybrid material, the most used and preferred is nanostructured hybrids because the nanosized fillers, improve the binding between the tissue and the organic matrices, once these materials alone cannot achieve [27]. The nanosized fillers allow an enhancement in some properties to the hybrids materials such as biocompatibility, osteoconductivity, cell adhesion and proliferation [28, 29]. The hybrid biomaterials that have been applied in TE are a combination of natural polymers such as collagen, gelatin, silk, chitosan, alginate, gellan gum; and synthetic polymers such as PEG, PLA, PGA, PLGA, ceramics, bioactive glasses [30-37]. Bioactive glass with PLA, PLGA, gelatin or chitosan has also been applied in bone tissue engineering [38]. There are other combinations that have been applied for bone tissue engineering such as hydroxyapatite/collagen, hydroxyapatite/collagen, silk/calcium phosphate, chitosan, calcium phosphates, hyaluronic acid/gelatin, gellan gum/hydroxyapatite [30-35, 37, 39].

Natural polymers are obtained from natural sources such as algae, plants, microorganisms, animals [40]. They present some advantages to other polymers such as high similarity to native ECM, cell adhesion and growth promotion, biological activity and biodegradability [14, 15]. Natural polymers used for various TE applications include proteins (e.g. silk, fibroin , collagen, gelatin, albumin, fibrinogen, elastin, keratin and actin [41, 42]), polysaccharides (alginate, gellan gum, chitosan [43, 44], chitin, konjac glucomannan) and glycosaminoglycans (e.g. hyaluronic acid [45-50]) as reviewed in [15].

Konjac glucomannan (KGM) is a natural water-soluble polysaccharide isolated from the roots and tubers of *Amorphophallus konjac* plant (Figure 2A). The back-bone chain of KGM is composed by D-glucose and D-mannose (1:1.6 molar ratio), linked by β-1,4 bonds and 5-10% acetyl-substituted residues at the side C-6 position [51-58] (Figure 2C). KGM molecular weight is between 500kDa and 2000kDa [57, 59].

KGM is approved as a safe food additive by Food and Drug Administration and by Health Canada [60-62]. The applications of KGM have been increasing due to the several reported health effects of its consumption. Reports of weight loss and satiety effects and prebiotic functions by improving the growth and viability of beneficial organisms in colon [60]. KGM is widely used in other areas such as biomedical and environmental areas, due to KGM characteristics such as good water binding ability [63], gelation capacity, film forming properties, biocompatibility, biodegradability and non-toxicity [56, 63, 64].
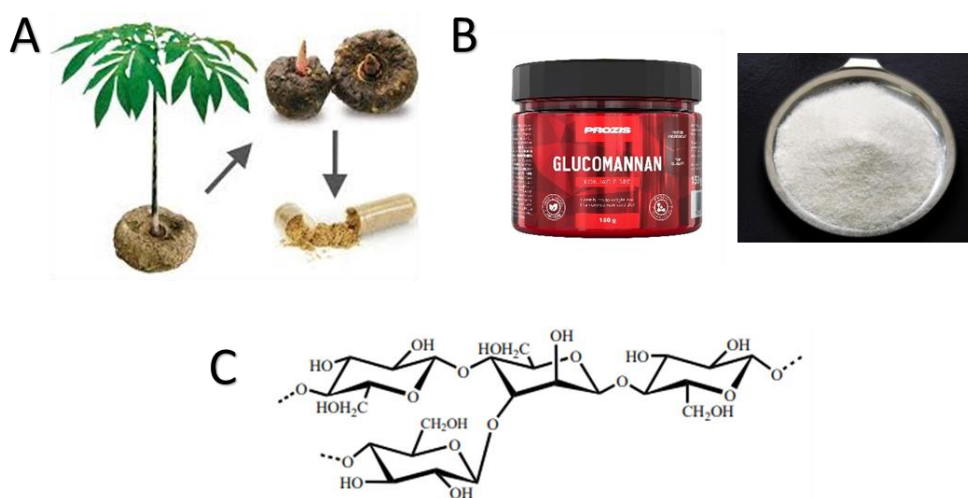
Figure 2- Konjac Glucomannan. A) Polymer extraction from plant and roots of the Amorphaillus konjac plant; B) Powder KGM used for different fields, inclusive food industry; C) KGM chemical structure. Adapted from[65].

KGM has been used in the development of strategies for drug delivery [56, 61, 66-83], wound healing [84-91], defect repair [92, 93]and biosensor production [94]. These KGM-based strategies have been developed to target a variety of tissues such as gastrointestinal tract [56, 66-68], colon [69-73], skin [84-91] and bone [92, 93] adopting different forms such as beads [66, 83], hydrogels [61, 68-70, 73-76, 84-86], film [67, 77, 87, 88, 95, 96] , nanoparticles [78, 80, 81], gels [56, 71, 72], aerogels [79], membrane [89], microspheres [82], nanospheres [94], cement [92, 93], sponges [90], cryogels [97], and nanofibers [98]. To

the extent of our knowledge, there is only one reported work where KGM was used as a biomaterial for Bone Tissue Engineering (BTE) [92, 93]. Table 1 summarizes the literature available of KGM use for Biomedical field applications.

Table 1- KGM for biomedical application. DD – Drug Delivery; WH – Wound Healing; DR – Defect Repair; TE – Tissue Engineering; RM – Regenerative Medicine. HUVECs- Human umbilical vein endotelial cells.

Biomaterials	Formulation type	Application	Assays	Reference
Alginate-KGM-chitosan	Beads	DD for gastrointestinal tract	*In vitro* BSA/Insulin release	[66]
Iron-providing material composed by KGM	-		-	[56]
KGM and poly(aspartic acid) (PAsp)	pH-sensitive hydrogels	DD for intestine	*In vitro* 5-Fluorouracil release	[68]
KGM-Chitosan	Film		*In vitro* diclofenac release	[67]
KGM and poly(methacrylic acid) (PMAA) as a carrier	Hydrogels	DD for colon	*In vitro* 5-Fluorouracil release	[69]
KGM copolymerized with acrylic acid	Gel		*In vitro* BSA and phosphate buffer solution with a certain quantity of Mannaway25L or Fungamyl 800L release	[71]
KGM/xanthan gum gel as an excipient	Gel		*In vitro* cimetidine release	[72]
KGM grafted acrylic acid	Hydrogel		*In vitro* vitamin B12 and cellulase E0240 release	[70]
Dialdehyde KGM as a cross-linking agent of chitosan	Hydrogel		*In vitro* ofloxacin release	[73]
Gelatin-oxidized KGM as crosslinker	Hydrogel	DD	*In vitro* ketoprofen release	[74]
KGM	Gel		Cell attachment assay using human fibroblasts	[56]
KGM grafted N-isopropylacrylamide	Thermos-sensitive hydrogels		*In vitro* BSA release	[75]
Light-responsive delivery system composed of TEMPO-oxidized KGM	Microspheres		Material characterization	[82]
Cholesterol-modified carboxymethyl KGM self-aggregates as drug carrier	Nanoparticle		*In vitro* etoposide release studies	[78]
KGM/Carboxyl-functionalized multiwalled carbon nanotube	Aerogels		*In vitro* 5-fluorouracil release studies	[79]
KGM/Poly(acrylic acid) blend	Films		*In vitro* ketoprofen release studies	[77]

Heparin-mimetic polyurethane cross-linked with sulphated KGM	Hydrogels		*In vitro* heparin release studies, haemolytic activity blood compatibility	[76]
Carboxymethyl KGM/Chitosan	Nanoparticles		BSA encapsulation efficiency	[80]
Glucomannan/Chitosan	Nanoparticles		*In vitro* insulin and P1 protein released studies	[81]
KGM and chitosan	Film		Hemostatic performance on rats	[87]
KGM and poly(N-vinylpyrrolidinone-co-poly(ethyleneglycol) diacrylate	Hydrogels		Tests: Live/dead (HaCaTs, fibroblasts) assay, scratch assay	[91]
Alkali-treated KGM	Film		MTT assay (HaCaTs, fibroblasts) Platelet absorption test	[88]
Carboxymethyl Chitosan/Collagen Peptide/Oxidized Konjac Composite	Hydrogel		Used: Human skin fibroblasts (NS -FB) and human hypertrophic scar fibroblasts (HS -FB) Tests: Cell survival rate	[84]
Oxidized KGM/carboxymethyl chitosan/graphene oxide	Hydrogel	WH	Cytotoxicity NIH-3T3 mouse cells	[85]
Acetylated KGM-based	Fibrous membrane		Macrophage polarization, cell proliferation and morphology, cell adherence rate, single-cell force spectroscopy. Wound healing on mice	[89]
KGM/Silver nanoparticle	Composite sponges		MTT assay on L929 cells Wound healing assay on rabbits	[90]
Xanthan Gum/KGM blend	Hydrogel		MTT assay proliferation and adhesion on fibroblasts Scratch assay	[86]
Anti-washout Calcium phosphate modified by KGM/k-carrageenan blend	Cement	DR of Bone	Used: mouse bone marrow mesenchymal cells Tests: Live and dead assay; Cell attachment and morphology and cell proliferation	[92]
Anti-washout Calcium phosphate modified by KGM/Guar gum blend	Cement		Used: Mouse bone marrow mesenchymal stem cells Tests: Live and dead assay; Cell attachment,	[93]

			morphology and proliferation	
Silk Fibroin/KGM	Beads	TE and DD	MTT assay using HaCaT and BALB/c 3T3 fibroblasts	[83]
Collagen/KGM composite	Hydrogel	TE, RM and DD	Cell proliferation and attachment (HUVECs)	[61]
Blend of KGM and chitosan	Films		Material Characterization	[99]
KGM and Chitosan blend	Films	Antibacterial	Antimicrobial assay (*E.coli, P.aeruginora and St.aereus*)	[95]
Nacre-like KGM-Montmorillonite composite	Films		Cytotoxicity assay on RAW264.7 cells	[96]
3D nanofibers cryogels of KGM/Poly (Lactic Acid)	Cryogels	*In vitro* cell culture	Material Characterization	[97]
KGM nanofibers	Nanofibers	*In vitro* cell culture	Tests: Live/Dead assay on L929	[98]
Carboxymethyl KGM grafted methoxy poly(ethylene glycol) copolymer	Graft copolymer	Biotechnology and pharmaceutical field	Material characterization Solubility determination	[100]
Carboxymethyl konjac glucomannan-g-poly(ethylene glycol) and (α-cyclodextrin)	Nanospheres	Enzyme encapsulation, bioreactors and biosensor devices	Encapsulation efficiency MTT assay	[94]

1.1.1. Polymer Extraction Methods

For a suitable application of the polymers, they need to be extracted, purified and modified to enhance their functionality [101]. The first step is the extraction and consists in the separation of the bioactive compounds from the raw materials (or biomass). There are several extraction methods such as chemical extraction (reduction, oxidation and ionic liquid), microbial and enzymatic extraction, soaking, sokhlet, distillation with water and/or steam, microwave-assisted extraction and supercritical fluid extraction. [101-103].

The conventional methods of solid-liquid extraction are soaking extraction, sokhlet extraction and distillation with water and/or steam [103].

Soaking is applied in an untreated or powdered raw material which is incubated with a solvent for defined time periods [103]. In **sokhlet extraction**, the soluble part of the material is transferred to the solvent

under controlled agitation, and depending on the raw material, high temperatures. The solid material left is pressed and it is re-extracted [103] and commonly used biomaterials as chitosan [104] and cellulose [105] benefit from this method of extraction. The process is repeated until the solute is separated from the solvent through distillation. The final step is solvent evaporation and retrieve of the solute [103]. When comparing soaking and sokhlet extraction to others solid-liquid extraction methods, these are less expensive and simpler although inappropriate to extract thermolabile compounds [103, 106].

Distillation is an extraction method applied for highly volatile compounds by exploiting the differences in the relative volatility of the mixture's components. [107]. Consists on the process of separating the substances from a liquid mixture by using selective boiling and condensation. Distillation may result in nearly complete separation (nearly pure components), or it may a partial separation that increases the concentration of selected components in the mixture. Keratin [102] is extracted using this method.

Microwave irradiation it is a solvent-free extraction method that can be used for the extraction of organic compounds [108]. The molecules of raw material absorb microwave energy and transform it into heat by two ways: ionic conduction and dipole rotation of the solvent and the sample [109]. Ionic conduction occurs when the application of an electromagnetic field causes the electrophoretic migration of ions [110]. The solution is heat due to the friction caused by the resistance of the solution to ions flow and molecules collisions [111]. Dipole rotation is due to the dipole moments of the alternative movement of polar molecules, which line up through an electric field. Compared with conventional methods have a rapid solute extraction from solid matrices and a better extraction efficiency [109]. A polymer that is extracted through this method is keratin [102] and pectin [112].

Supercritical fluid extraction method is based on the use of temperatures and pressures above critical points of the solvents [113]. This method of extraction has two steps, first, the supercritical fluid extracts from the matrix the soluble substances, second, after the extraction, the extracted compounds present in supercritical solvent is separated or fractionated. This method eliminates the disadvantages of the other abovementioned techniques such as intensive labour, low selectivity, low extraction and low reproducibility. When the substance reaches its critical temperature, the gas and liquid phases meet resulting in one phase at the critical point forming a homogeneous supercritical fluid. Many compounds can be used as supercritical fluids such as ethane, water, methanol, carbon dioxide, ethane, nitrous oxide, sulphur hexafluoride, *n*-butene, *n*-pentane [114]. Poly(methyl methacrylate) is extracted using this method [115].

Chemical methods of extraction can be divides in reduction, oxidation and ionic liquid. Chitin and chitosan are extracted by chemical methods [116] The **reduction** method uses reducing agents, such as chemicals that contain a thiol group, in combination with different processing conditions such as denaturing agents and different pH [117, 118]. The **oxidation** method allows the extraction of α-Keratin from wool and

hair in the presence of peracetic acid or performic acid [119, 120]. **Ionic liquids** are molten salts composed by a combination of organic cations with organic or inorganic anions. They present many advantages compared with solvents used in the conventional methods such as thermal stability, great dissolving and extraction ability, the capability of tuning the optimal viscosity and miscibility, low volatility and combustibility [121-123]. These salts melt at temperatures below 100˚C [124]. The ionic liquids with a good solubility and temperature of the solution allowed to dissolve the wool and the dissolved keratin was then separated from the solution using precipitation with water, methanol or ethanol [125]. Lignin is extracted using this last method [101].

Microbial and enzymatic methods are based on the use of enzymes and microorganisms such as bacteria and fungi to separate the bioactive compounds from the raw material. Microorganisms can produce proteases which are able to digest and hydrolyse the raw material. Using enzymes as catalysts presents some advantages regarding the resultant the functionality of the obtained polymer when comparing with chemical methods [102]. The polymer that is extracted using this method are keratin and chitin [101, 102].

Literature reports that KGM can be extracted by dry processing or wet processing. In the first, dried konjac chips is milled into crude konjac flour, which will be further purified. In wet processing, the KGM is extracted from the konjac flour using salt, 2-propanol coupled with starch hydrolysing enzymes, ethanol and acetate as reviewed in [126].

1.1.2. Polymer Purification Techniques

The process of extraction of the majority of the polymers is not able to render a compatible polymer for the use in biomedical engineering approaches. Normally, immediately after extraction, polysaccharides are mixed with various other substances such as proteins, nucleic acids, lipids, secondary metabolites such as toxins or even inorganic components. If on one hand pure polysaccharides could allow accurate and reproducible usage for safe experimental or therapeutic applications, on the other hand, polysaccharide purity will allow the accurate determination of structure and composition [127]. Different polysaccharides have different properties, hence different methods of extraction, isolation and purification will have to be implemented for the different polysaccharides.

The evaluation of polysaccharides' suitability for TE approaches is highly dependent on the presence of common contaminants such as heavy metals, protein, polyphenols, endotoxins, nucleic acids, cell debris. The polymer by itself can be immunogenic thus uncoupling the effects of the polymer by itself and the contaminants is a very important step to use polysaccharides in biomedical applications. Polymers must be biotolerable, meaning they must have "the ability of to reside in the body for long periods of time with only low degrees of inflammatory reaction". Biotolerability is used to describe the performance of more traditional slow-degrading/non-degrading implant materials and importantly, biocompatibility is by

definition "the ability of a material to locally trigger and guide nonfibrotic wound healing, reconstruction and tissue integration," which is more adequate for TE products [128].

Polysaccharides represent an attractive source of biomaterials for TE but have to go through a screening process and they will only be considered safe for implantation if they first prove to be cytocompatible (i.e., compatible with cells) in vitro. The classical measurements that validate what is usually referred to as "biocompatibility" in vitro consist in assessing the effect that a biomaterial, or its leachates, has on living cells [129]. Several guidelines are described in international standard protocols and, among the available tests, the evaluation of the materials' toxicity (defined as cytotoxicity) is the most common and the most widely used, as it is an effective indicator of the biomaterial in vivo toxicity potential. The absence of cytotoxic leachates and endotoxins, along with an adequate level of sterility are key requirements for implant materials [130]. Other important biosafety tests, also covered in standards, include the evaluation of the mutagenic and carcinogenic potential [131, 132]. Most regulatory agencies require in vitro tests to be performed prior to in vivo tests.

Purification methods are of utmost importance. Depending on the type of contaminants, different kind of purification methods are described in the literature.

Some of the impurities present after extraction are inorganic salts, mono- and oligosaccharides, non-polar substances, and organic impurities (e.g. proteins). Inorganic salts, monosaccharides, oligosaccharides and low molecular weight non-polar substances can be removed using dialysis. The choice of dialysis duration and dialysis bags molecular weight cut-off is very important [133].

The dialysis process is a passive method based on the diffusion of small molecule passing through a semipermeable membrane with a suitable molecular weight cut-off, i.e., from a higher concentration solution to a lower concentration solution. Diffusion coefficients are higher in small molecules (Figure 3). If larger molecules are able to pass through the pores of the membrane, their rate of transportation is low due to the lower diffusion coefficients [133]. This method is used to purify poly(vinylpyrrolidone) [134].
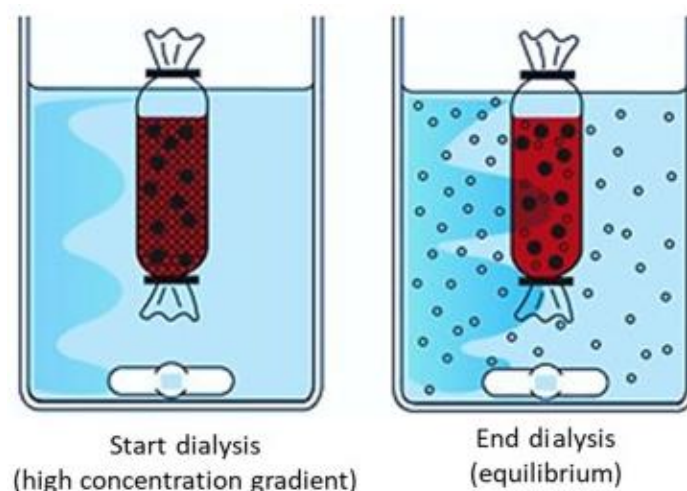
Figure 3- Schematic representation of the dialysis purification method. The small molecules pass through a semipermeable membrane from higher concentration to lower solution until the equilibrium is established. Adapted from [135].

Inorganic salts can also be removed by ion exchange resins normally in the form of beds of ion exchange resin [136].

The methods available for protein separation are diverse. One of the approaches is the use of enzymes such as proteases (e.g pronase) to hydrolyse/digest the proteins in the extracts [137]. The Sevag method can also be used though rendering low efficiency of removal. Briefly, Chloroform is used to denature the proteins in aqueous solution and after agitation with n-butanol, the proteins can be removed by centrifugation. Trichlorotrifluoroethane (CCl_2FCClF_2) and Trichloroacetic acid (TCA) methods can be applied to eliminate proteins from the polysaccharide's solution. The first has a low boiling point being highly volatile thus must be performed at 4°C which can restrict its use. TCA is widely used but as has a relative acidity can damage the polymer´s structure [136, 137].

Polyphenols can be decreased by introducing activated charcoal washes and centrifugations though some polymer loss can be expected. Adsorption by activated charcoal consists in the adsorption ability of activated charcoal for low-molecular weight organic substances as polyphenols. There are two types of adsorption: powder-like and granular. The adsorption ability to polyphenols, in liquid-phase solution, will depend on the pore structure, ash content, functional groups of the adsorbents; pKa, functional groups present, polarity, molecular weight, size of the adsorbate; and the pH, ionic strength of the solution; and the adsorbate concentration [138, 139]. The adsorption by activated charcoal is used to purify pectin [140]. Other alternative to reduce polyphenols is the Hydrogen peroxide (H_2O_2) method and functionalized resins with imidazole and pyridine [141].

Ultracentrifugation is a physical method that allow not only the elimination/decrease of contaminants but also the isolation of specific fractions of polysaccharides since different polysaccharides

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with different molecular weight have different sedimentation speed [142]. Ultracentrifugation isolate the fractions of polysaccharides or eliminate the contaminants based on the fractioning of the particles mixture according with their size and shape, separating them in two fractions –sedimenting particles/ pellet (which are the large particles) and unsedimenting particles/ supernatant (which are the smaller ones) and density gradient centrifugation. Nevertheless, there are two modes – rate zonal centrifugation and isopycnic centrifugation. Rate zonal centrifugation separates particles according with their different sedimentation coefficients, while isopycnic centrifugation separates particles based on their distinct effective buoyant densities [143]. This method is usually used for the purification of pectin [140].

Filtration and ultrafiltration are based on the different size and shape of polysaccharide molecules in a solution (molecular sieve), however depending on the material of the filters used, since they can adsorb polysaccharide, high amount of polysaccharides can be retained decreasing the yield of the purification [144]. A driving force must be applied to accelerate the process (e.g. vacuum) (Figure 4) [145]. This technique is widely used for pectin, alginate and chitosan purification [101, 140, 146].
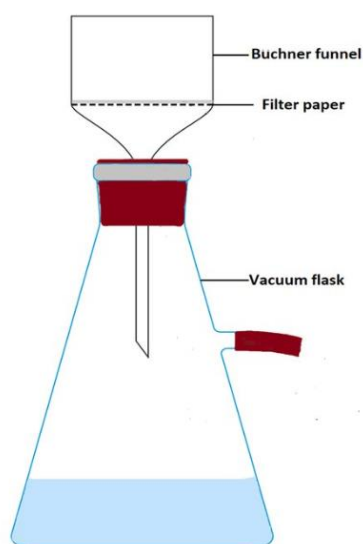
Figure 4- Vaccum filtration apparatus. The polymer to purify is put into Buchner funnel and the contaminants will be trapped in the filter paper. At the end of the process, in the vaccum flask it is the purified polymer. Adapted from [147].

Other methods are available to differentially separate the polysaccharides according to their characteristics such as affinity chromatography, Gel column chromatography, anion exchange column chromatography, Cellulose column chromatography, Quaternary ammonium salt precipitation method, Metal coordination method, Graded precipitation methods and preparative zone electrophoresis method as reviewed in [148] are primarily used to isolate mixed polysaccharides in order to obtain different fractions of homogeneous polysaccharides but not primarily to decrease contaminants though some contaminant decrease can be obtained. Furthermore, the use of polymers in TE must be cost effective, scalable and easy to use and these last methods can be expensive.

In chromatography methods, a porous solid loaded in a tube or a column constitutes the stationary phase. The mobile phase is a polymer dissolved mixture, which passes through the column or tube. The different constituents of the sample will be distributed in both phases, according to the substance's proportions. The nature of the stationary and mobile phase and the nature of sample constitutes are influenced by the coefficients of distribution which in turn will affect the rate of the reaction [149]. The chromatography is used for poly(methyl methacrylate) and polyethylene oxide [150]. Crystallization is a solid-liquid separation and purification process. The solid compound that is dropped in the adequate liquid will dissolve until the saturated point is reached and the solid is not added anymore. As the saturation point depends on temperature, when the temperature is low, the solubility decreases and the solid will precipitate, however, when the temperature is higher, the solid will continue to dissolve. Therefore, the purification and separation of the samples can be controlled by the conjugation of saturation and temperature control [151-154]. Moreover, precipitation is used, at the same time that crystallization is used. The difference between them is a formation of an insoluble compound in the solution due to a chemical reaction. Many substances precipitates due to a reaction, and for that to happen the concentration of the precipitating agent needs to be controlled [155]. This method is used to purify pectin and chitosan [101]. Electrophoresis is used to separate and purify biopolymers, depending on the size and charge of molecules. By combining the size and charge, molecules are separated in a polymeric gel, when an electric field is applied [156-158]. Hemicellulose is purified by electrophoresis [159].

Endotoxin contamination is one of the major issues to solve before a biomaterial can be considered safe to use. Routine endotoxin testing on biomaterials should be made and this must be the responsibility of every scientist to ensure the safety and validity of any biomaterial, however it is of utmost importance to prevent its contamination in the first place. This is especially important in polymers since endotoxins are highly heat-stable, standard autoclaving will not destroy endotoxin, however, autoclave cycles of 250˚C for more than 30 min or 180˚C for more than 3 h will although few polymers can withstand these conditions [160].

Endotoxins (lipopolysaccharides -LPS), are an integral part of the outer cell membrane of Gram-negative bacteria. Endotoxins consist of a lipid component, Lipid A, a core oligosaccharide and a long heteropolysaccharide chain, the O-specific chain that represents the surface antigen (O-antigen) [161].

Lipid A is the most conserved part of endotoxin and is responsible for most of the biological activities of endotoxin. Endotoxins are shed upon cell death but also during growth and division. It has been clearly shown that endotoxin can significantly affect the biological response since when endotoxin contaminated samples were tested in vitro with cells before and after removal of endotoxin contamination, the presence of endotoxin induced significant production of cytokines or cell maturation [162, 163]. Current FDA limits are such that eluates from medical devices may not exceed 0.5 EU/mL, or 0.06 EU/mL (in cerebrospinal fluid) [164]. In humans and animals, endotoxins have very strong biological effects. Fever and shivering,

hypotension, adult respiratory distress syndrome, disseminated intravascular coagulation, endotoxin shock and ultimately cases of sepsis result from Gram-negative bacteria and its pathophysiology is initiated by LPS, which stimulates the synthesis of inflammatory mediators such as the cytokines IL-1b, IL-6 and TNF-a as reviewed in [163]. Different methods (ultrafiltration, extraction, adsorption, are available to remove endotoxin from contaminated solutions [161]. However, the viscosity of the (polymer) solution or its acidity (such as the case with a collagen solution) prevents the use of many of the available techniques. Finding a supplier that offers an endotoxin-free product is often the only solution.

2. Bone Tissue

Bone is a complex organ composed by a specialized, mineralized and vascularized tissue. Bone main functions are providing mechanical support for locomotion structural support and protect of soft tissues mineral homeostasis for metabolic processes and haematopoiesis [165-168].

In an adult human skeleton, bone tissue consists of cortical and cancellous bone [166]. Cortical bone, or compact bone, represents 80% of the skeletal mass and is composed by repetitions of osteon units and is mostly calcified. Cancellous bone, or trabecular bone, represents 20% of the skeletal mass and consists of an interconnecting framework of trabeculae (Figure 5) [165, 166, 169].

Cortical bone provides supportive and protective function to the skeleton, whereas cancellous bone provides metabolic function [170, 171] .

Figure 5- Bone structure from the nanoscale to the macroscale and its morphology. Bone is composed by cortical bone, which is repeated osteon units, and cancellous bone, which is interconnecting framework of trabeculae. Structural differences between them leads to different functions. Cortical bone provides mechanical support and protective function to the skeleton, whereas cancellous bone provides metabolic functions. At sub-nanoscale is observed the two principals components of the bone: collagen (organic phase) and hydroxyapatite (mineral phase). Adapted from [172].

Bone tissue is composed approximately by 90% of bone matrix and 10% of bone cells [165]. Bone matrix is comprised of a 65% mineral phase, 35% organic phase and a small amount of water [173, 174]. The

mineral phase of bone tissue is mainly constituted by hydroxyapatite (HA), however small amounts of citrate, fluorine, chorine, sodium, potassium, magnesium, strontium, zin and iron can be found in its composition [175, 176]. The organic phase is comprised of 90% type I collagen, approximately 5% of non-collagenous proteins and 2% of lipids. Type I collagen provides elasticity to bone tissue, stabilizes the ECM, binds to macromolecules and act as a template for initial mineral deposition [177]. Non-collagenous proteins are osteocalcin (OC), osteonectin (ON), osteopontin (OPN) and alkaline phosphatase (ALP), which are not exclusive to the bone mainly providing proteins structural, and mechanical strength, also modulating the cell behaviour [170].
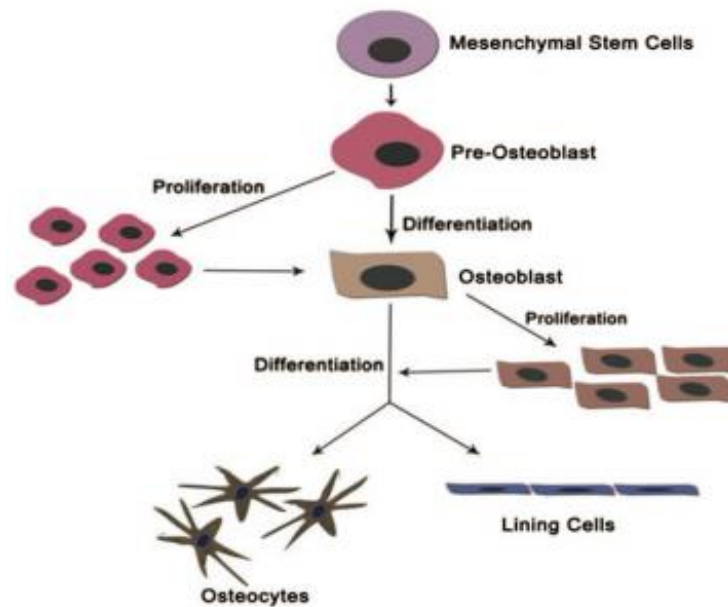
2.1.1. Bone Cells

Bone cells populations are osteoblasts, osteoclasts, osteocytes and bone lining cells; and they represent a small volume of the bone tissue. Osteoblasts are cuboidal cells comprising 4 to 6% of total bone cells and are found along the bone surface [170]. These cells differentiate from the osteoprogenitor mesenchymal stem cells of the bone marrow stroma (Figure 6) being responsible for the synthesis of the organic matrix and the regulation of calcium and phosphate fluxes. Under specific stimuli, osteoblasts can produce various growth factors such as insulin-like growth factors (IGF), platelet-derived growth factors (PDGF), basic fibroblast growth factors (bFGF), transforming growth factor-beta (TGF-β) and bone morphogenic proteins (BMPs) [165, 171, 178-181]. Young osteoblasts are active, whereas, old osteoblasts are inactive, thin and elongated lining cells that are present in the bone surface. Differentiation and proliferation of these cells are required for the formation of new bone and repair processes [182].

Osteoclasts are giant multinucleated cells that can contain one or more nuclei, and their main role is bone resorption [165, 166, 178, 183]. These cells originate from mononuclear cells of hematopoietic stem cells (Figure 6) [184]. Osteoclasts extremely mobile which allow them to move to different locations in the bone surface [182].

Osteocytes represent 90 to 95% of total bone cells in adult human skeletons [183]. Osteocytes are mature osteoblasts localized inside the bone matrix (Figure 6). They have uniform shape and size and are responsible for the detection of microfractures, and bone remodelling, being also involved in the regulation of osteoclast and osteoblast activity [170, 182]. When compared to osteoblasts, young osteocytes have lower cell volume, protein synthesis and are more structurally similar to osteoblasts, whereas, old osteocytes have reduced cell volume and accumulation of glycogen in the cytoplasm. During osteoclastic bone resorption, these cells are phagocytosed and digested [185].

Bone lining cells are thin, elongated and inactive cells that are localized along of bone surface (Figure 6) [182]. Bone lining cells play a crucial role in the coordination between bone resorption and bone formation, as well in the control of mineral flow between bone tissue and the extracellular fluid [182, 186].

Figure 6- Illustration of mesenchymal stem cells lead to different types of bone cells. The differentiation of mesenchymal cell precursors result into pre-osteoblasts, which will proliferate and differentiate into osteoblasts. The proliferation and differentiation of mature osteoblasts result into osteocytes and lining cells. Adapted from [187].
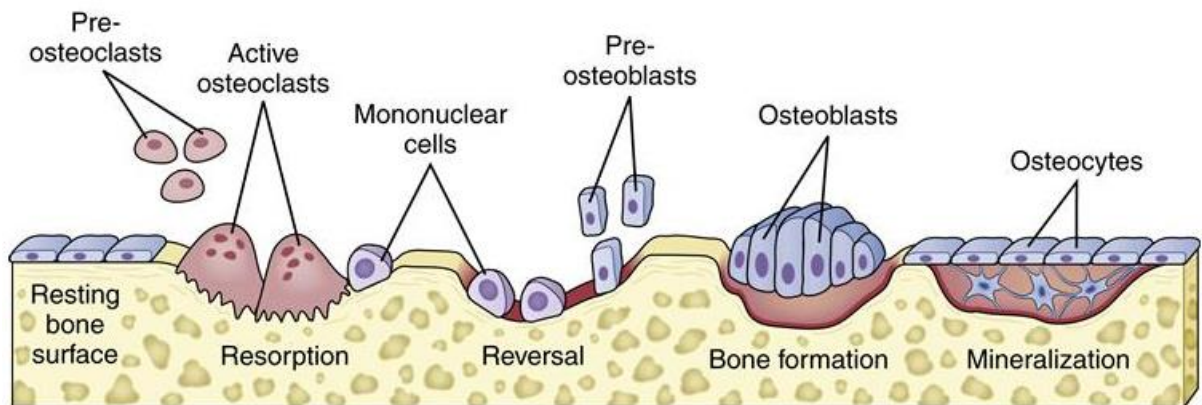
2.2. Bone Remodelling

Bone is constantly remodelling throughout life, as it is a highly dynamic tissue. It can regenerate itself allowing the healing of bone tissue injuries and fractures without scar tissue formation. This process is important for the maintenance of the adequate bone mass, suitable mechanical properties, and skeleton integrity [178].

The remodelling process is the result of the coordination between osteoclasts and osteoblasts cells. The remodelling cycle consists of four consecutive phases: activation, resorption, reversal, and formation [170]. During activation phase, there is a mechanical request of osteocytes (e.g. recognition of suffered lesions), leading to the bone lining cells retraction and the osteoclasts cells recruitment derived from the cytokines release [188]. Resorption begins when the differentiated mononuclear preosteoclasts start to migrate towards the bone surface, forming a multinucleated osteoclast. During this phase, bone matrix demineralization occurs, where crystals of HA are dissolved and the organic phase of the bone tissue is degraded by proteolytic enzymes, resulting in gap formation [170, 178].

After the resorption phase is complete, the reversal phase begins when the mononuclear cells arrive to the bone surface, prepare it for bone formation, and provide molecular signals for the recruitment of osteoblasts precursor cells, which will proliferate and migrate and differentiating in to osteoblasts [170, 178]. In the formation phase, osteoblasts lay down until the bone that was resorbed is replaced by new bone [178, 186, 188]. Bone formation can be divided in two steps: production of the bone matrix and its consequent mineralization [166]. When bone is completely formed, the remodelling process finishes and osteoblasts

undergo apoptosis or terminal differentiation into osteocytes or bone lining cells (Figure 7) [178, 186]. The different phases of the remodelling cycle have different lengths, i.e, resorption last approximately 2 weeks, reversal phase can take up to 4 to 5 weeks, and formation can last up to 4 months [178].

In homeostasis, there is an equilibrium between bone resorption and bone formation, however, when an imbalance between these two processes occur several diseases can develop [170, 183, 189].

Figure 7- Bone Remodelling Cycle. Bone remodelling starts when are osteocytes' mechanical request or there is suffered lesion. Resorption phase begins when resorb bone mineral and matrix. Then, reversal phase begins when the mononuclear cells prepare the resorbed surface for osteoblast, and consequently a generation of new synthesized matrix. The formation phase consists in the replacement of resorbed surface to a newly bone. When bone is completely formed, the remodelling process finishes. Adapted from [190].

3. Bone Cancer and Current Treatments

A genetic or environmental deregulation in the bone cells or in the tissue microenvironment can cause bone cancer, including primary bone tumours and bone metastasis (Figure 8) [191].
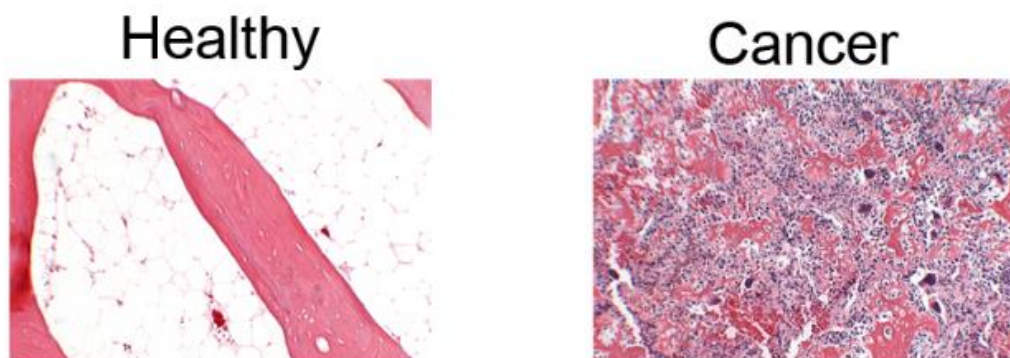
Figure 8- Histology of a healthy bone tissue versus a bone with cancer. Adapted from [192].

Primary malignant tumours are rare, with a 1% of diagnosed bone cancers. These tumours can be categorized in three different types: osteosarcoma, chondrosarcomas and Ewing's sarcoma [193, 194]. Osteosarcomas and Ewing's sarcoma are frequent in adolescents and young adults, whereas chondrosarcoma is present in adults and elderly population [193, 195]. Osteosarcoma arise from malignant

mesenchymal cells and develops in the metaphysis of long bones, although it can also develop in the distal femur, proximal tibia, proximal humerus and can metastasize to the lungs or extremity bones [194]. It is still not known which cell gives rise to Ewing's sarcoma, but it has been supposed that develops from primitive neuroectodermal, undifferentiated or neural crest cells, although, recent research proposed that this type of cancer derives from primitive stem cells [196]. Chondrosarcoma occurs in soft tissue near bones, and the affected cells produce cartilage [194].

Bone metastasis is the most commonly malignant bone tumour and are derived from multiple myeloma, prostate, breast, lung, kidney, renal, gastrointestinal and thyroid cancers which have several foci and usually prefer the hematopoietic marrow sites in the proximal long bones and axial skeleton to metastasize. The tumour uses available sources, such as multiple bioactive factors and resident cells within the bone marrow, to promote its localization and proliferation in the bone [197]. The equilibrium between bone resorption and bone formation is affected by the presence of cancer cells in the bone microenvironment, causing patients' excessive bone resorption and increasing formation of low-quality bone, leading to lytic, sclerotic or mixed lesions [198]. When multiple myeloma, breast, lung, thyroid, renal and gastrointestinal malignancies; metastasize in bone with a lytic form, there is an occurrence of focal bone destruction. Otherwise, when there is an enhancement of osteoblastic activity, the metastasis have a sclerotic appearance and develop usually from prostate cancer, but also, from breast and lung carcinoid. Mixed lesions have an enhancement of osteoblastic activity and bone resorption rate [199]. Depending on the nature of these lesions, their site and their treatment may lead to serious complications such as pain, pathological fractures, spinal cord compression, bone marrow repression and a decrease of performance status [200]. Lytic and osteoblastic lesions develop complications such as pathological fractures and hypercalcemia, however, in the osteoblastic lesions, the frequency of these complications decreases [193]. These complications lead to significant consequences for the patients such as impairment or loss of functionality, decrease of quality of life and a decline of survival rate [191].

There are several approved treatments for bone cancer, such as therapeutic agents, such as bisphosphonates and denosumab; radiopharmaceuticals/radioisotopes; hormonal therapy; chemotherapy; immunotherapy; percutaneous minimally invasive techniques; radiation therapy and surgery [201-206]. Nonetheless, the gold standard treatments used, in clinics and hospitals, are radiotherapy, chemotherapy and surgery [207, 208]. Radiotherapy is used for patients with localized metastatic bone pain, Ewing sarcoma and osteosarcoma. It consists in a short treatment schedule of one to five fractions of external beam radiation. Adversities of this treatment are, the structural quality of bone maybe being affected by the different fractioning schedules, which is not completely understood, but is important to take this in consideration in a weight-bearing site [209]. Surgery can be used in patients with metastasis in long bones, however surgical intervention is usually not the first option in patients with bone metastasis, so others treatments such as radiotherapy and chemotherapy are prioritized [201, 210, 211]. Surgery is also used for

chondrosarcoma, because this cancer has limited vascular connections in malignant cartilage cells, which can lead to inefficiency of commonly utilized chemotherapeutic agents [194, 202]. Chemotherapy is used in patients with advanced bone metastasis disease and in patients with osteosarcoma and Ewing sarcoma, where chemotherapeutic agents are utilized, like docetaxel and cabazitaxel. In high dosages chemotherapy treatments are known to cause side effects such as neurotoxicity, nauseas and vomiting, bone marrow suppression and temporary alopecia [194, 201, 212]. Nonetheless, unconventional treatments such as hyperthermia are being explored, since it was shown to reduce both the number of doses of chemotherapeutic agents and the radiation levels that are needed to maintain or enhance the efficiency of treatment. Moreover, hyperthermia can reduce side effects caused by chemotherapy and radiotherapy; however, this therapy can be used in concomitance with radiotherapy and chemotherapy [207, 208].

4. Hyperthermia

Since the 1940s, hyperthermia has explored as a treatment for different types of advanced cancer [213]. Hyperthermia therapy is being increasingly either alone or as an adjuvant therapy with chemotherapy or radiotherapy [213]. By definition, hyperthermia consists of using high temperatures to heat the target cells causing cell death or making them more sensitive to chemotherapy and radiotherapy [214]. Cancer cells are sensitive to relatively higher temperatures than healthy cells [215]. Low pO_2 and pH conditions are characteristic of tumour site and together with induced hyperthermia (between 40 to 45 ˚C) on localised treatments revealed to be cytotoxic for cancer cells [213]. Cancer cell death occurs by apoptosis, however, the specific efficient temperature depends on the patient condition [216]. Hyperthermia causes several changes at the cellular level, and there are two ways that cells can respond to stress: apoptosis and the heat shock response. In the former response, cells activate the signalling pathways, which comprehend coordinate action of caspases that will cleave the targets provoking cell death. The latter leads to an activation of heat shock protein causing an unfold of proteins provoking a loss of functionality [217, 218]. Hyperthermia therapy is utilized for variety of cancers such as rectal, vaginal, cervical, brain, bladder, cervix, rectum, lung, esophageal, vulval, stomach, bladder, esophagus, prostate, pancreatic and breast cancers. There are several clinical trials performed testing hyperthermia therapy alone or in combination with radiotherapy and chemotherapy [217].

There are three types of hyperthermia therapy: local, regional or whole-body (Table 2) [213]. Local hyperthermia therapy is utilized for patients with solid and localized disease near the skin surface or body orifices. Regional hyperthermia therapy is used for patients with diseases localized in deeper tissues and can be performed by various methods, such as non-invasive methods (radiofrequency), irrigation of body cavities, blood heating and increasing of organs or limbs perfusion. Whole-body hyperthermia therapy is mainly used for metastatic disease and can be achieved by an infrared chamber or heating a patient's room or covering the patient with heated blankets [214, 217, 218].

Table 2- Techniques of hyperthermia. Adapted from [214].

Type of hyperthermia	Source of Energy	Limitations	Applications
Local hyperthermia	Microwave; Radiofrequency; Ultrasound	Therapeutic depth; Ultrasound may cause patients discomfort; Cannot approach irregular surfaces.	Superficial tumours
Regional hyperthermia		Systemic side effects; Heating of healthy surrounding tissue.	Deep seated tumours
Whole-body hyperthermia	Near infrared radiation	Maximum 42˚C; Deep analgesia and sedation or general anaesthesia; Systemic toxicity; Thermal lesions due to their superficial overheating.	Cancer with distant metastases
Magnetically induced hyperthermia	Alternating magnetic field and magnetisable particles	Limited range of action due to being a localized approach; Possibility of particle toxicity.	Superficial and deep seated tumours

Different techniques and energies sources are used to induce hyperthermia by transforming the energy sources into heat deposition. Some of them are microwave (MW), radiofrequency (RF), near-infrared light (NIR) or ultrasounds (US) [219]. The specific absorption rate depends on the rate of electromagnetic energy that is transformed in heat energy. The main goal is to preserve the heathy tissue, while homogeneous heat deposition occurs at the tumour site, however, the challenge is to have a deep homogenous deposition but also achieve the target, when the tumour is located deeply in the tissue [214]. Microwave is applied in hyperthermia therapy for patients with tumours localized in tissues that have high amounts of water or in tissues with high electric current resistance [220]. It has some advantages such as deep penetration of energy source into tissue, fast deposition of heat into tumour, great reduction of tumour volume, low damage in the surrounding normal tissue and low pain through the procedure [221]. Radiofrequency cannot be used for patients with tumours located in specific sites alone, since this energy is attenuated through the tissues [222,

223]. However, this technique has an appropriate penetration depth, is safe and less expensive [223, 224]. Near Infrared presents advantages since tissue chromophores softly absorb near infrared protecting healthy tissue from damage [223]. Ultrasound causes a mild hyperthermia and has some advantages such as low costs, a great penetration and for drug delivery it is a precise method; however this technique has a disadvantage because ultrasound cannot transform its energy into heat deposition in some parts of the body [225]. Thereby, depending how deeply is the tumour to treat, the hyperthermia method to be used is different [214].

Additionally, another technique utilized is magnetically induced hyperthermia (MIH). Magnetic materials are heated by applying an external alternating magnetic field. This technique has a better heat deposition at the tumour and is majorly safe to the surrounding healthy tissue [214]. There are three different approaches to administrate hyperthermia using magnetic materials: arterial embolization hyperthermia, direct injection hyperthermia or intracellular hyperthermia [226]. The former, uses magnetic nanoparticles that are coated to target the cancerous cells. In direct injection hyperthermia, the magnetic nanoparticles can migrate to other parts of the body that may causes an accumulation of nanoparticles in sensitive organs (Figure 9) [214].
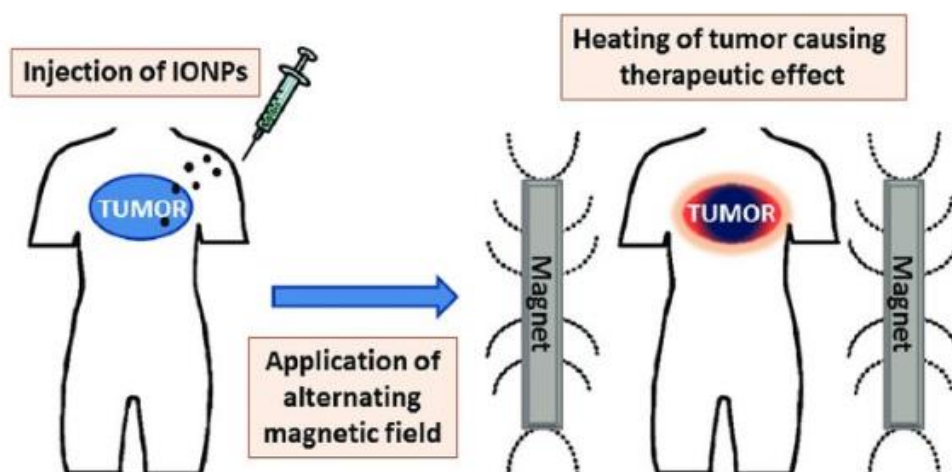
Figure 9- **Scheme of magnetically induced hyperthermia.** The Iron Oxide Nanoparticles (IONPs) fluid was directly injected into the tumour. Therefore, an external alternating magnetic field was applied and the magnetic energy was converted into heat deposition, leading to ablation of the tumour. Adapted from [227].

When comparing all the previously discussed techniques, the currently attractive technique is photothermal therapy, which uses NIR as a source of energy [228]. This therapy has numerous advantages such as, being a minimally invasive therapy, healthy tissue that surrounds the tumour is kept intact (in other words, does not damage the healthy tissue), the electromagnetic radiation can penetrate deeply in the tissue, slight side effects and expensively specificity; because there is a control in the laser irradiation parameters (time of irradiation, light intensity, location and the wavelength of the laser) [228-231]. Photothermal therapy is a local treatment with minimal toxicity and it is based on absorption energy through pulsed laser irradiation

at NIR, which will activate the photosensitizing agents which will convert this radiation into heat, provoking cancer cell death due to temperature increase, consequently leading to the ablation of tumour (Figure 10) [229, 232, 233]. The photosensitizers agents needs to fill some requirements such as, high photostability; a considerable absorption cross-section; high conversion efficiency; nontoxicity; an extended biosafety and a great biocompatibility [228, 234].
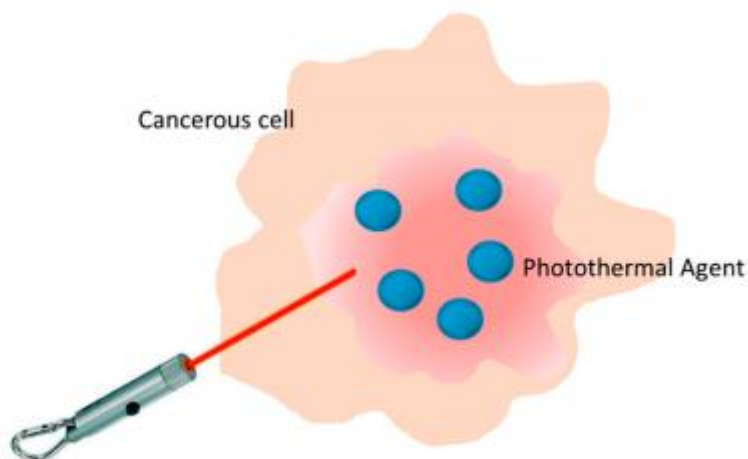
Figure 10- Basis of Photothermal therapy. The photosensitizer is accumulated on the tumour and a pulsed laser irradiates it. The radiation absorbed is converted into heat deposition leading to cancer cell death. Adapted from [235].

5. Materials used in Hyperthermia

Biomaterials, nanoparticles and photosensitizer agents are used in hyperthermia therapy [225, 232]. Biomaterials have good properties to be used as hyperthermia agent such as its stout NIR absorbance, great thermal conductivity, low toxicity, its cytocompatibility and great photothermal conversion efficiency [236]. Nanoparticles are dispersions or solid particles with a nanoscale size between 10-1000nm and they present some advantages to be applied in hyperthermia therapy such as high surface to volume ratio, noninvansive, their photothermal conversion efficiency and maximum absorption wavelength [223, 225, 237, 238]. Carbon nanotubes is being used due to this absorption in an extensive electromagnetic range and they are used for a combination with radiofrequency to treat deep tumours [222, 239, 240]. Graphene is a good biomaterial for hyperthermia therapy because it displays high stability, great thermal conductivity and notable mechanical and electronic properties [241]. Moreover, graphene is also a good biomaterial for hyperthermia therapy since it is low cost, great surface to volume ratio, great dispersibility with organic solvents and water [242]. Gold nanoshells are nanoparticles and present good alternative characteristics such as thermal stability and a benign toxicity profile [225]. Gold nanorods have a good absorption coefficient and can be used in combination with chemotherapy [243]. Gold nanoparticles are also being used in this field because of their characteristics such as nontoxicity; responsive to radiofrequency, microwave and visible light; and an ease synthetize with a well-defined structure [244, 245]. Magnetic nanoparticles are being used for magnetically induce hyperthermia therapy because of their ability to respond and modulate when a magnetic force is

applied and it is used also as carrier for anticancer agents [237, 240]. Iron oxide nanoparticles have good properties such as low cost, biocompatibility, superparamagnetism and ease synthesis; however, it is not efficiency in terms of heating. These nanoparticles are being tested for treatment of brain, breast and prostate tumours [237]. Silica nanoparticles usually need to be combined with other materials for a great action but they are used due to their high surface area. Conjugates with silica nanoparticles cannot be used in hyperthermia therapy, that uses near infrared radiation or magnetic field radiation [246, 247]. Small molecules such as organic dyes (IR780, IR783, IR88, IR825 and indocyanine green) are being used for hyperthermia therapy. IR780 dyes have some characteristics such as stout NIR absorbance a good conversion efficiency, however present low tumour specificity [225]. Several types of materials have this capacity and have been developed to be applied in bone cancer treatment [225, 232].

5.1. Photosensitizer agents

The photosensitizers agents converts infrared radiation into heat. Usually, a good photosensitizer agent to photothermal therapy requires: biocompatibility, nontoxicity, high conversion efficiency, good absorption cross-section, absorb NIR radiation capability, 30-200 nm diameter for a long circulation and a improve tumour accumulation. There are various materials with different structure shape and composition that can be used. These photothermal agents can be inorganic materials or organic materials. The former can be metallic nanostructures (gold, silver), carbon based materials (carbon spheres, carbon nanotubes, graphene oxide), nanoscale metal chalcogenides (nanostructures based on iron, cupper, sulphur, cobalt and selenium), transition metal dichalcogenide nanostructures, metal oxide nanoparticles. The organic materials can be dyes such as cyanines, squarines, phtalocyanines, porphyrin derivates; and polymers nanoparticles (polydopamine) [228]. Polymers nanoparticles have been attractive for photothermal therapy, because polymers shun long-term side effects and augment metabolic degradation, but also nanoparticles because of their size, have a high surface to volume ratio [225, 248].

5.1.1. Polydopamine

Polydopamine (PDA) is an insoluble biopolymer and it is a synthetic analogue of natural melanin (eumelanin). It is formed through autoxidation monomer dopamine (DA) (Figure 11) [249, 250]. DA is a catechol neurotransmitter in the brain and is altered in conditions such as schizophrenia, deficit hyperactivity disorder, movement control and in the symptoms and biology of Parkinson's disease. Melanin is a natural pigment, produced by epidermal melanocytes, which is responsible for the skin colour and acts like an antioxidant and photoprotectant. The tyrosine or 3, 4-dihydroxyphenylalanine (L-DOPA) naturally gives rise to DA and eumelanin. PDA and L-DOPA are structurally related. There are three methods to synthetize PDA: solution oxidation, enzymatic oxidation and electropolymerization [249]. This polymer is very promising for biomedical application due to their advantages such as biocompatibility, low cytotoxicity, biodegradability, adhesiveness, paramagnetism and eletromechanical, electrochemical and optical properties. Polydopamine-

derived nanostructures have been developed for drug delivery, photothermal therapy, biomolecule immobilization, bone and TE applications, molecular imprinting technique, cell adhesion patterning, antimicrobial applications, catalyst support and adsorbents [249, 251]. Liu et al developed DA-melanin controlled sized nanospheres for in vivo cancer therapy and showed that these nanospheres are better photosensitizer when compared with Au nano-rods because their photothermal transduction efficiency was 40% [252]. Gao et al showed that nanoparticles of sweet PDA, synthesized by oxidative polymerization of lactosylated, have great characteristics for photothermal therapy such as great photothermal conversion efficiency, great photostability, and a good NIR absorption [253].
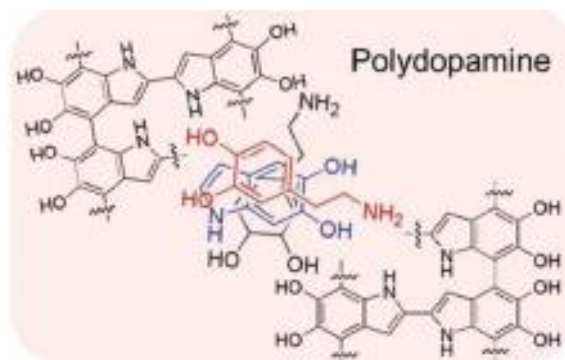
Figure 11- Polydopamine structure. Adapted from [249].

Experimental Strategy Rationale and Aim of the thesis

The initial goal of the thesis was to develop a strategy where Konjac Glucomannan as used as an injectable carrier of polydopamine nanoparticles to induce hyperthermia at bone tumour sites. Due to time constrains our experimental approach resumed to KGM purification optimization. This step is of utmost importance since the commercially available "purified" KGM contains elevated levels of contaminants to use in TE.

KGM should be validate in terms of safety upon biomaterial implantation in the host to avoid an acute immunogenic response and consequently a rejection of the implant by the human body [254]. The presence of common contaminants such as proteins, polyphenols and endotoxins cannot able to render a compatible polymer for the use in TE [127]. Of the available purification methods to purify a polymer, we chose to test dialysis, filtration, adsorption by activated charcoal and ultracentrifugation since these methods are known to preserve polymer structure and the equipment used for these methods is available at the majority of labs. Moreover, these techniques are simple and reproducible.

To achieve a successful strategy, one of the next experimental steps after purification may include the development of an injectable KGM purified hydrogel [61, 255]. This route could present important advantages since hydrogels are easily integrated into the defect site (which leads to no need for open surgery with a difficult recovery), adjustable and easy to manipulate for cell, growth factors or nanoparticle delivery due to their great water content [15]. Injectable hydrogel composed by silk fibroin was used for bone and cartilage tissues defects [255].

The last experimental step after the development of injectable KGM purified hydrogel may include the incorporation of polydopamine nanoparticles to induce hyperthermia at bone tumour sites. Polydopamine can be used due to their characteristics such as biocompatibility, biodegradability, adequate long-term toxicity during their retention in rats [250, 252]. Moreover, is a promise photothermal agent for tumour therapy because it was demonstrated that has a great photothermal conversion efficiency of 40% [252].

Material and Methods

1. Materials

KGM was purchased from Prozis, Portugal. For KGM purification: NaCl (VWR, Pennsylvania, USA), MWCO 3.5 kDa dialysis membrane (Spectra/Por®, SpectrumLabs), 3 μm and 0.8 μm filter membranes (mixed cellulose esters, MCE, Millipore) and Norit (Sigma-Aldrich, Missouri, USA) were purchased and used as received. For contaminants quantification: Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). was purchased. For characterization of purified KGM: deuterated water (Euriso-top, Derbyshire, UK) and 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (Euriso-top, Derbyshire, UK) were purchased.

2. KGM Purification

The purification of the polymer was performed applying different separation methods, including: 1) filtration using mixed cellulose ester filters (MCE, Millipore) with 3 μm and 0.8 μm pore sizes, 2) washing with activated charcoal (Norit) and 3) dyalisis. Briefly, a 0.25% KGM was prepared in dH$_2$O, under constant stirring, overnight at room temperature (RT). The obtained solution was then purified by different procedures and divided in batches for subsequent analysis. Briefly, the purification of KGM procedure involved an initial filtration of the polymer solution through a 3 μm pore size membrane filter, followed by a second filtration through a 0.8 μm pore size membrane filter, both assisted by using a vacuum bomb. The filtered polymer could be further purified applying two additional procedures. The solution passed through the 0.8 μm membrane could be stirred in the presence of activated charcoal or alternatively, it could be just dyalised. The first method involved the addition of activated charcoal (2 wt%, Norit SX Plus, Norit) to the KGM filtered at 0.8 μm, and stirring for 1h at room temperature (RT). The resultant suspension was centrifuged for 1h at 120 000 × g using a Ultracentrifuge Beckman Optime XE 100 (Beckam Coulter, Carnaxide, Portugal), the supernatant decanted and then passed through a 0.8 μm membrane filter to remove possible small amounts of activated charcoal particles that remained in suspension . Alternatively, the filtered KGM solution could be transferred to dialysis membrane (MWCO 3500 Da) and dyalised against decreasing NaCl concentrations (30g, 25g, 20g, 15g, 10g, 5g, 0g per 4L H$_2$O) along 3 days. Overall, 4 batches of purified polymer were obtained, two of them resultant from filtration of the KGM through a 3 μm and 0.8 μm pore size membrane filter, one obtained from filtration through 3 μm and 0.8 μm pore size membrane filter followed by purification with activated charcoal, and another corresponding to the dialysed fraction. The purified solutions were frozen and freeze-dried to obtain the polymer as a white fluffy solid, which was stored at -20 °C until further use. The diagram illustrating the adopted purification steps is represented in Figure 12.
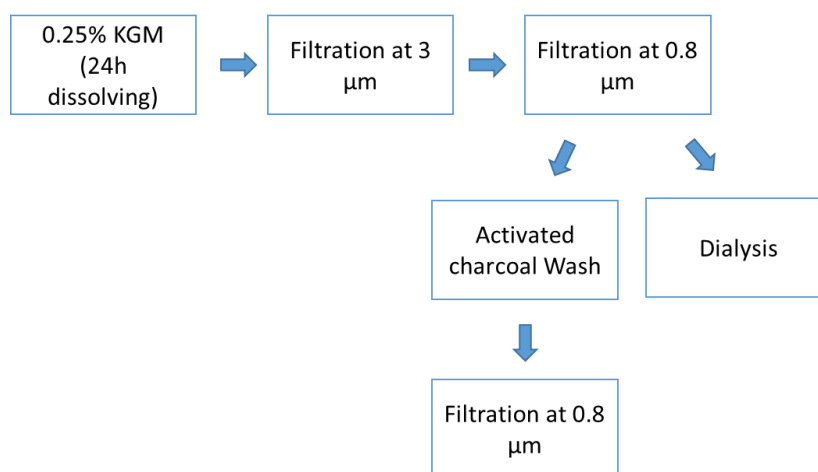
Figure 12- Scheme of KGM purification. The process includes filtration with membrane filters with different sizes, followed by washings with activated charcoal or dialysis. The polymer purified in the presence of activated charcoal should be additionally passed through a 0.8 μm membrane filter.

3. Measurement of Konjac Glucomannan

3.1. Proteins quantification

The amount of protein contaminants present in KGM was measured using Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Briefly, KGM was dissolved in miliQ water at a concentration of 0.5% w/v, and the solution sonicated using a Branson Sonifier 250 (Brason Ultrasonics, Carouge, Switzerland) for 40s with a constant Duty Cycle and an output control of 6. Then, it was incubated for 30min at 37˚C with Working Reagent (mix of BCA Reagent A and BCA Reagent B). The protein concentration was determined by measuring absorbance at λ= 562 nm using a micro-plate reader (Synergy MX, BioTek). A calibration curve was performed from standard bovine albumin solutions with concentrations ranging from 0 mg/mL to 2 mg/mL.

3.2. Polyphenols quantification

The fluorescence spectra of KGM dissolved in miliQ water at a concentration of 0.5% w/v were obtained using a micro-plate reader (Synergy MX, BioTek) working in fluorescence mode. The sample was excited at λ= 366 nm and emission recorded at 445 nm. The amount of polyphenols was measured in arbitrary fluorescence units (AFU).

4. Characterization of purified KGM

4.1. Rheology

Rheological measurements of the purified polymer were performed using a Kinexus Pro Rheometer (Malvern, Pennsylvania, USA). The viscosity was measured in viscometry mode using a cone and plate sensor system (4˚/40 mm) and 150 μm gap size. Samples were placed in the plate and it was, subsequently, analysed

in manual viscometry mode applying different shear stress (0.1, 1, 5, 10, 20, 50, 75, 100, 200, 300 and 400 Pa).

4.2. Proton Nuclear Magnetic Resonance (^{1}H-NMR)

KGM was dissolved (8 mg/mL) in deuterated water (D_2O, Euriso-top) and transferred to NMR tubes. An internal standard ($\delta=0$), 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP-d4, Euriso-top) was used and the spectra were acquired using a 400 MHz spectrometer AVANCE II (Bruker).

4.3. Attenuated total reflectance- Fourier- transform infrared spectroscopy (ATR-FTIR)

The spectra was recorded using a PerkinElmer Frontier FTIR spectrometer (Massachusetts, USA) from 4000 to 400 cm^{-1} and with a resolution of 4 cm^{-1}.

5. Statistical Analysis

GraphPad Prism 7.0.3 was used for statistical analysis. A Mann-Whitney test was used to compare the results of the different methods of purification.

Results and Discussion

1. Evaluation of protein and polyphenols content

The most common contaminants present in polysaccharides extracted from plants are proteins, polyphenols and endotoxins [127]. They must be removed before the implantation of the biomaterial in the human body because polyphenols can accumulate in the human body once they are biorecalcitrants and according with World Health Organization it is considered dangerous for humans. Moreover, the presence of endotoxins in the implant injected into bloodstream cause an increase of body temperature and the presence of proteins in implants lead to an immune reaction [146, 254]. The presence of contaminants present in the implant that was not purified leads to an immune reaction, which the implant is rejected by the human body [254]. The foreign body reaction and fibrous capsule are end-steps of the immune reaction but they are extremely important because the surface and the properties of the biomaterial that will determinate the intensity and extent of these end-steps and consequently the biocompatibility of the implant, therefore, the presence of contaminants in the surface of the biomaterial will cause a wide extent and intensity of foreign body reaction and fibrous capsule. The foreign body reaction recruits macrophages and foreign body giant cells to the local, which leads to a formation of a fibrous encapsulation around the implant leading to a rejection of the implant [256]. The presence of such contaminants was assessed for each purification technique (filtration, activated charcoal wash and dialysis), but there are many other methods (chromatography, crystallization and precipitation, distillation and electrophoresis) to remove these contaminants from the biopolymers for biomedical applications [146, 257-259]. However the purification was carried out by these methods and not others due to the fact that they are easy to reproduce and low cost. At the end of each purification, its efficiency was calculated, that is, the percentage of polymer obtained at the end of each purification method starting from a nonpurified solution with 63.5mg of polymer (Table 3). It was observed that filtration, activated charcoal wash and dialysis have a great efficiency when compared with nonpurified polymer despite the efficiency of activated charcoal wash is slightly less than filtration and dialysis.

Table 3- Efficiency of each purification method.

	Weight (mg)	Yield (%)
Nonpurified	60	96
Filtered at 3 μm	56	89.60
Filtered at 0.8 μm	55	88
Activated charcoal wash	48	76.80
Dialysis	51.5	82.40

The amount of proteins decreased independently of the purification method when compared with initial nonpurified polymer, exhibiting a statistically significant difference (p < 0. 0001). Comparing the different purification techniques, the filtration method using a membrane with 3 μm pore size reduces 1.42 mg of the amount of proteins by 1 g of polymer, while the filtration method using a membrane pore size of 0.8 μm decreases 1.62 mg of the amount of proteins in 1 g of polymer. Applying filtration followed by washing with activated charcoal followed by filtration again using a membrane with 0.8 μm pore size, a higher decrease in protein content was observed (3.57 mg/g of polymer), whereas the alternative dialysis process decreases the protein content by 3.29 mg/g of polymer (Figure 13 A). Overall, the purification process that shows a higher reduce in the protein content that exhibits a higher decrease in the amount of proteins is the combination of filtration using 3 μm and 0.8 μm pore size membranes followed by washing with activated charcoal. This is in accordance with other results in literature for purification of polysaccharides, where it was reported that Pectin also showed a decrease in the proteins content in 70% by applying a similar purification procedure based on filtration followed by activated charcoal washing [140].
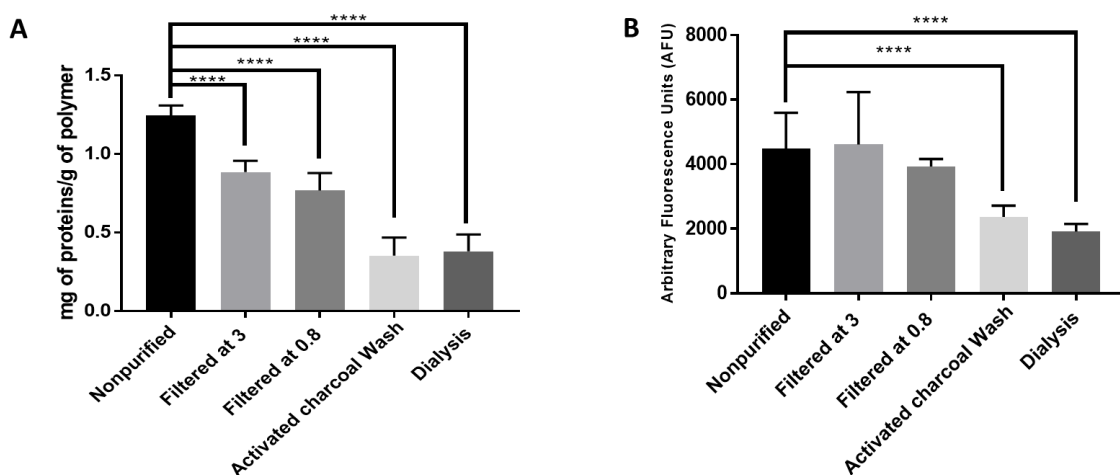
Figure 13- Quantitative evaluation of contaminants in Konjac Gucomannan purified by different methods. A) Protein quantification, the results are presented in grams of protein per gram of dry polymer. B) Polyphenol quantification, the results are presented in arbitrary fluorescence Units (AFU). Statistically significant differences are marked with **** (p < 0.0001), when there is nothing indicated imply the absence of statistically significant differences.

The amount of polyphenols remaining in the polymer after treatment through the different purification procedures was also analysed. Comparing non-purified polymer with the ones filtered through a 3 μm and 0.8 μm pore size membrane, there was no significant difference in the polyphenol content. The filtration method using a membrane pore size of 3 μm reduces the amount polyphenols by 0.97 AFU compared to the non-purified polymer, while the one using a 0.8 μm pore size membrane decreases the amount polyphenols by 1.14 AFU. A statistically significant (p < 0. 0001) lower amount of polyphenols was

observed in samples washed with activated charcoal and purified through dialysis, reducing the content in polyphenols by 1.89 AFU and 2.34 AFU, respectively (Figure 13 B). Thus, it can be considered that the purification process that more decreases the amount of polyphenols present in the polymer is the combination of filtration with dialysis. On the other hand, other polymers such as pectin showed a 52% decrease in the polyphenols contents when purified through filtration and washing with activated carbon [140]. This is controversial with the results because the filtration and activated charcoal wash does not remove enough polyphenols as filtration and dialysis, this happened maybe because the activated charcoal utilized. The functional groups present in the surface of activated charcoal influences the adsorption ability. The activated charcoal utilized is an acidic carbon, meaning that these charcoals have oxygen in the acidic sites, leading to a weak or an inexistent adsorption of phenols, because the phenol is adsorbed on the surface of activated carbon through donor-acceptor complex mechanism, which the group with oxygen is the electron donor and the aromatic ring in the solution is the acceptor [260, 261]. Nonetheless, it has been shown that activated charcoal is good for polyphenols and endotoxins, however, due to what was abovementioned that method was not enough to remove polyphenols [138, 260]. Probably, for a better removal of polyphenols another activated charcoal should be used or another efficient method to remove polyphenols or maybe repeat two times the procedure (that was done in the pectin purification using the same method). Klöck et al developed an in-house procedure that has been showed efficient to remove polyphenols from alginate which maybe can be applied in KGM. The procedure consists in adsorption with acidic charcoal followed by neutral charcoal, then a filtration and after an acidic treatment and then a dialysis [262].

In this thesis, we evaluated the efficiency of three purification methods for KGM, and the main goal was to establish a simple a low cost purification method. Taking into account the results obtained in this section, it can be concluded that the purification method involving filtration and wash with activated carbon showed to be a good purification method to remove the proteins, although it did not demonstrate to be as efficient in the removal of polyphenols as filtration followed by dialysis. Nevertheless, the first purification methodology is cheaper than the latter. Despite of this method is not extremely efficient in lowering the amount of polyphenols, this can be overcomed by performing the procedure twice (that was done in the pectin purification using the same method) or by using another type of activated charcoal because of the mechanism of donor-acceptor complex between the surface of activated charcoal and the polyphenols, which lead to a weak or inexistent adsorption, which was explained above. The activated charcoal has been shown to be a great adsorbent of different types of compounds such as phenolic compounds [138, 263].

2. Structural characterization of Purified Konjac Glucomannan

The KGM structure was characterized by ^{1}H-NMR analysis before and after application of the different purification methodologies (Figure 14 A-D). As mentioned previously, it is known that KGM is composed by mannoses and glucoses monomores, in which some of them may be acetylated [64]. In all ^{1}H-NMR spectra it is possible to identify the peaks corresponding to protons from monomeric units that compose the polymeric chain. According to the literature, the broad peaks between 5.5 and 3.1 ppm correspond to the glycosidic moiety [100]. The peak at 2.2 ppm is attributed to the methyl protons (CH_3) of acetylated moieties [100, 264]. The acetylation degree may be calculated by the ratio of the integral between the peak corresponding to acetyl moiety and the peak appearing at 4.52 ppm, which corresponds to the proton from C8 of the glycosidic ring. For nonpurified polymer, the acetylation degree of KGM was calculated to be 16%, which is also similar for the purified polymer, suggesting that the purification methodologies applied in this work did not introduced significant changes on the structure of the polymer. The remaining peaks presented in the spectra do not belong to the backbone of KGM polymer, meaning that it could correspond to impurities present in the polymer. In the spectrum belonging to non-purified KGM (Figure 14 A), it is well observed these unidentified peaks at δ= 6.52 ppm, 3.14 ppm, 2.70 ppm, 2.40 ppm and 1.91 ppm. Some of these peaks may be characteristic of aliphatic impurities such as proteins, which also may present characteristic peak at δ= 6.52 ppm corresponding to the proton belonging to amide moiety. Peaks around δ= 2.3-2.7 may also be related to the presence of impurities bearing ester moieties [265], and thus the presence of certain lipidic contaminants cannot be ruled out. The purification of the polymer by filtration through 0.8 µm membrane filters allowed the disappearance of peaks at δ= 6.52, 2.70 and 2.40 ppm (Figure 14 B). Additional purification through washings in the presence of activated charcoal did not produce significant changes in the ^{1}H-NMR spectrum (Figure 14 C), while purification through dialysis promoted the disappearance of all the peaks corresponding to contaminants, remaining only evident the presence of peaks corresponding to the monomeric units of KGM (Figure 14 D).
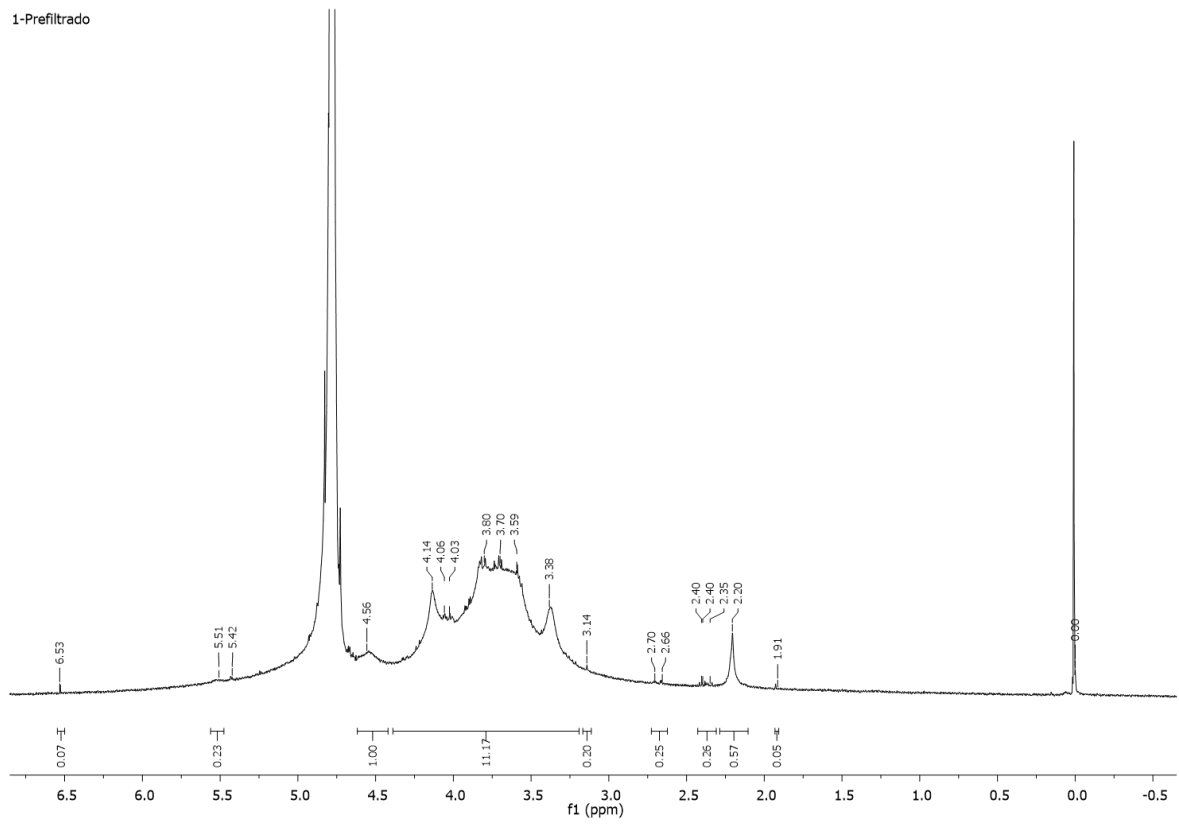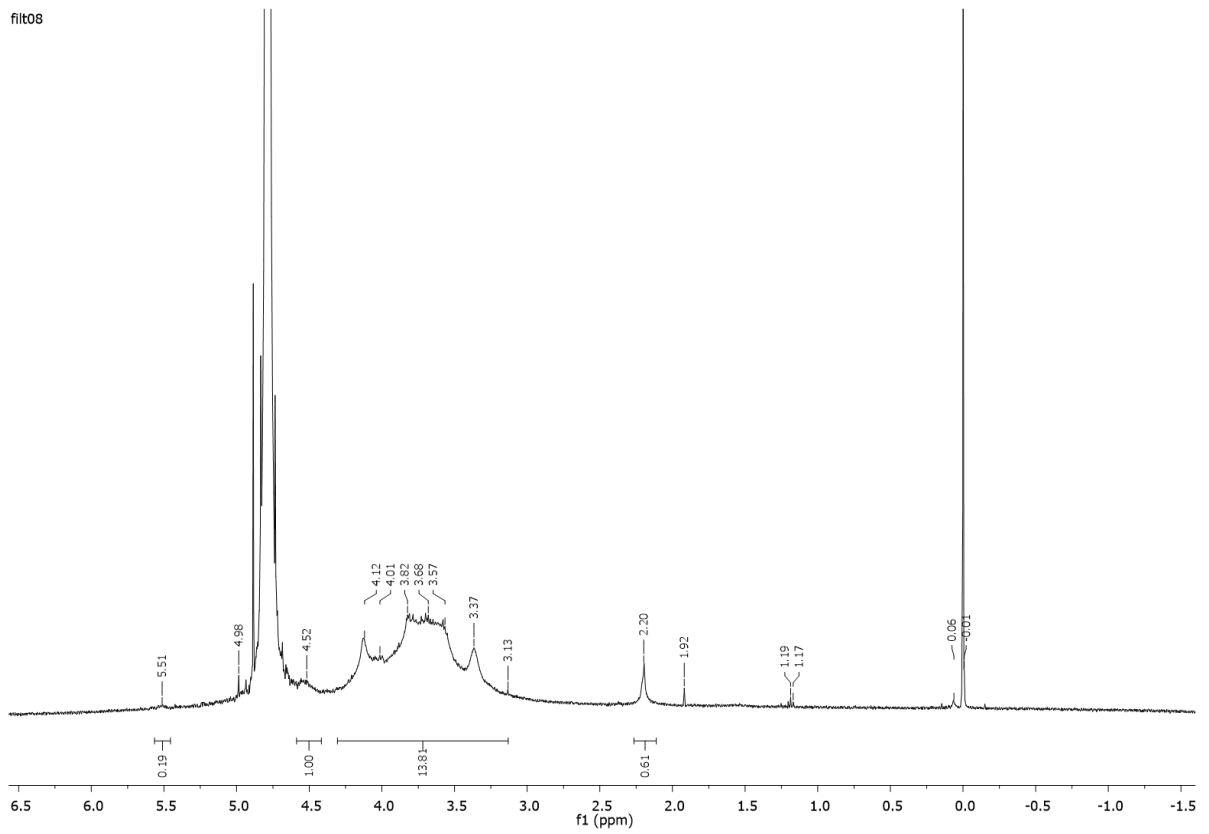
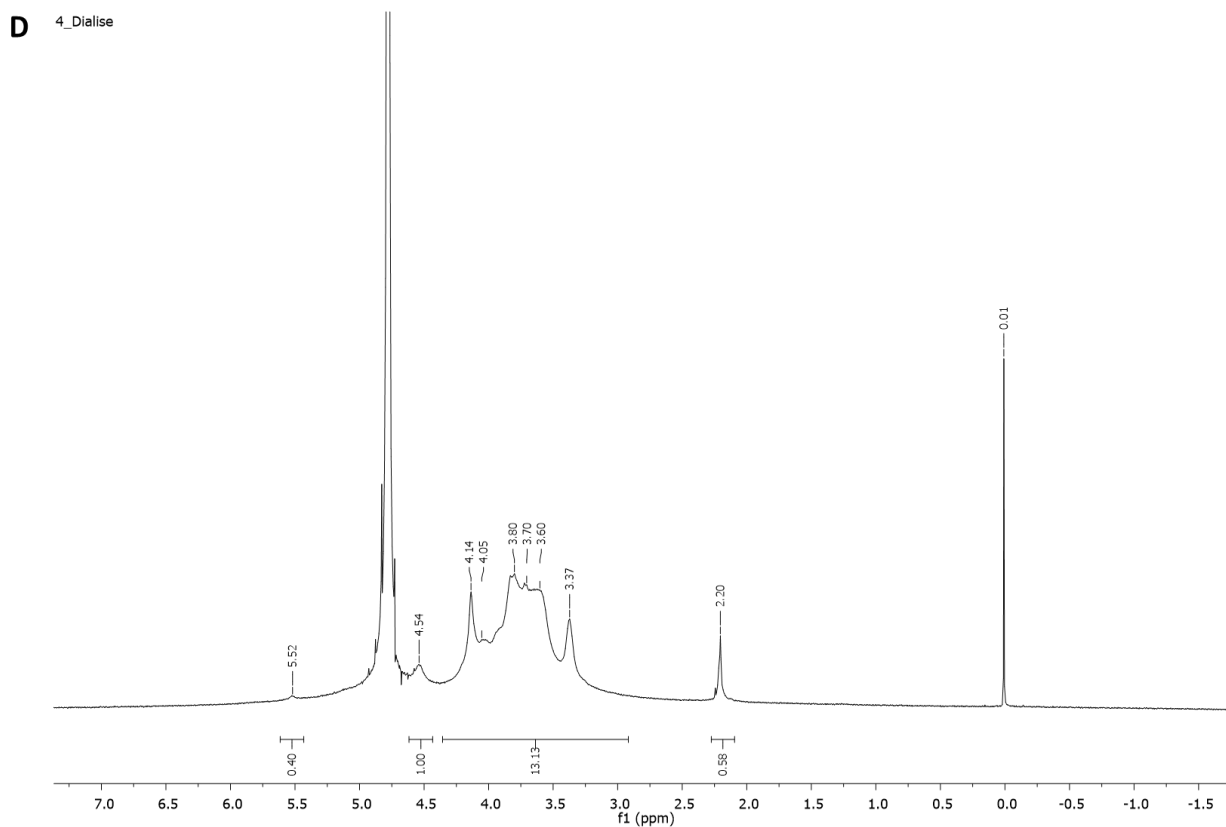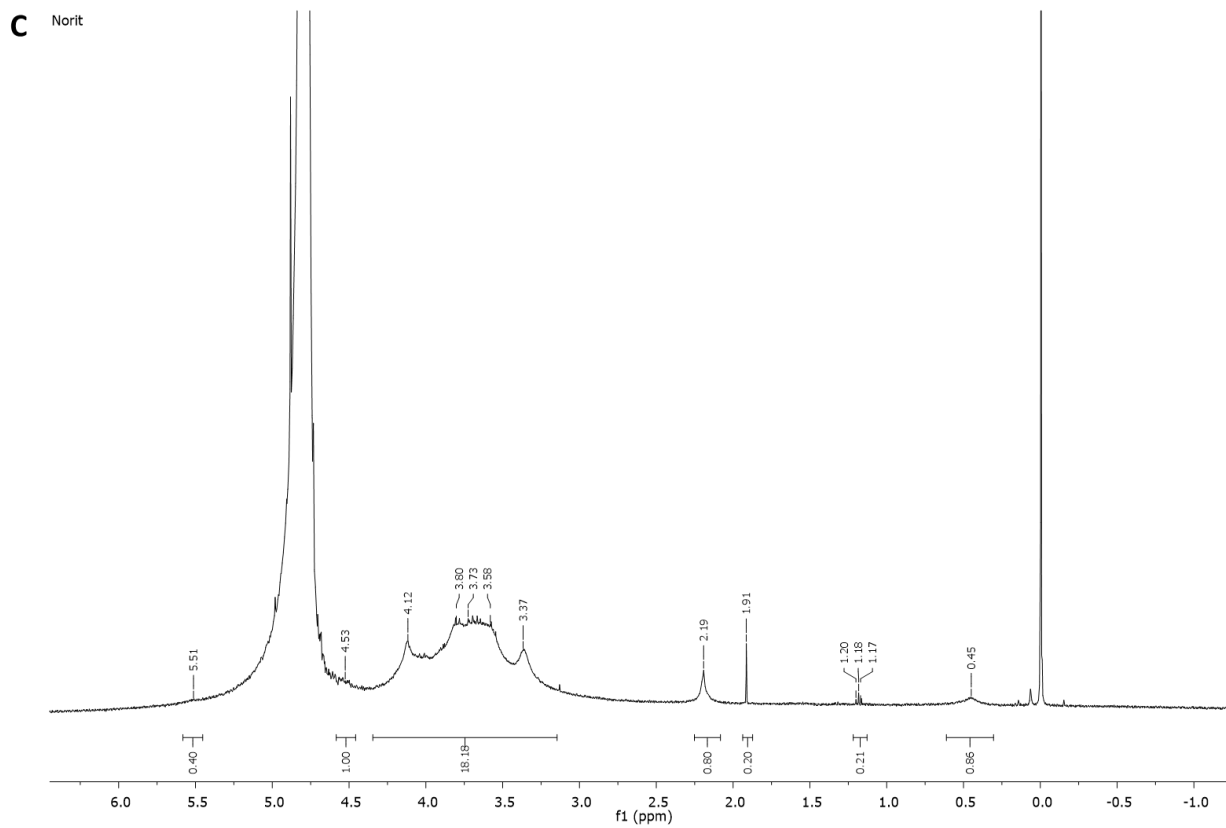**Figure 14- ¹H-NMR spectrum of Konjac Glucomannan.** ¹H NMR spectra of Konjac Glucomannan before (A) and after being submitted to different purification procedures, including filtration (B) followed by washing with activated charcoal (C) or dyalisis (D).

Although it has been identified the presence of proteins and polyphenols in previous tests (Figure 13), it is not clear that the contaminant peaks present in ^{1}H-NMR spectra correspond to such compounds. For example, it has been observed a significant decrease in protein content after the application of the different purification protocols, which could be in accordance with the disappearance of the peaks located at δ= 6.52 ppm, 3.14 ppm, 2.70 ppm, 2.40 ppm in the ^{1}H-NMR spectra of purified KGM. Although there are still proteins present in these purified polymers, its content may not be detected by ^{1}H-NMR due to characteristic sensitivity of the technique, which impose problems on detecting compounds present in low amount (< 5 mol%). This could be also in line with the absence of peaks characteristic of polyphenols in all the ^{1}H-NMR spectra. On the other hand, there may also be contribution from the problems with the homogeneity of the solution and its viscosity [266]. Overall, by analysing the ^{1}H-NMR spectra it seems that the best purification procedure seems to be the combination of filtration with dialysis.

ATR-FTIR may provide additional structural information on the polymer, particularly through identification of certain functional groups present in the polymer backbone and, probably, on the contaminants. With this aim, the non-purified and purified polymers were analysed by ATR-FTIR (Figure 15 A-D).
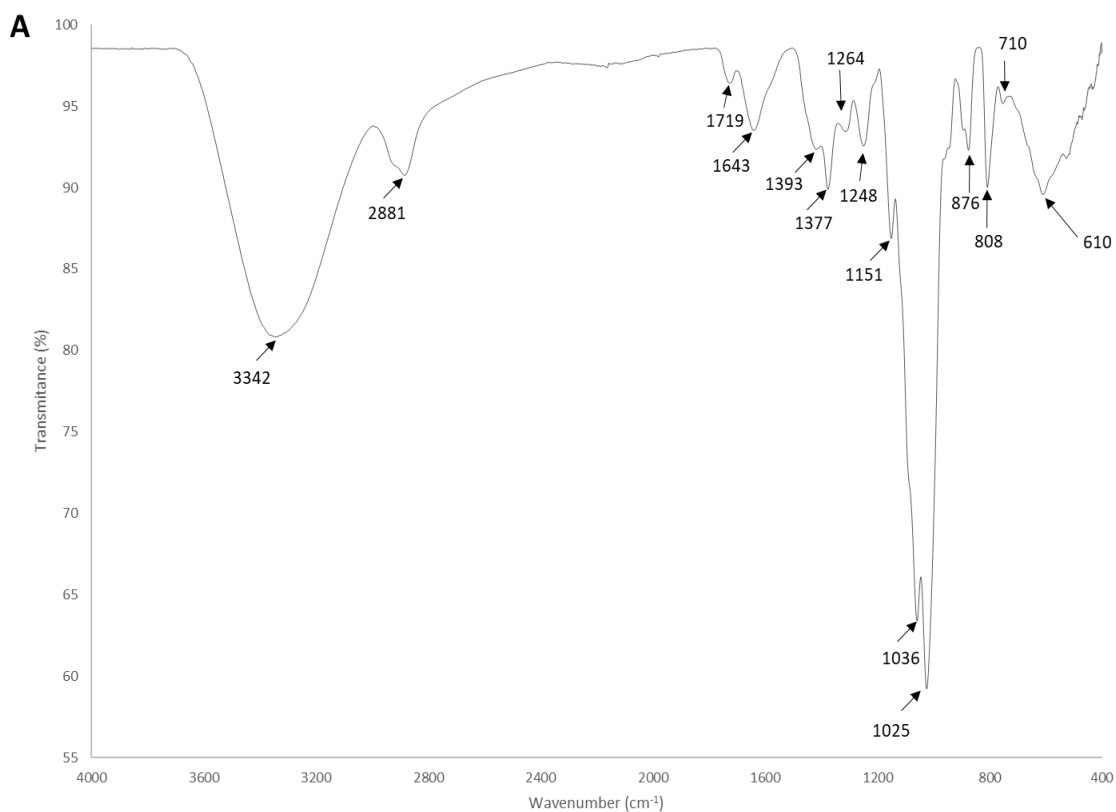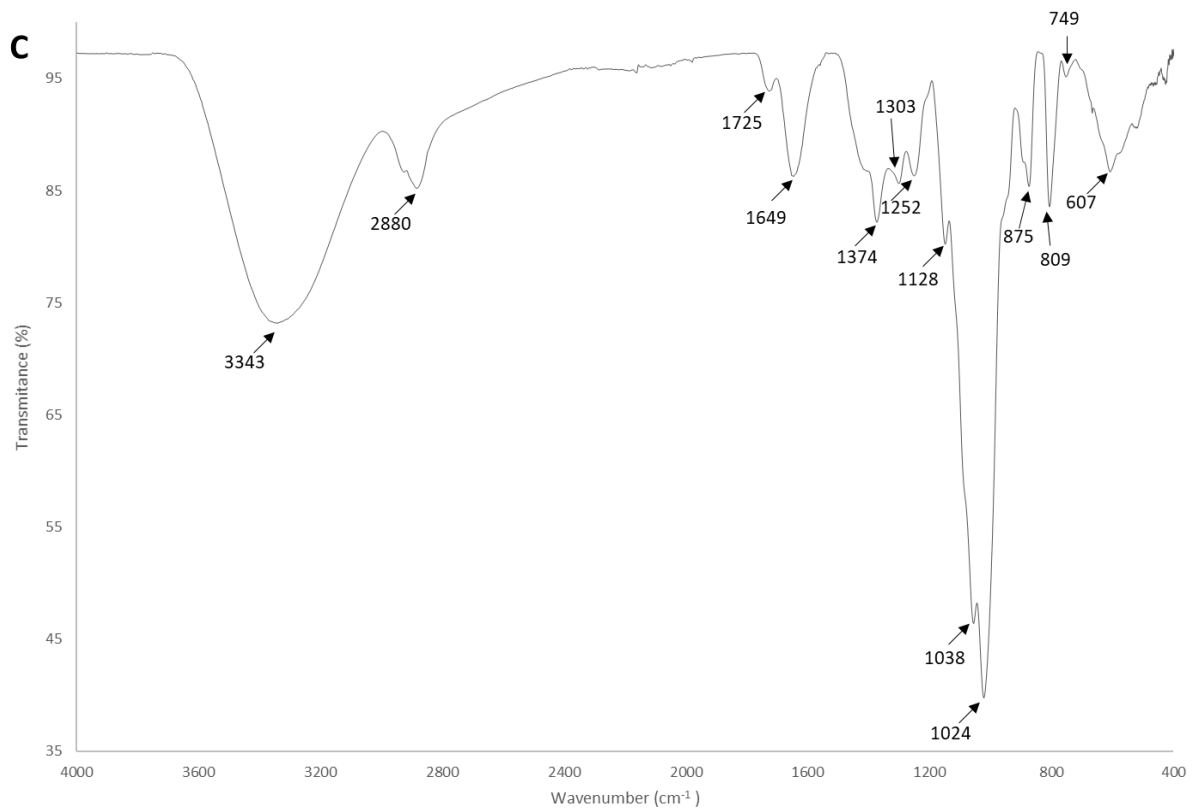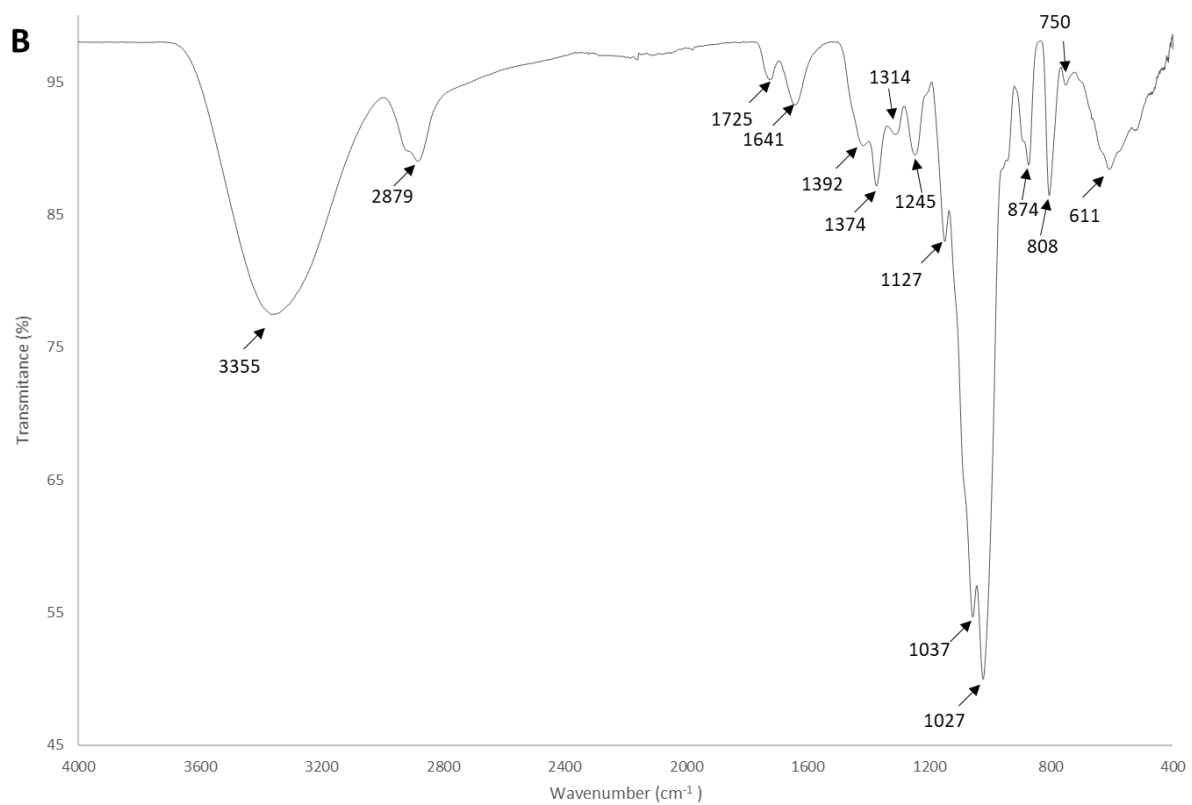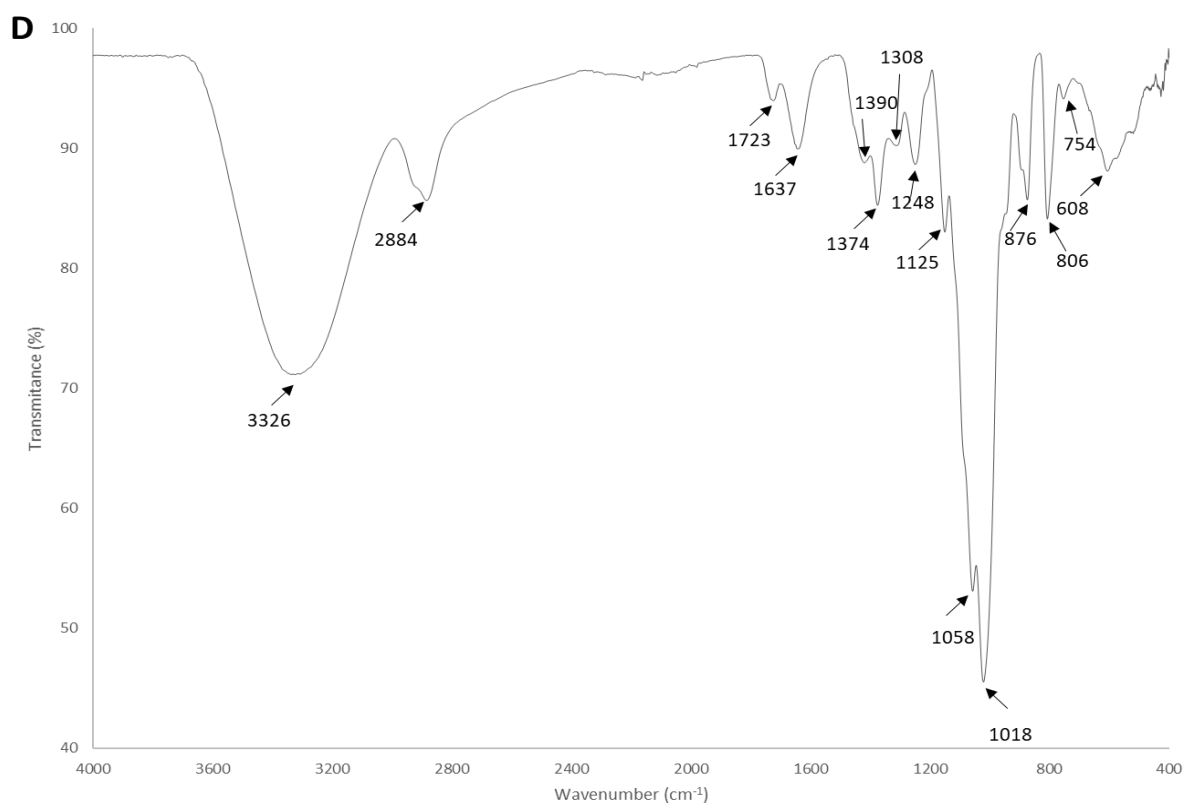
Figure 15- ATR-FTIR spectrum of Konjac Glucomannan. ATR-FTIR spectra of Konjac Glucomannan before (A) and after being submitted to different purification procedures, including filtration (B) followed by washing with activated charcoal (C) or dyalisis (D).

Regarding to the literature and analysing all FTIR spectra (Figure 15 A-D), the peaks between 3600-3000 cm^{-1} may correspond to the stretching vibration of O-H groups, while the peaks around 2920-2850 cm^{-1} can be attributed to the asymmetric and symmetric stretching in CH$_2$ of the glycosidic ring [100, 267-269]. In addition, the peak around 1730 cm^{-1} may be characteristic of the C=O stretching vibration in carbonyl from acetyl moieties [268, 269]. In the region of 1500-1000 cm^{-1}, the peaks can be attributed to the stretching vibration of C-C, C-O and C-H of the glycosidic units of the polymeric backbone [100, 269]. The possible presence of mannose units may be also identified by the presence of peaks around 875 and 805 cm^{-1} [268].

The remaining peaks appearing in the spectra may not belong to the backbone of KGM polymer, and may probably be attributed to contaminants. It is observed in all FTIR spectra (Figure 15 A-D) not only peaks between 1250-1300 cm^{-1} that can be attributed to C-O aromatic stretching vibrations in phenolic moieties or to N-H bending coupled with C-N stretching of proteins (amide III), and also peaks between 1650-1630 cm^{-1} corresponding to the C=O stretch in proteins (amide I) [270-272]. The presence of phenolic moieties may also be supported by the appearance of peaks between 1390-1400 cm^{-1} that could correspond to O-H bending in phenols [273]. However, no significant differences were found on these peaks independently of the purification methodology applied to each KGM batch. Comparing with others biopolymers described in

literature, minor differences were also observed in the FTIR spectra of non-purified and purified pectin [140]. However, it is important to highlight that the spectra of purified polymers exhibit a similar pattern to the one of initial KGM, supporting that the purification procedures do not introduce significant structural changes in the polymer. The rheological behaviour of non-purified and purified polymers was also investigated in order to understand the influence of the different purification methodologies on the viscosity of the corresponding polymeric solutions (Figures 16-17).
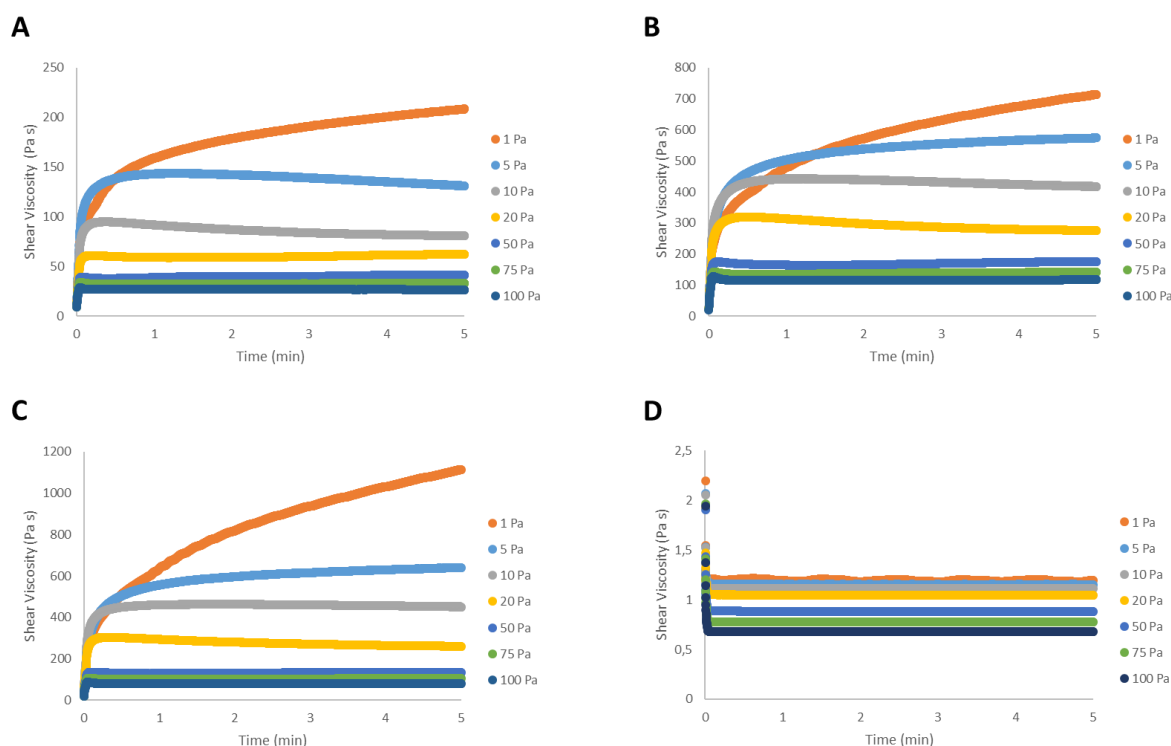
Figure 16 – Rheological characterization of KGM solution. Evaluation of shear viscosity with time for different shear stress values of 2 wt% non-purified KGM solution (A), 2 wt% KGM solution resultant from the polymer purified by filtration through a membrane with a pore size of 0.8 µm (B), and by filtration followed by washings with activated charcoal (C) or dialysis (D).

In 2 wt% nonpurified KGM solution, the shear viscosity increases for a shear stress of 1 to 5 Pa and decreases for a shear stress of 10 to 100 Pa (Figure 16 A). In 2 wt% KGM solution purified by filtration using a membrane pore size of 0.8 µm, the shear viscosity increases from a value of shear stress of 1 to 20 Pa, and decreases for values of shear stress of 50 to 100 Pa (Figure 16 B). In 2 wt% KGM solution purified by washings with activated charcoal, the shear viscosity increases for a shear stress of 1 to 20 Pa and decrease for a shear stress of 50 to 100 Pa (Figure 16 C). In 2 wt% KGM solution purified by dialysis, the shear viscosity decrease in all range of values of shear stress of 1 to 1000 Pa, (Figure 16 D). Regarding to the literature, when the shear stress decreases, the shear viscosity increases, leading to a shear thinning behaviour of KGM, which it is observed in 2 wt% KGM solution purified by filtration using a membrane pore size of 0.8 µm, in 2 wt% KGM

solution purified by activated charcoal wash and in 2 wt% nonpurified KGM solution, while for in 2 wt% KGM solution purified by dialysis has a Newtonian fluid flow characteristics [274]. A similar test was performed to 1% KGM solutions, where it was observed for the nonpurified polymer an increases of shear viscosity for a shear stress of 1 to 20 Pa and further decreases for a shear stress values between 50 to 100 Pa (Figure 17 A). In 1 wt% KGM solution purified by filtration using a membrane pore size of 0.8 μm, from a value of shear stress of 1 to 20 Pa, the shear viscosity increases, however the shear viscosity decrease for values of shear stress of 50 to 100 Pa (Figure 17 B). In 1 wt% KGM solution purified by washing with activated charcoal, the shear viscosity increases for a shear stress of 1 Pa and decrease for a shear stress of 5 to 100 Pa (Figure 17 C). In 1 wt% KGM solution purified by dialysis from a value of shear stress of 1 to 100 Pa, the shear viscosity decreases (Figure 17 D). Thus, a shear thinning behaviour of KGM is observed in 1 wt% KGM solution purified by filtration using a membrane with pore size of 0.8 μm, and in 1 wt% non-purified KGM solution, while the 1 wt% KGM solution resultant from the polymer purified by dialysis has a Newtonian fluid flow characteristics [274]. The 1 wt% KGM polymer resultant from purification by dialysis has a behaviour of Newtonian fluid because the solution was in non-optimal conditions.
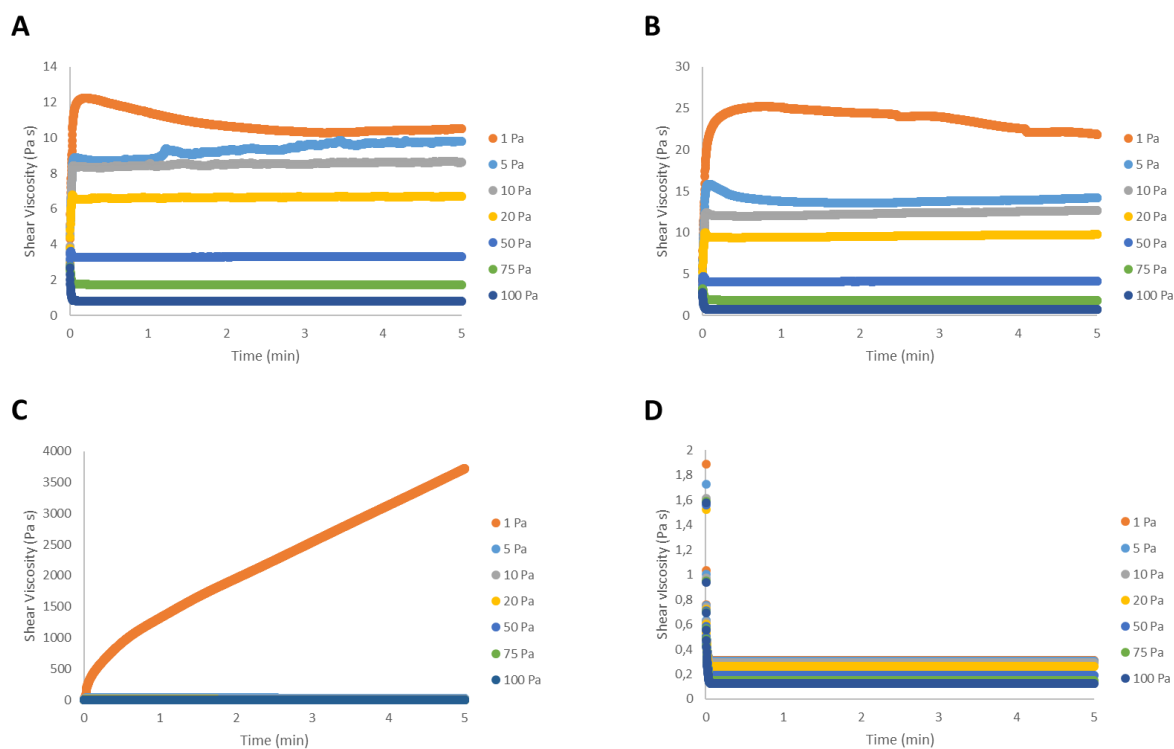
Figure 17– Rheological characterization of KGM solution. Evaluation of shear viscosity with time for different shear stress values of 1 wt% nonpurified KGM solution (A), 1 wt% KGM solution resultant from the polymer purified by filtration through a membrane with a pore size of 0.8 μm (B), and by filtration followed by washings with activated charcoal (C) or dialysis (D).

The injectable hydrogels for tissue engineering should be shear thinning, i.e, at low shear stress, the shear viscosity should be high, whereas for higher shear stress values the viscosity should decrease. Thus, this rheological study allowed to conclude that the KGM polymers purified through filtration using a

membrane with pore size of 0.8 μm (Figures 16B and 17B), or combination of this procedure with washings with activated charcoal (Figure 19A), may be used as injectable hydrogels for tissue engineering, similarly to non-purified KGM solution. However, the KGM purified through filtration followed by dialysis did not exhibit this characteristic shear thinning behaviour neither in 1 wt% or 2 wt% KGM solutions, although according to ^{1}H-NMR results, it appears that this methodology is probably the most efficient on removing all the contaminants, being this result also supported by the assays regarding protein and polyphenol contents (Figure 15). Altogether, the obtained results suggest that the purification procedure involving filtration followed by washings with activated charcoal appear to be the most adequate method for KGM purification, since it removes most of contaminants and still preserve the mechanical properties of the polymer. Furthermore, it is important to highlight that the shear viscosity of solutions of KGM purified through this methodology is significantly higher than the one from solutions resultant from the non-purified KGM, supporting that the applied purification procedure appears to efficiently remove a significant fraction of contaminants present in the initial polymer. The removal of contaminants often results in an increase of the viscosity on solutions resultant from the purified polymer.

3. Economic viability of purification processes

To establish a connection between the efficiency *vs* economic viability of each purification process, a study of the total cost of each purification protocol was analysed. One of the main goal of this thesis was to establish a simple and low cost purification method for KGM. Thus, it was found that the protocol including two filtration steps followed by washing with activated charcoal was cheaper than the alternative procedure including dialysis (Figure 18).
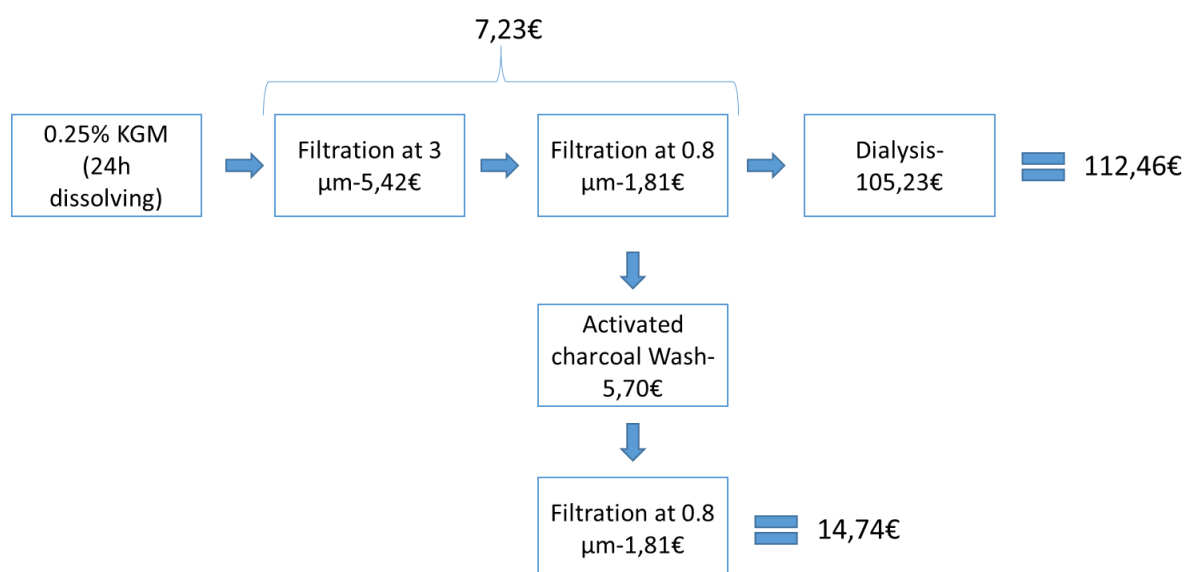

Figure 18- Total cost calculated for the purification of one gram of KGM.

This study further supports the use of the purification protocol including filtration combined with washings with activated charcoal as the most efficient and cost-effective method for purification of KGM, while it preserves structural properties and shear-thinning behaviour of the polymer.

Conclusions and Future Work

KGM has many attractive features which making this natural polysaccharide an interesting target to be applied in tissue engineering such as bone cancer. However, the polymer to be applied in the human body needs to be free of contaminants to not cause a rejection of the implanted biomaterial by the human body. For that, the polymer was purified by two different ways: filtrations followed by activated charcoal wash and the combination of filtration with dialysis. The presence of contaminants, such as proteins and polyphenols were assessed and it was showed that filtrations followed by washing with activated charcoal decrease more significantly the proteins amount, whereas, the combination of filtration with dialysis decrease more significantly the polyphenols content. Concerning to the KGM structure in the different purification techniques, ^{1}H-NMR, ATR-FTIR and rheology were performed. Only the ^{1}H-NMR of KGM purified by filtration and dialysis presents only peaks corresponding to KGM, which means that there no impurities, although the acetylation degree is the same in all purification techniques demonstrating that any of these purification methods change the structure of the polymer. In all ^{1}H-NMR spectra of purified polymer using the different techniques, peaks corresponding to polyphenols do not appear maybe because of the homogeneity and viscosity of the polymer and maybe due to the ^{1}H-NMR sensitivity. In ATR-FTIR spectra of KGM purified by filtration, filtrations followed by activated charcoal wash and filtration with dialysis, it was showed besides peaks corresponding to the polymer but also peaks corresponding to impurities such as phenols, amide III and amide I. However, all of ATR-FTIR spectra showed no changes in the polymer structure demonstrating that the purification techniques do not affect the KGM structure. Regarding to rheology, in 2wt% purified polymer by filtration and filtrations followed by activated charcoal wash, but also 1 wt% purified polymer by filtration have a shear thinning behaviour, whereas, 2 wt% purified polymer by dialysis and 1 wt% purified polymer by filtrations followed by washing with activated charcoal and combination with filtration and dialysis have a Newtonian fluid flow characteristics. The better performing method is the filtrations followed by activated charcoal washes because the resultant KGM displayed higher shear viscosity comparing with non-purified polymer due to the removal of the contaminants. As the main goal of this thesis is define a purification protocol for KGM that is efficient, low cost and easy to reproduce, but, at same time a purification method that does not change the structural properties and the shear-thinning of the polymer, which is filtrations followed by washing with activated charcoal. However, this method was not as effective in removing the polyphenols content. This fact can be overcome using a neutral activated charcoal or to do the same procedure twice (that was done in the pectin purification using the same method). Afterwards, the same procedure but with a neutral activated charcoal and the same procedure performed twice needs to be compared between them and compared with the same methods discussed here. After establish a purification protocol that decreases the major contaminants and preserves the structure properties and shear thinning behaviour, an injectable KGM purified hydrogel should be developed. Test on biocompability, *in vitro* cytotoxicity and the ability to promote bone formation should also be addressed.

Viscoelastic properties measurement with polydopamine nanoparticles will be necessary to assess the injectability of the KGM hydrogel.   Hyperthermia induction upon irradiation should be tested.

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