UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ

NATAN WIELE

OPTIMIZATION AND EVALUATION OF THE DECELLULARIZATION OF A BIOHYBRID MATERIAL BY SUPERCRITICAL CO_2 TREATMENT

PONTA GROSSA

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Optimização e avaliação do processo de descelularização de um material biohíbrido através de tratamento com CO_2 supercrítico

Course conclusion paper as a requirement to obtain the bachelor's degree in Bioprocesses and Biotechnology Engineering at the Federal Technological University of Paraná (UTFPR).
Advisor: Juliana Vitória Messias Bittencourt.

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ABSTRACT

Recently, decellularized extracellular matrix (ECM) has been reported to provide important components to be used as a scaffold for cell delivery on damaged tissues after ischemic heart diseases. Among different decellularization techniques, the supercritical fluids are being increasingly used in living and synthetic tissues, offering a fast and less toxic decellularization process to further biological applications. The supercritical carbon dioxide is the most commonly candidate to replace or compensate the large use of toxic detergents like the Sodium Dodecyl Sulfate (SDS) due to its extraction properties. However, in this work, the synergetic effect between both SDS and supercritical CO_2 was used to decellularize the biohybrid material developed by the Laboratory of Vascular Translational Science coated with gelatin to mimic the ECM and with 14 and 7 days (G14 and G7) of cell culture, for later use as a cardiac graft. After confocal analysis of the fluorescent labeled biopolymers after treatments, it was seen the potentialized decellularization effect of SDS 0.5% + $scCO_2$ only in the G14 since almost no DNA was found. DNA quantifications were performed within the immersion liquid (SDS) and the biopolymers after treatment to check the amount of DNA that was managed to remove. Finally, this condition appeared to have a decellularization effect in a much shorter time span than the standard decellularization process and with a much smaller amount of solvent.

Keywords: decellularization; supercritical carbon dioxide; scaffold.

RESUMO

Recentemente, a matriz extracelular descelularizada tem sido utilizada como provedora de importantes componentes quando usada como enxerto para recomposição celular em tecidos danificados após doenças isquêmicas do coração. Entre as diferentes técnicas de descelularização, os fluidos supercríticos estão sendo cada vez mais utilizados em tratamentos de tecidos vivos e sintéticos, oferecendo uma descelularização rápida e menos tóxica. O gás carbônico supercrítico é o candidato mais comum na substituição ou compensação do uso abrangente de detergentes tóxicos como o *Sodium Dodecyl Sulfate* (SDS), graças às suas propriedades de extração. No entanto, neste trabalho, o efeito sinergético de ambos SDS e CO_2 supercrítico foi usado para descelularizar o material biohíbrido desenvolvido pelo *Laboratory of Vascular Translational Science,* o qual é revestido com gelatina para mimetizar a matriz extracelular, com 14 e 7 dias de cultura celular (G14 e G7), para posterior uso como enxerto cardíaco. Após as análises realizadas em microscópio confocal dos biopolímeros marcados fluorescentemente após tratamentos, foi observada uma descelularização potenciada quando usados SDS 0,5% + $scCO_2$ apenas no G14, tendo em vista a baixa quantidade de DNA encontrada no polímero. As quantificações de DNA foram realizadas nos líquidos de imersão (SDS) e nos biopolímeros após os tratamentos afim de verificar a quantidade de DNA que foi possível retirar com cada tratamento. Finalmente, a condição de SDS 0,5% + $scCO_2$ apresentou um melhor efeito de descelularização em menos tempo e utilizando uma quantidade menor de solvente quando comparado com o processo padrão.

Palavras-chave: descelularização; gás carbônico supercrítico; enxerto.

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ABBREVIATIONS

CBMN	Institute of Chemistry & Biology of membranes & Nano-objects
G7	Biopolymer coated with gelatin and with 7 days of cell culture
G14	Biopolymer coated with gelatin and with 14 days of cell culture
IHD	Ischemic Heart Disease
LVTS	Laboratory for Vascular Translational Science
$scCO_2$	Supercritical Carbon Dioxide
SCFs	Supercritical fluids
SDS	Sodium dodecyl sulfate
TE	Tissue Engineering

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1 INTRODUCTION

One of the major contributors to the global disease burden is the ischemic heart disease (IHD), which has caused more than 7.0 million deaths in 2010, reason why is the leading cause of death worldwide. The absolute number of years of life with disability has increased 29% in 20 years (1990 – 2010) even if the age-standardized IHD mortality has decreased (KHAN *et al*., 2020).

Furthermore, it was projected that IHD-related healthcare costs would rise from $126.2 billion to $177.5 billion between 2010 and 2040 in the USA, thus being categorized as the major cause of devastating health expenditure in the country. Nevertheless, by age group, in 2017 the age distribution of IHD worldwide shows higher occurrence between 75 and 95 years (Annex A) and, as shown in annex B, the highest age-standardized incidence of IHD in 2017 were seen in North Africa, Middle East Europe and Central Asia (KHAN *et al*., 2020).

Over the last years, lot of studies about the regeneration of diseased heart valves have been reported. Tissue regeneration demands a series of solutions to be accomplished, for example acute rejection, the degradation of the graft without losing its properties, cell recolonization of the new recipient, right recipient penetration in the graft, etc. According to those studies, heart valve replacements are commonly carried out using biomaterials like synthetic biodegradable polymers, being polylatic acid and polyglycolic acid examples (SAWADA *et al*., 2008).

Whether derived from synthetic or natural materials, tissue engineering (TE) scaffolds must possess certain characteristics for their specific applications, such as sterility, be porous and mechanically strong, have biocompatibility, etc. Additionally, in order to the patient's cells be able to access this material for its proliferation, the scaffold fabrication process must be aware of some characteristics, including durability of the mechanical strength, not dehydration of the scaffold and preservation of the extracellular matrix (ECM) during decellularization (CASALI *et al.,* 2018)**.**

Decellularization can be performed using different techniques such as physical, chemical, enzymatic and detergent treatments. Although these methods prove efficacy, novel methods have been tested to reach a more functional decellularization process, preserving the material properties, in shorter times and without using solvents for long periods. One of those new methods is the supercritical carbon dioxide ($scCO_2$) application (HALFWERK *et al*., 2018).

1.1 Biomaterials

1.1.1 Biomaterials generalities

Established in the mid twentieth century, the word Biomaterial is defined as “any materials that are coming in contact with human or animal biologic systems in order to perform their intended function”. Its definition has been developed over the years based on the new applications. The biomaterials used are constantly growing over the areas of biology, chemistry, material engineering and mainly in medicine. Usually, it is not the final product, but the interaction means with the medical product (KULINETS, 2015).

The classification of a biomaterial can be given by the degree of their interaction with the body, labeled as “bioinert”, “biocompatible” and “bioactive”. As demonstrated in the annex C, biomaterials can also be classified by its level of “smartness”, divided in four groups: Inert, defined as “the ability to be just biocompatible/bioinert and not exert any additional biological benefits”; Active, defined as “the ability to release a one-way bioactive therapy to provide an interaction with biological processes or with the surrounding environments”; Responsive, when the biomaterial has the ability to sense a stimulus (internal or external), such as pH levels, antigens or temperature, and react to it by releasing specific therapeutic agents; and Autonomous, the highest degree of smartness nowadays, when the biomaterial can independently adjust its properties and functions depending on changes in its environments (MONTOYA *et al*., 2021).

According to their function classifications, biomaterials can be made by different materials as metals, ceramics, polymers, carbons, or composite materials (Annex D) (RATNER, 1996). Besides those synthetic biomaterials, they can also be nature derived like plant or tissue-derived, and semi-synthetic or hybrid. Metals biomaterials are commonly used as orthopedic implants while ceramics ones are used in dental restorations and composites ones in a wide range of medical applications. Additionally polymeric biomaterials are gaining ground in the medical field, more specifically in tissue engineering sector acting as prosthesis, skin, or cartilage. Other forms such as liquid, films and hydrogels are seen in the modern classes of polymeric biomaterials (KULINETS, 2015).

1.1.2 Biopolymers

Polymers are a class of molecules consisting of a long chain of repeat units of building blocks called monomers. Biopolymers or natural polymers, therefore, are defined as “polymers formed under natural conditions during the growth cycles of all organisms”. A vast number of research and production of biodegradable polymers have been made due to their environmental importance, since microorganisms and enzymes can degrade them. Those polymers can be synthetized by different microorganisms as bacteria and fungi and among them, complex hydrocarbon polymers, polysaccharides in particular, such as xanthan, curdlan, pullulan, chitosan and hyaluronic acid have drawn attention in view of their properties and applications, principally in the medical sector (RAO; BHARATHI; AKILA, 2014).

This type of materials has been developed for different uses like packing, cosmetics, food additives, industrial plastics, biosensors, medical materials, etc. Once implanted they do not induce any immunological rejection or reaction, they have great potential to be used in the development of therapeutic devices, acting as prosthesis, scaffolds (3D porous structures), sustained release drug delivery vehicles, etc. (REBELO; FERNANDES; FANGUEIRO, 2017).

1.2 Supercritical fluids

1.2.1 Supercritical fluids generalities

Discovered in 1822 by Baron Charles Cagniard, supercritical fluids (SCFs) are substances for which both pressure and temperature are above the critical values (Annex E). These substances belong to the relatively new high-pressure technology, used as a tool for the development of processes with minimal environmental impact by using fewer toxic residues and energy in its procedures. SCFs presents gas and liquid properties, such as diffusivity and density, what makes them great solvent for different applications. One of the main advantages lays on the fact that the SCFs, usually a gas at atmospheric pressure and temperature, can be separated, recovered and recycled after treatment (KNEZ *et al*., 2014).

Since Pression and Temperature are the main factors, small variations can easily change significantly the SCF’s properties, like the density or viscosity. The high power of diffusivity of SCFs combined with the low viscosity provides an interesting

transportation and dragging capacity, which can be implemented in extraction processes (BRUNNER, 2010).

1.2.2 Supercritical carbon dioxide

Supercritical carbon dioxide ($scCO_2$) is frequently used in processes with supercritical fluids. Besides being abundant, inexpensive, nonflammable, and nontoxic, it has low critical temperature and pressure values, 31ºC and 7,3 MPa, respectively, which can be easily reached. Nonpolar organic compounds are highly soluble in $scCO_2$, therefore it is used to extract hydrocarbons, essential oils and phospholipids, among others. (NOYORI, 1999).

However, the removal of polar molecules like proteins and DNA by $scCO_2$ can also be done by using polar cosolvents like ethanol (CHOU *et al*.,2020). By all of its characteristics, $scCO_2$ is a subject increasingly studied in tissue engineering for the decellularization process, removing nucleic acids and cell debris while preserving the ECM and replacing detergent methods (SEO; JUNG; KIM, 2018). This article demonstrates the $scCO_2$ technology effect to obtain an ECM decellularized from a heart tissue using an immersion test in ethanol 100%.

1.3 Excalybur Project

The Excalybur project is an ANR (Agence Nationale de la Recherche) young researcher project detained by Teresa Simon-Yarza at the LVTS (Laboratory for Vascular Translational Science) whose purpose is to develop a biohybrid material which will be used to repair the damaged heart tissue after heart attack. In this project, the LVTS is responsible for the formulation of polysaccharides-based 3D hydrogels with controlled and tunable physico-chemical properties, mimicking the native cardiac tissue to reinforce the myocardial wall, providing a healthy environment to the cardiac cells to favor tissue repair, using a specific biological composition of a cell secreted ECM coating produced by fibroblasts grown inside the pores of the polymer.

Once the fibroblasts have secreted enough ECM, after 10 to 21 days of culture, these cells must be completely removed before the patient cells colonize the obtained biohybrid material. CBMN (Institute of Chemistry & Biology of Membranes & Nano-objects) is responsible for the different supercritical tests to decellularize the biomaterial without removing the extracellular matrix in order to minimize later immune

response and replace the golden standard and long (5 to 7 days) Sodium dodecyl sulfate (SDS) decellularization protocol used commonly.

SDS is considered as a very effective solvent to cellular components removal when compared to other detergents, it has a higher yield in the complete removal of nuclear remnants and cytoplasmic proteins. Another effect of this solvent is the decrease of glycosaminoglycans (GAGs) concentration due to its disruptive effect on the native tissue structure (GILBERT; SELLARO; BADYLAK, 2006). Nonetheless, for the same reason, SDS is more disruptive to the ECM, causing a loss of collagen integrity (CRAPO; GILBERT; BADYLAK, 2011).

Also, this solvent has been cited in several publication on tissue decellularization, such as the ovine aortic root, where favorable effects were demonstrated after the $scCO_2$ decellularization within the detergent solution. The assisted process was made using a pressure of 250 bar, leading to a satisfactory removal of endotheliocytes and fibroblasts from the aortic walls, a low toxicity of the tissue, slights alteration of the ECM of the aortic valve and an increase in the porosity (GAFAROVA *et al*., 2020).

The biohybrid and decellularized material is later used as a graft and soft tissue repair in hearts after IHD which helps to increase the patient life quality and alleviate heart failure symptoms. This study, therefore, focuses on the preparative conditions for the decellularization of a biopolymer produced by the LVTS using the $scCO_2$ technique. Determining the optimal conditions for obtaining biologic scaffold without damaging the ECM and high decellularization was one of the goals of this study. For this purpose, the best decellularization treatment and technique using different approaches were investigated in detail along with the effect of parameters such as pressure, time and cosolvent.

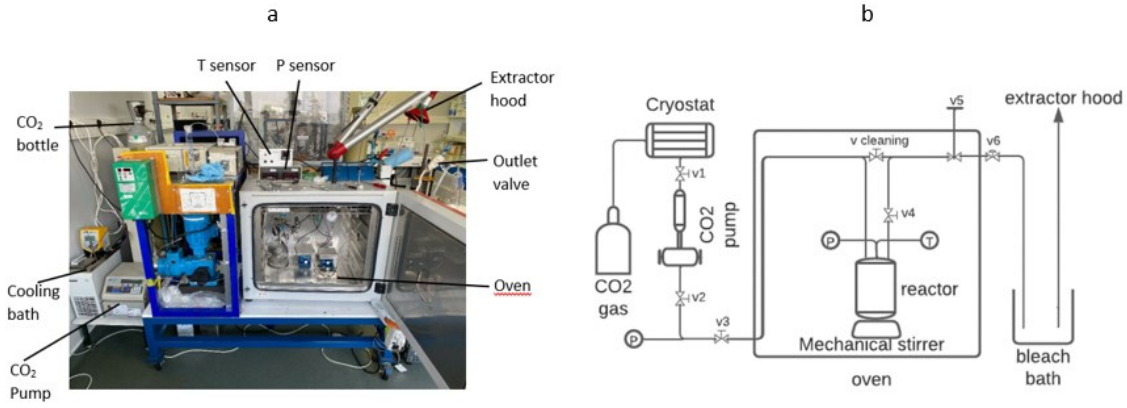
2 MATERIALS AND METHODS

2.1 Supercritical CO_2 decellularization

1 cm circular polymeric membranes were obtained by LVTS after homogeneous high-density cell seeding (2 million) and were stored at -21ºC before experiments. The biohybrid polymers were made by a combination of Pullulan, Dextran, and a protein coating made either of gelatin or collagen. Decellularization was conducted on membranes coated with gelatin after 7 (G7) or 14 (G14) cell culture days.

Decellularization assays were carried out using a device specially set up in the CBMN laboratory, represented in the figure 1. The CO_2 was first liquified at -1ºC (Lauda Eco RE420, France) and then introduced into the reactor via a CO_2 pump (Teledyne ISCO 260D, Serlabo, France) at a rate of 5ml/min. Reactor is hold in a regulated oven (Binder GmbH, Tuttlingen, Germany). Experiments were conducted at 170 bar and 45ºC for 1.5 hours. All tests using $scCO_2$ were called EXCAL and they were coded in the laboratory according to the parameters used.

Figure 1 - Representation of the decellularization device, (a) real picture, (b) plan



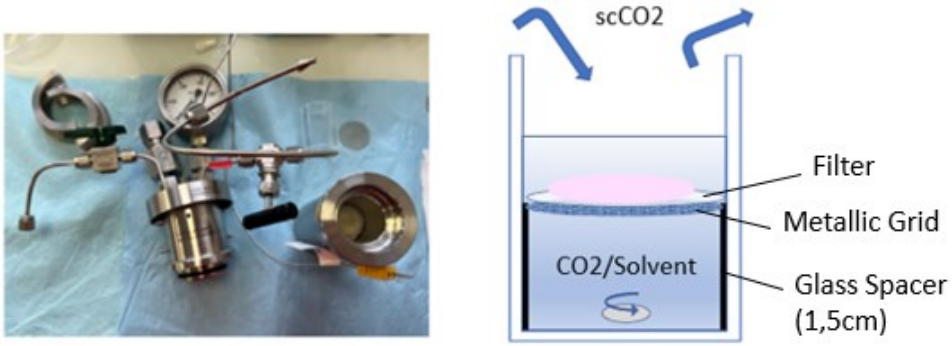
Source: Own authorship (2022)

A needle valve (20SC4082, Autoclave Engineers, Unites States) allows to depressurize the system. Two types of experiments were conducted: with CO_2 flow or in immersion.

For the immersion experiments, the reactor consisted of a quick-opening stainless steel reactor with capacity of 50 mL and an internal diameter of 3 cm (ROR reactor, Autoclave, France). Reactor was agitated by a magnetic stirrer at 500 rpm. 15 mL of solvent (water, SDS 0.5 or 1 %) were introduced in the reactor as the 1.55 cm magnetic bar. The membranes were deposed in the solvent on a metallic grid and a

filter paper, a 1.5 cm glass spacer ensure that magnetic bar do not touch the membrane. The setup is shown on figure 2.

Figure 2 - ROR Reactor, assembly and representation

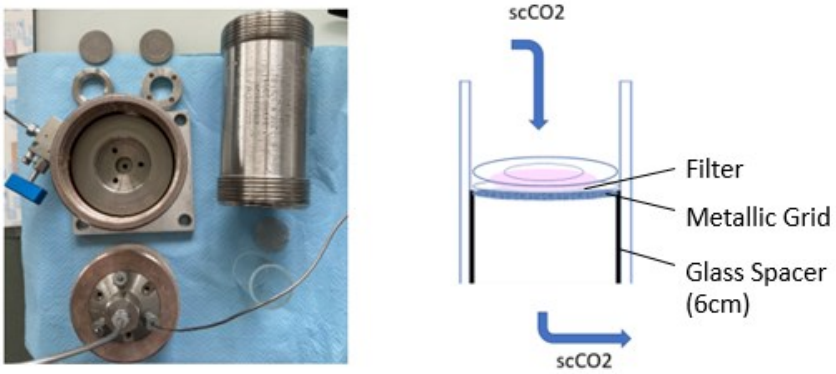


Source: Own authorship (2022)

For immersion experiments 1 membrane was treated at a time except for replication experiments of G14 where 2 membranes were treated at a time. For each immersion test, a control is performed in the same conditions but without $scCO_2$, those tests were called AIR (with 1 or 2 membranes as described before).

For flow tests, the EFS 100 mL reactor (3 mL internal dimeter) diameter (EFS Reactor, Autoclave, France) was used. The membranes were deposed on a metallic grid and a filter paper, a 6 cm glass spacer ensure the positioning of the membrane as shown in figure 3. The CO_2 flows from top down in the reactor. 1 membrane was treated at a time.

Figure 3 - EFS reactor, assembly and representation



Source: Own authorship (2022)

2.2 Membrane analysis

2.2.1 $scCO_2$ post treatment

Before and after each experiment, diameter and weight measurement were taken for each membrane for analysis of physical consequences of each treatment, like shape and color. Furthermore, the solvents in immersion were collected and frozen at the end of each experience for pH measurements and DNA indirect quantification.

The $scCO_2$ treated biopolymers were immersed 30 minutes in PBS solution if they belong to flow conditions, then dried and frozen in 6 well plates until the fluorescent labeling performed for the confocal microscopy analysis.

For the experiments using SDS as a solvent, biopolymers were immersed 1 hour twice in PBS solution under agitation at ambient temperature, then a last PBS washing was performed over the night before being frozen. At the same time, the 3 washing baths were grouped together then frozen for further analysis. Also, in the same experiments, a replication of tests was done to check its effectiveness.

2.2.2 Confocal analysis

Differently from the conventional microscopy, where the samples are illuminated in an entire field, producing background “noise”, even if the brightest and highest intensity light is at the focal point of the objective lens, the confocal microscopy uses the fluorescent light coming back from the sample after projecting an excitation beam of incoming light. The objective of this microscopy allows to focus the incoming light on a small spot in the sample (about 0.5 µm) (NWANESHIUDU *et al.*, 2012).

Fluorescence confocal microscopy uses fluorophores, molecules with a specific design to target and identify subcellular structures such as cytoplasm, nuclei

or mitochondria. The excitation light in fluorescence confocal microscopy is usually provided by a laser at a wavelength that will also excite a specific fluorophore. The specificity of these molecules promotes a better detection of different parts of cellular samples since different fluorochromes can be used at the same time once using different wavelengths (NWANESHIUDU *et al*., 2012).

After $scCO_2$ treatment, membranes were thawed, cut in half, and stained using the LVTS protocol, in which the samples were fixed with 500 µl of Formol Aldehyde 4% for 1h at 4ºC, later washed three times with 500 µl of PBS 1X (5 min each wash under stirring), then the samples were permeabilized with 500 µl of TRITON 0.1% for 45 min at room temperature and stirring, followed by another washing process. At last, the samples were colored with 500 µl of a solution with 2 different fluorophores (DAPI for nuclei and DNA visualization in blue and TRITC for cytoskeleton visualization in red) for 1 h at room temperature, under stirring and sheltered from light. Finally, the samples underwent a final wash and were stored in PBS 1X at 4ºC until confocal analysis. The polymeric membranes have already FITC in their composition, a fluorophore for hydrogel visualization in green.

Microscopy visualizations were made using the ZEISS confocal microscope and the Zen (blue edition) software ®, where the same fluorophores for nuclei and hydrogel was used in 400-500 nm and 505-557 nm, respectively. The fluorophore TRITC (547-572 nm) was visualized using the Alexa Fluor 568 parameters, containing a range of 553 to 568 nm. The stained polymeric membranes were analyzed in 3 different zones, center, right edge and left edge and at an average depth of 180 µm with a number of photos per sample between 100 to 250.

For each type of cellularization duration, to see how the biomaterial was cellularized before treatment, a control biopolymer was analyzed, that had undergone neither solvent nor $scCO_2$ treatment. This condition is also used to validate the labeling protocol of experimental biopolymer.

This analysis is a qualitative methodology where a visual observation is made in order to estimate the efficiency of different treatments. The success of the decellularization experiments is determined when the fewer number of blue (DNA) and red (cytoskeleton) molecules are present inside the biopolymer pores.

2.2.3 Nucleic acids quantification in supernatants

To complete the analysis with confocal microscope of the membranes after immersion assays, the supernatants were removed from the ROR reactors and stored in the freezer to further nucleic acids quantification by Nanodrop after precipitation.

The measurement of a sample absorbance at 260 nm is one of the most commonly used method to estimate nucleic acid concentrations, also the 260/280 and 260/230 ratios are used to determine the presence of chemical and biological contaminants in the samples during DNA extraction process (GARCÍA-ALEGRÍA *et al*., 2020).

The 260/280 ratio (R) is a method to determine if the DNA samples are contaminated by proteins or RNA, where an increase in the ratio means a RNA contamination, since the RNA is a single strand of DNA, so increasing the average absorption coefficient. A DNA sample with R coefficient between 1,8 and 2,0 is considered pure, when R < 1,7 it means a protein contamination and a R > 2 means an RNA contamination (Nadir s. d.). This parameter is used in precisely methodologies where DNA purity is fundamental.

To extract the DNA from the supernatants, a volume of 10 ml of the samples were transferred to a clean tube, where 2.5 V (25 mL) of cold ethanol 96% and 0.1 V (1 mL) of sodium acetate 3M were added. The mixtures were left 30 minutes at -20 ºC and then centrifuged at 17000 RCF at 4ºC (Rotanta 460 RF, Hettich). The supernatants after centrifugation were eliminated and the pellets were dried at 37ºC for 10 minutes. Finally, the precipitates were dissolved in 20 µl of pure water and stored in 4ºC. The dissolved precipitates were analyzed using the Nanodrop system (Thermo Scientific Nanodrop), which gives the concentration of DNA based on a spectrophotometry method. This methodology consists of an indirect diagnosis of the nucleic acids remaining in membranes after treatment complementary to confocal analysis.

For the replications tests using the G14 an additional washing step was added, the pellets after drying were then dissolved in 2 mL of ethanol 70% and transferred to a 2 mL Eppendorf tube, which were centrifuged for 8 minutes at 14500 rpm. The supernatants were eliminated and another drying step at 37ºC were made until got rid of the ethanol traces and finally 20 µl of pure water was used to dissolve the precipitate after elimination of ethanol traces.

2.2.4 Nucleic acids quantification in the biopolymer

With the aim to quantify the DNA remaining in the biopolymer after the replicate's treatment with the G14, a biomaterial degradation and a cell digestion was performed.

The tablets were cut in half and weighed. The half tablets were cut in smaller pieces to facilitate the degradation and were put in 2 mL Eppendorf tubes. A volume of 250 µl of an enzyme solution with Pullulanase and Dextranase was put inside the tubes and they were incubated for 2 hours at 37ºC under agitation.

When the polymer was completely degraded, 250 µl of a solution of Proteinase K and NaCl/EDTA (1:1) was put in the tubes and the digestion was carried out for 2,5 hours at 55ºC under agitation.

After the digestion time, 100 µl of a saturated solution of NaCl (6M) was put in the tubes to stop the digestion, the tubes were vortexed for 30 seconds and centrifuged for 5 minutes at 20800 G. The supernatants were transferred into new 2 mL Eppendorf tubes, where 350 µl of isopropanol 100% was added. The samples were vortexed for 30 seconds and centrifuged for 15 minutes at 20800 G. Finally, the supernatants were eliminated without removing the precipitate and a washing step were performed.

The supernatants were resuspended with 500 µl of ethanol 70%, the solutions were vortexed for 30 seconds and centrifuged for 30 minutes at 20800 G. If in case the precipitate is not resuspended after vortexing, an ultrasound bath can be performed for 15 minutes. The supernatants were removed and a second washing process was made.

After the second washing process, the tubes were left in the chemical hood overnight to evaporate the supernatants and in the morning the precipitates were resuspended with 40 µl of pure water.

The final solutions were analyzed by the Nanodrop system (Thermo Scientific Nanodrop), in the same way as immersion liquids.

2.2.5 Electrophoresis

After nanodrop analysis of the first assay in immersion tests, an electrophoresis was put into practice to confirm that the spectrophotometer lecture was capturing DNA and RNA.

An agarose gel 0,8% was made using 0,4g of agarose and 50 mL of TBE 0,5 X tampon. The solution was melted in the microwave until homogenized, avoiding boil. After cooling, 10 µl of SYBR safe™ was added. SYBR safe™ is used to replace ethidium bromide in stain process, a potent mutagen and moderately toxic agent after acute exposure. Besides SYBR safe™ being a non-hazardous fluorescent nucleic acid stain, this component was developed to be visualized using a blue light, harmless to DNA, unlike ultraviolet light used in ethidium bromide processes (MARTINEAU; WHYTE; GREER, 2008).

The final solution was poured into the support, without forming bubbles and the comb was added. After gelation, the support was added in the electrophoresis vat, which was covered with TBE 0,5 X.

The samples were homogenized with the loading buffer, applied in the wells and settled at a voltage of 50 V. Two measures were made after 30 minutes and 45 minutes under blue light.

2.3 Statistical analysis

Tests were realized once except for replication study where experiments are reproduced at least 4 times. Number of replicates (N) will be indicated for each result.

Statistical analysis were performed using the Kruskal and Wallis multiple comparisons test available for Excel by Anastats (Rilly sur Vienne, France). This test is a frequently nonparametric statistical procedure used to compare a variety of independent populations with more than 2 conditions and with less than 30 samples each (VARGHA; DELANEY, 1998).

For the biomaterial's DNA quantification after treatments the Outlier Graphpad software was used to detect significant outliers values, which have been removed for further data processing.

3 RESULTS AND DISCUSSION

Aiming to find the best decellularization methodology with the available resources, a few previous tests were made using the flow and batch reactor. All previous tests were made using the G14, due to its greater availability in number of membranes.

Both flow and immersion tests were found in the literature for different applications such as the decellularization of pulmonary arteries for transplant purposes (GIL-RAMÍREZ *et al*., 2020) and the decellularization of heart ECM hydrogel (SEO; JUNG; KIM, 2018).

3.1 Flow experiments

After experiments performed in CBMN laboratory, it was decided that temperature, pressure and time values would be fixed in 45ºC, 170 bar and 1.5 h, respectively.

An added flow experiment was also tested performing in the same conditions to the standard but at 100 bar to study the influence of pressure using G14.

First visual analysis of the physical characteristics of the polymeric membranes after treatment showed that the flow process causes a retraction of the membrane (Figure 4). The polymers present an average mass worth 0.158 g and an average diameter of 1.1 cm. After treatment, the membrane treated at 170 bar had a mass of 0.02 g and a diameter of 0.6 cm while the membrane treated at 100 bar had a mass of 0.01 g and a diameter of 0.56 cm.

A rehydration process with PBS was performed for 30 min in ambient temperature, recovering the mass and diameter of 0.11 g and 0.82 cm for the 170 bar treated membrane and 0.09 g and 0.9 cm for the 100 bar treated membrane.

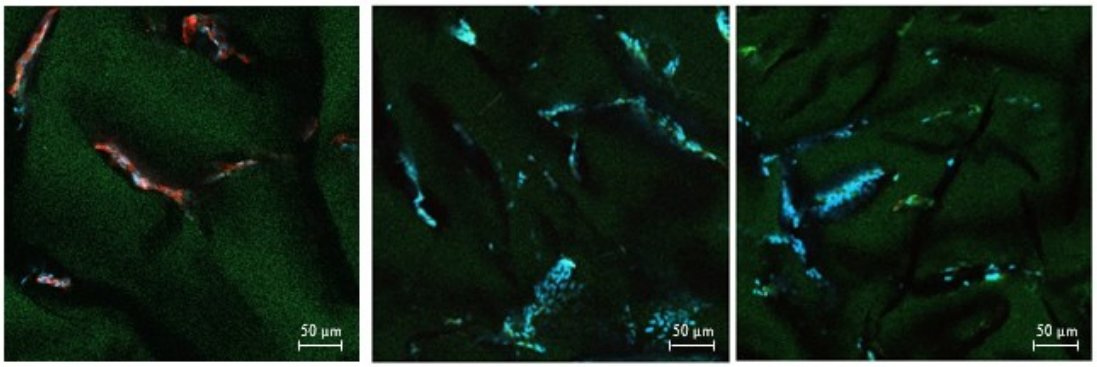
Photography 1 - Physical aspects of membranes before and after flow treatments

Source: Own authorship (2022)

Also, a change in color was observed, untreated membranes have a light pink color due to the phenol red present in culture medium, after flow treatment the polymeric membranes present a yellow coloring due to the CO_2 dissolution, what causes the formation of carbonic acid (thus acidification), producing the yellow color when in contact with phenol red.

Later confocal analyses for both membranes showed that, although they did not have the cytoskeleton any longer, they presented plenty of DNA inside the pores compared to the control membrane as shown in figure 5, demonstrating an ineffectiveness of the flow treatment. The retraction and acidification (explained in following acid pH experiments) caused by the CO_2 flow prevents cells from leaving the membrane, making it impossible to use them later, independently of the pressure used.

Figure 4 - Physical aspects of membranes before and after flow treatments



Source: Own authorship (2022)

3.2 Immersion experiments

Based on non-relevant flow test results, it has been decided to change the reactor condition, a batch reactor replaced the flow one, where the membranes could be deposited in a closed recipient where the decellularization could be made in a $scCO_2$ and co-solvent environment.

In all immersion tests, whatever the solvent, it was noticed a different effect from the flow tests in the physical aspects of the membranes after treatment. Once in contact with a solvent, the membranes swelled, increasing their weight and size. The weight of membranes after immersion tests increased by 115.19 %, from 0.158 g to 0.34 ± 0,11 g of average mass, also the diameters increased by 29.09 % from 1.1 cm to 1.42 ± 0,13 cm average size.

A drop in pH, however, was also noticed in EXCAL tests (tests performed under $scCO_2$), the supernatants were used to measure the pH values after each experiment. AIR experiments (control tests without $scCO_2$) had a pH average of 6.97 ± 0.40 while EXCAL experiments had 4.79 ± 0.34, meaning that the $scCO_2$ causes an acidification in solvent when dissolved.

3.2.1 Water immersion

The first co-solvent tested was pure water, two experiments using the temperature and pressure standard values (45ºC and 170 bar) were carried out with different durations, 1.5 h and 24h treatment to study the influence of time in $scCO_2$ decellularization by the way of a longer dissolution in water. Polymers G14 were used.

First analysis of confocal microscope results (Figure 6) showed that in both polymeric membranes cytoskeleton was not present. Comparing the AIRs experiments with different duration of immersion time, it was seen that 24h treated membrane presented fewer nucleus than 1.5 h treated one, demonstrating a synergistic effect of time in decellularization process. Nonetheless comparing the respective AIR and EXCAL treatments it was observed that $scCO_2$ combined with water did not have the desired effect, both EXCAL after 1.5h and 24h treatment presented lots of nucleus, even more than AIR, visually (Figure 6). Using water as solvent, therefore, the time influence was bigger than $scCO_2$ effect.

Nanodrop results confirmed visual confocal analysis. Higher values of genetic material extracted were seen in the immersion solvent of AIR treatments than in EXCALs' one (Table 1). The DNA and RNA reading by the Nanodrop system was confirmed by the electrophoresis.

Table 1 -DNA quantification of immersion liquid for water immersion tests

Treatment	Time treatment (h)	DNA concentration (ng/µL)	260/280 (R)
AIR	1.5	61.7	1,9
EXCAL	1.5	12.4	1.45
AIR	24	224.1	1.42
EXCAL	24	40.5	1.25

Source: Own authorship (2022)

The absorption coefficient showed that, besides the 1.5 h with no-$scCO_2$ treatment, all immersion in water assays presented protein contamination, which means the conditions extracted an amount of the coating. The purity of the DNA is not relevant for this study, since in the first immersion tests Nanodrop were performed to visualize if nucleic acids were obtained in the supernatants, complementing the confocal analysis. However, for further treatments, the effect of long treatments using $scCO_2$ in the maintenance of the ECM must be analyzed by the LVTS to adapt the treatment to extract the less coating possible.

3.2.2 SDS immersion

Although the longer treatment time presented itself a good option for decellularization when combined with pure water as solvent, all polymeric membranes

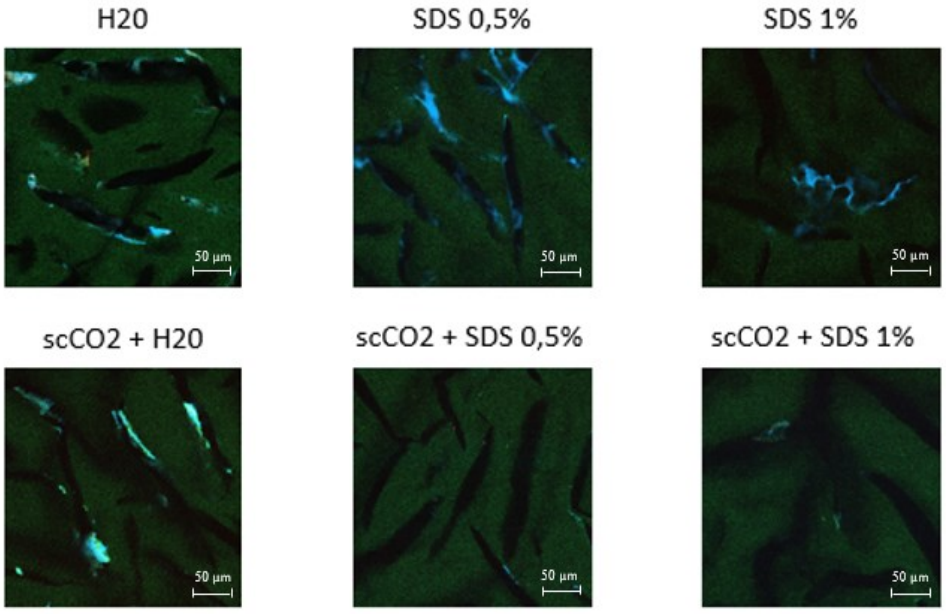
had a large qualitative number of nuclei, making the use of this treatment unfeasible for further applications.

For this reason, it was decided to test the SDS for the new immersion experiments with G14 (170 bar, 1.5h and 45ºC), the same solvent used in the standard treatment but in a lower concentration and in a shorter time treatment.

Based on that, two tests were made using the standard pression, time and temperature parameters, but with two different concentrations of SDS, 0.5% and 1%.

Confocal analysis showed that although after both types of treatments, with and without the presence of sc-CO_2, treated biopolymers didn't present cytoskeletons inside the pores, the AIR treated ones still had a great number of marked DNA. However, treatments combining scCO_2 with both SDS concentrations showed a great synergetic effect, resulting in an almost complete decellularization, where few nuclei were seen with difficulty after traversing the surface of the polymeric membranes, different from the immersion test in water, as seen in figure 6.

Figure 5 - AIR and EXCAL representative images after treatments using different solvents



Source: Own authorship (2022)

Nanodrop quantification also presented higher concentrations of nucleic acids, as demonstrated in table 2. The DNA presence in the samples was also confirmed by an electrophoresis performed with the same immersion solvent.

The only test with any protein contamination was the SDS 0.5% alone, proving that even in the absence of drastic conditions such as supercritical fluids, the SDS 1%

can be already compromising for the ECM. The SDS in both concentrations when combined with the $scCO_2$ can also present a negative effect in the ECM stability.

Table 2 - DNA quantification of immersion liquids for SDS immersion tests

Treatment	SDS concentration (%)	DNA concentration (ng/μL)	260/280 (R)
AIR	0.5	294.3	1.86
EXCAL	0.5	899.2	1.02
AIR	1.0	116.35	1.42
EXCAL	1.0	660.4	1.14

Source: Own authorship (2022)

The great effect using SDS seen in the confocal microscope in addition to the elevated concentration of nucleic acids quantified in Nanodrop contributed to SDS be chosen for further study.

3.2.3 Replicate tests

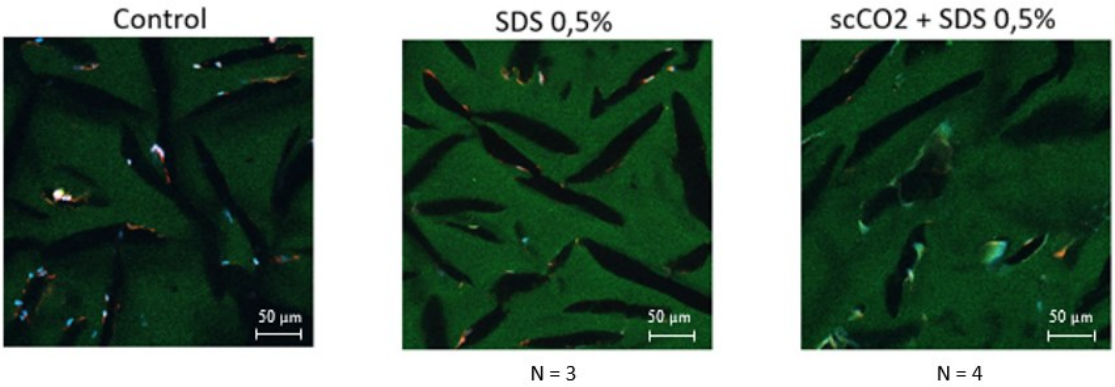
Due to the more efficient results observed using SDS as a solvent, the time treatment reduction and since the initial goal was to reduce the use of detergent, the SDS with a 0.5% concentration was chosen to carry out the replicate tests in order to confirm the good results obtained previously.

The first tests were performed using the G7 to check the effectiveness of this treatment in a different membrane.

According to the availability of the polymeric membranes, 4 tests in different days under the same condition used in immersion tests (45ºC, 1.5h and 170 bar) were performed within this biopolymer and analyzed under the confocal microscope, as shown in the figure 7.

The confocal analysis showed that, for this polymer with a different cellular culture time, the parameters used in this treatment are not enough to decellularize it, also it was seen in all AIR and EXCAL reproduction tests that the cytoskeleton marked in red was present in all replicates. After the $scCO_2$ treatments, it was observed that the nuclei weren't no longer attached to the pores walls but dispersed inside the pores, so a PBS washing was performed after each treatment to check if it would be obtained more DNA later.

Figure 6 - Comparison between different treatments in 7 culture cell days polymers



Source: Own authorship (2022)

Nanodrop analysis confirmed the inefficacy of this treatments for this type of polymeric membranes, even though it was observed more extracted DNA in $scCO_2$ treatments, it was not observed a significant difference. The average DNA quantification for AIR tests were 60,47 ± 42.49 ng/µl when 102.56 ± 58.13 ng/µl for EXCAL tests, proving that there was a great variability in the results.

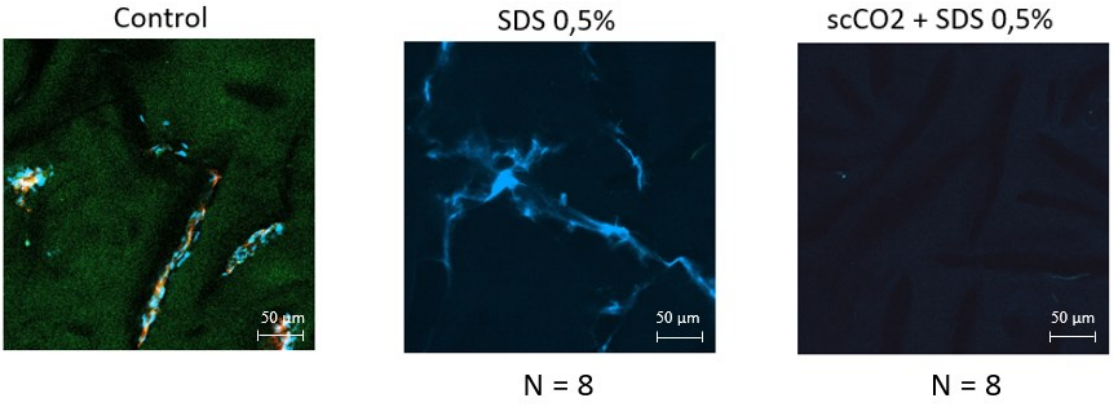
A nucleic acid quantification was performed in the washing baths of PBS where it was able to see the extraction of DNA that were detached from the pore walls but did not get out of inside the polymer. The PBS washing baths, then, were maintained for the next experiments as an ally to the removal of the remaining loosened DNA and also of the detergent inside the polymers.

The next step was focused on the G14 since greater results were obtained previously.

The 4 replications in duplicates (4 testes performed on different days treating two membranes each time) were made also under the same conditions as the first essays in SDS 0.5% immersion obtaining the visual results in confocal microscopy as seen in figure 8. The green absence on the photos is due to the absence of the FITC fluorophore in the initial membrane.

After pictures analysis it was seen that for all replications, the same effect visualized in the first immersion essays was confirmed, comparing AIR experiments to the control tablet, it was seen the extraction of the cytoskeleton but lots of remaining DNA (blue) inside the pores all over the polymer. Yet the EXCAL treatments presented a great decellularization finding some nuclei attached to the pore's walls with difficulty. The pore' stability was also observed, even after the $scCO_2$ extraction and the medium acidification, the pores maintained their conformation.

Figure 7 - Comparison between different treatments in 14 culture cell days polymers



Source: Own authorship (2022)

Even though in a first observation the decellularization was well performed only in EXCAL condition, hypothesis about AIR fluorescence was proposed. The DAPI fluorophore marks the double strand DNA, observed in the control membrane as blue points, which shows that the DNA is folded by protein inside the nucleus.

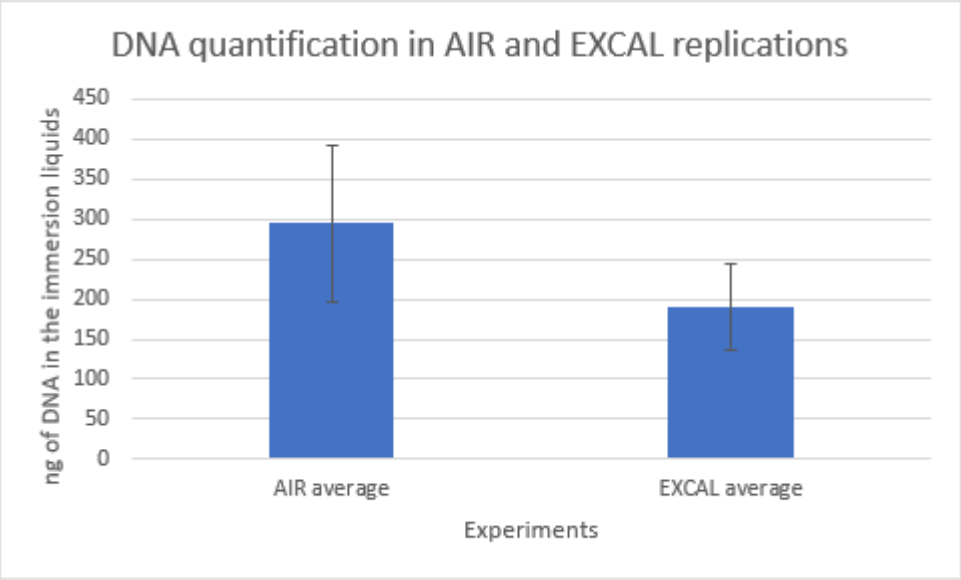
However, after AIR tests the cytoskeleton is removed and the DNA could be spread all over the pores since they are no longer coiled. So, the AIR treatment could have a decellularization effect despite the remaining DNA seen in the pores of the biomaterial.

Although the best decellularization results obtained in confocal analysis, the nanodrop DNA quantification of the immersion liquids proved different.

All immersion liquids of AIR and EXCAL replications were quantified 5 times of 3 analysis each in the Nanodrop system, then a media for each treatment was made and a final average was made for the test with and without $scCO_2$, represented in the figure 9.

In first place, treatments without $scCO_2$ presented more DNA in the immersion liquid compared to those that used the supercritical fluid, showing that the SDS combined to the $scCO_2$ present any effect in the decellularization, inversely than what was observed in confocal examination and in the first immersion tests.

Graphic 1 - DNA quantification of immersion liquids after treatments of G14 (170 bar, 1.5h, 45ºC)

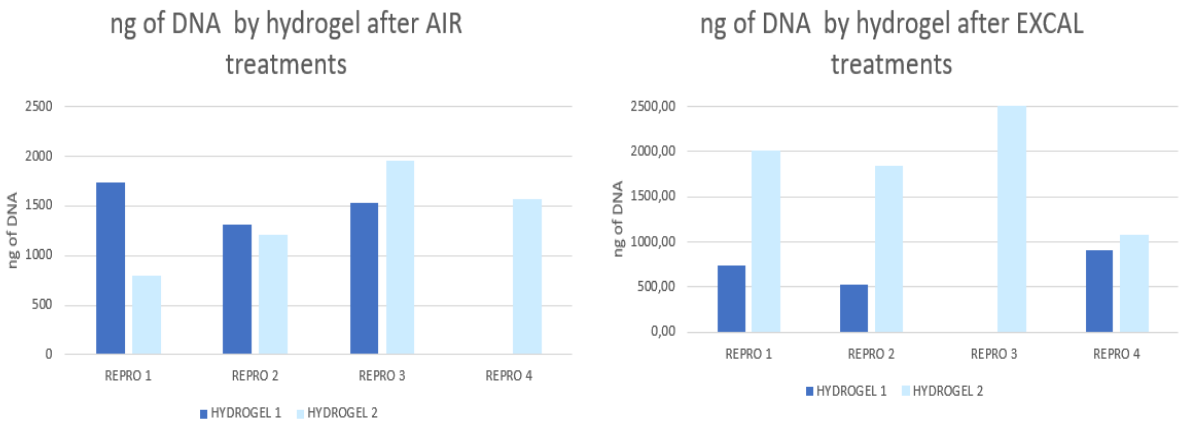


Source: Own authorship (2022)

The variability of the data (figure 9) shows non-homogeneous results and different from what was observed in previous analysis. More assays must be done to lead to a better validation.

Also, a DNA quantification test was performed with each polymeric membrane used in the replications of G14 and with 4 controls membranes (without any treatment). It was expected to obtain a value around the control DNA quantification when adding the data of the immersion liquids with the data of the post treatment polymers, the results are presented in figure 10.

Graphic 2 - DNA quantification of G14 after treatment



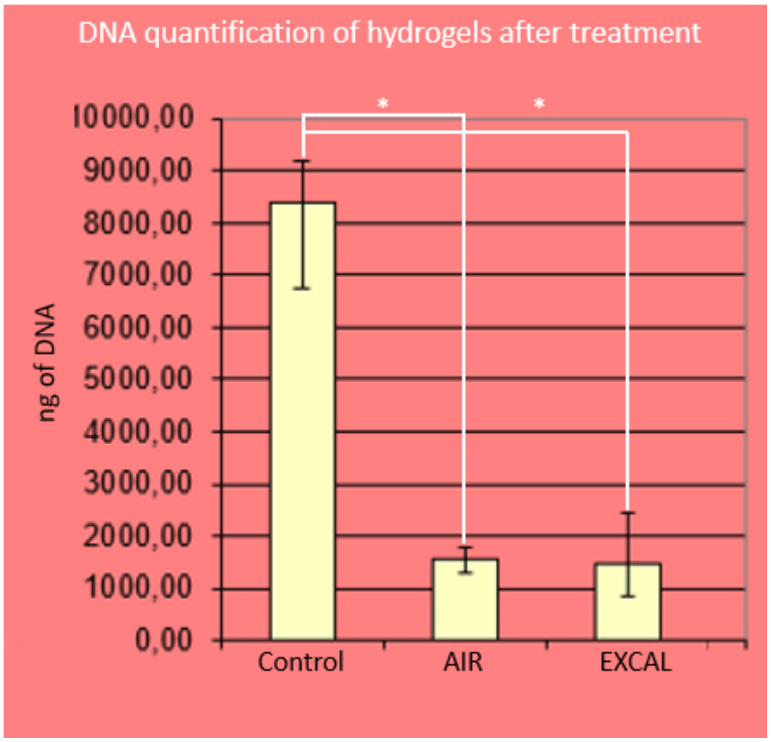
Source: Own authorship (2022)

When comparing the polymeric hydrogels that were in the same reactor and between the different treatment days, it was seen a certain homogeneity in the DNA quantification between with and without $scCO_2$ tests. After handling outliers using the outlier graphpad software, it was seen that the level of DNA quantity for both treatments was similar. These results appeared different from what observed in confocal analysis. Also, after outliers' exclusion, it was observed a homogeneity in the AIR reproductions, with a DNA quantity average of 1443.4 ± 350.4 ng, what was not observed in EXCAL biopolymers DNA quantification, which presented a great variability even in the biomaterials treated together with a DNA quantity average of 1554.1 ± 1049.43, equally different from microscopic analysis.

Nonetheless when joining these two quantifications information (DNA quantification on supernatants and polymers post treatment), similar values to the DNA quantification in control membranes are not obtained, proving that nucleic acids are lost in the DNA precipitation process.

Finally, the effect of supercritical carbon dioxide was compared to the air effect and both treatments were compared to a control polymer using the Kruskal and Wallis multiple comparisons test, illustrated in the figure 11.

Graphic 3 - DNA quantification of non-treated polymer compared with G14 after treatments



Source: Own authorship (2022)

This test informed that the experiments are significative (represented by a "*"), when compared to the nucleic acid quantification in control biohybrid material, both AIR and EXCAL treatments are statistically significant, proving the pronounced effect of the SDS in the decellularization. However, when comparing AIR and EXCAL the software pointed that their differences are not statistically significant, mostly due to the large variability in the samples, demonstrating that the $scCO_2$ does not present a synergetic effect with the SDS in the decellularization.

3.3 Influence of pH

Since after treatments using $scCO_2$ plus SDS 0.5%, there were still few visible nuclei inside the pores of some biohybrid polymer in G7, it was decided to evaluate the influence of the pH towards the decellularization process. After all types of immersion treatments, the pH was measured and compared to the original pH of the solvent and in all cases, it was seen that in EXCAL treatments the pH was way lower than in treatments with the absence of $scCO_2$, due to the dissolution of the compound in water.

For the reproduction tests within the G7, the AIR tests presented an average pH of the immersion liquids after treatment was in the value of 7.0 ± 0.6 and the EXCAL

presented an average of 4.6 ± 0.17 compared to an average pH of 7.6 ± 0.17 of the SDS 0.5% solution. The same pH reduction was obtained for the reproductions of the G14. An average of 7.0 ± 0.15 was obtained in no-$scCO_2$ treatments and 4.82 ± 0.28 for $scCO_2$ ones.

The pH has a direct influence on the molecules' charges, principally for proteins, whose charges are given by the sum of their amino acids. When the suspended particle has a surface electric charge equal to zero at a certain pH, this pH is called the isoelectric point of that molecule, when in pH lower than the isoelectric point, the proteins present a positive charge and when in higher pH they present themselves negatively charged (DINGER, 2006).

The biohybrid polymer presents a negative charge in any pH while collagen and gelatin present an isoelectric point around 4.7 (HIGHBERGER, 1939), since the pH after treatments using $scCO_2$ with SDS 0.5% is around 4.6 it is expected an interaction between the positives charged cell molecules and the negative charged biomaterial, reason why there are still few cells remaining inside the pores seen under confocal analysis after these treatments.

4 CONCLUSIONS AND PERSPECTIVES

The decellularization process using supercritical fluids technology is a great way to optimize long processes that use disruptive solvents. The SDS has been cited over the years in the decellularization on living and artificial tissues, what was confirmed in this study. Its effect, however, can be potentialized using a supercritical fluid under the right conditions.

For the G14 provided by the LVTS it was visualized in the microscopy a pronounced effect in the almost complete decellularization combining SDS 0.5% as a solvent and $scCO_2$ in a much shorter time span than the standard decellularization process and with a much smaller amount of solvent, what wasn't seen in the G7 that went into the same treatments and washing conditions.

The DNA quantification tests pointed an opposite effect of this combination, however when comparing the immersion liquid and post treatment polymers quantification it was proved that lots of DNA are lost in the process until the Nanodrop analysis, which allows to conclude that these protocols are not suitable and should be optimized for the analysis to which the samples are being submitted. Also, there was seen protein contaminations in the final precipitates, which means that certain elements are recovered that further distort the result.

In the next step of this study, the tested gelatin coated polymers with the 2 different cell culture time are going to be analyzed in the LVTS to check if the decellularization process did not interfere with the stability and the structure conservation of the extracellular matrix. Also, the same conditions are going to be tested for the biomaterial coated with collagen with 7 and 14 days of cell culture to check if the synergetic effect with SDS and $scCO_2$ obtained in gelatin coated membranes are the same in a different coating, since the interactions of cells with collagen are stronger than with gelatin.

New immersion tests with ethanol up to 30% as a solvent are going to be performed in all formulations of the biopolymer. Higher percentages of this solvent (>30%) impact on the physical and mechanical properties of membranes.

Once the best solvent is defined, pressure, temperature and time parameters could be tested to achieve the best decellularization model for all formulations. To do that an experimental design of a three-level incomplete factorial can be put into practice, such as the Box-Behnken design, a symmetrical and response surface

design. Experimental designs are used to improve a variety of processes by optimizing the operating conditions and identifying the significant factors to a specific process (SAHU *et al*., 2018).

Using these resources, it is expected to find a fast and effective methodology to decellularize the LVTS biohybrid material so that, in this way, it can apply its functions and collaborate with medical and cardiological practices implementations.

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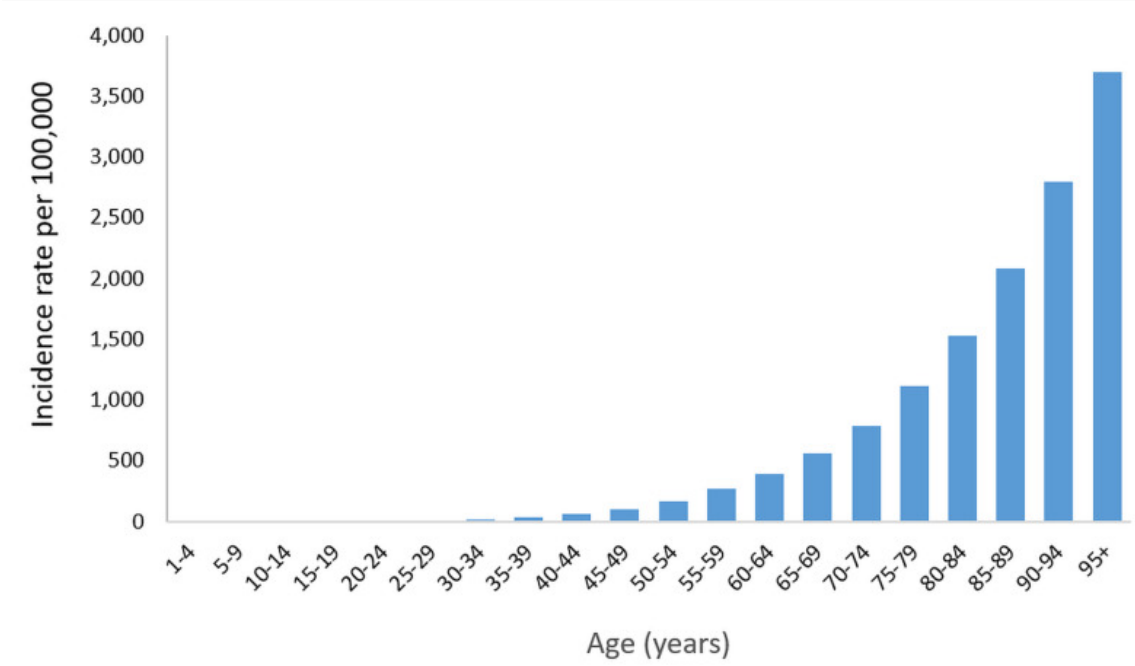
ANNEX A - Age distribution of ischemic heart disease incidence worldwide graphic

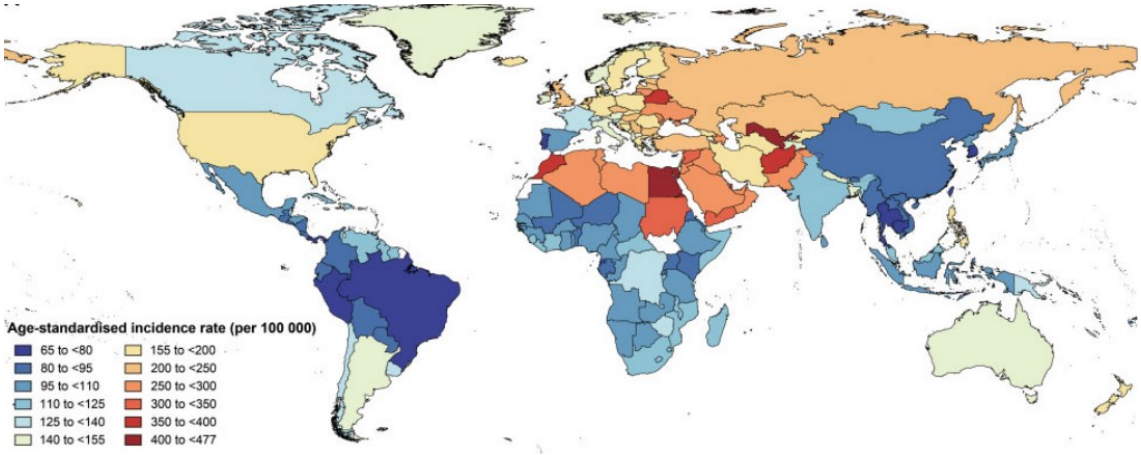
Annex A - Age distribution of ischemic heart disease incidence worldwide graphic



Source: Khan *et al*. (2020)

ANNEX B – Global distribution of ischemic heart disease

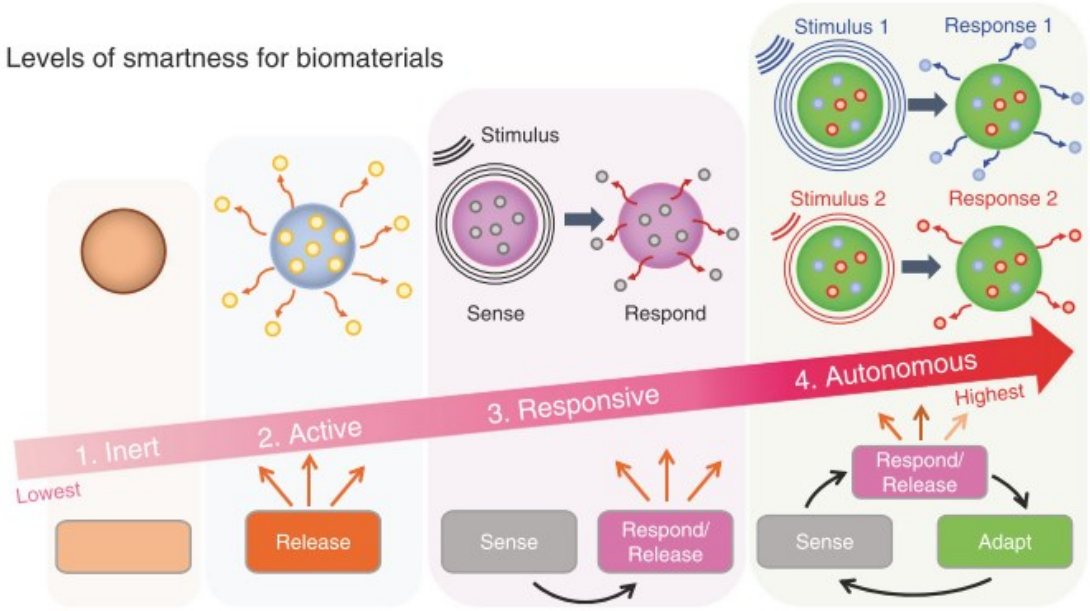
Annex B - Global distribution of ischemic heart disease



Source: Khan *et al*. (2020)

ANNEX C – Levels of smartness of biomaterials

Annex C - Levels of smartness of biomaterials



Source: Montoya *et al*. (2021)

ANNEX D – Applications and raw material of biomaterials

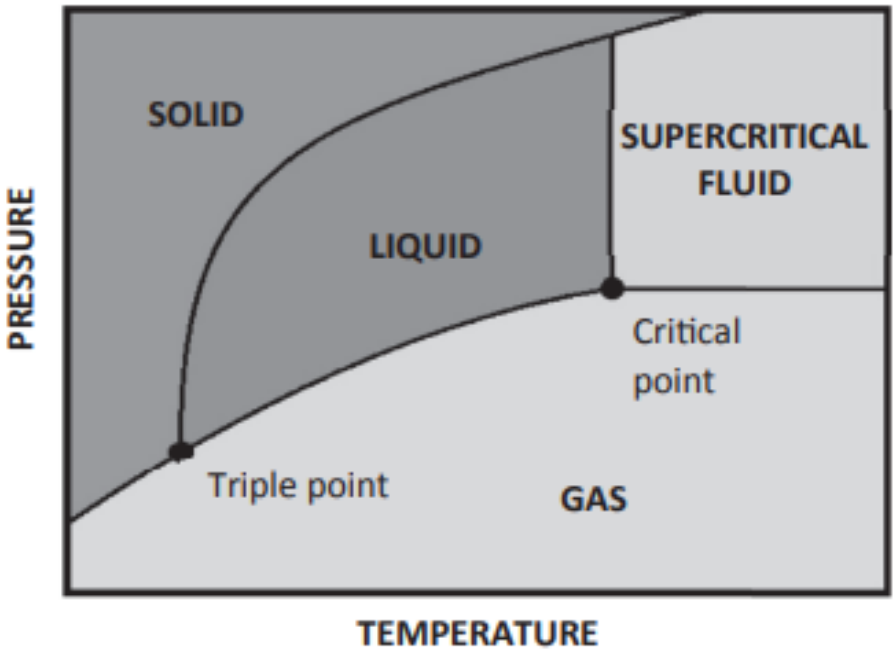
Annex D - Applications and raw material of biomaterials

Application	Types of materials
Skeletal system	
Joint replacements (hip, knee)	Titanium, Ti–Al–V alloy, stainless steel, polyethylene
Bone plate for fracture fixation	Stainless steel, cobalt–chromium alloy
Bone cement	Poly(methyl methacrylate)
Bony defect repair	Hydroxylapatite
Artificial tendon and ligament	Teflon, Dacron
Dental implant for tooth fixation	Titanium, alumina, calcium phosphate
Cardiovascular system	
Blood vessel prosthesis	Dacron, Teflon, polyurethane
Heart valve	Reprocessed tissue, stainless steel, carbon
Catheter	Silicone rubber, Teflon, polyurethane
Organs	
Artificial heart	Polyurethane
Skin repair template	Silicone–collagen composite
Artificial kidney (hemodialyzer)	Cellulose, polyacrylonitrile
Heart–Lung machine	Silicone rubber
Senses	
Cochlear replacement	Platinum electrodes
Intraocular lens	Poly(methyl methacrylate), silicone rubber, hydrogel
Contact lens	Silicone–acrylate, hydrogel
Corneal bandage	Collagen, hydrogel

Source: Ratner (1996)

ANNEX E – Pressure and Temperature phase diagram

Annex 1 - Pressure and Temperature phase diagram



Source: Knez *et al*. (2014)