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DEVELOPMENT OF OCULAR IMPLANTS USING A SUPERCRITICAL FLUID FOAMING/ MIXING METHODOLOGY

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UNIVERSIDADE DE COIMBRA

To Duarte for all the playing time we missed…

To Camila for all the basketball games I didn't attend to…

To Bruno for all the travelling we had to hold over…

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Abstract

Despite advances in ophthalmological pharmacology, the treatment of eye diseases have limitations since conventional methods of drug delivery are not completely effective. A possible solution for the maintenance of therapeutic levels within the eye includes the controlled release systems such as implants, capsules, liposomes and iontophoresis.

Supercritical carbon dioxide ($scCO_2$) foaming/mixing methodology (SFM) is an alternative processing method that has unique advantages over standard techniques, including porosity control, absence of organic solvents and reduction of the melting (T_m) and glass transition temperatures (T_g) which is an important condition in what concern the incorporation of thermally and chemically sensitive drugs. In this type of processing, the polymer phase melts due to the dissolution of the fluid phase, and pores are formed upon fluid release.

Thus, the main propose of this work was the development of poly (ε-caprolactone) (PCL) ocular implants loaded with drugs of ophthalmological interest (dexamethasone (DXMT), used to prove the concept and due to its lower price and frequent use in the treatment of ophthalmological disorders, and 2-Chloro-N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (2-Cl-IB-MECA), a promising drug for future *in vivo* application) using SFM process. To achieve viable implant dimensions and to evaluate the effect of material porosity in drug release were also defined objectives. Pure PCL, PCL with glycofurol and PCL with glycofurol and the selected drugs were processed by $scCO_2$ at 45 ° C, 200 bar, for two hours. Depressurization rates tested were 10, 20 and 30 bar/min. Produced implants have similar dimensions to ophthalmologic implants currently marketed (length 2 mm and diameter ≤ 0.464 mm).

Glycofurol was shown to be important to ensure compatibilization of the polymer/drug mixture because, when used, there was greater drug incorporation. The ideal percentage of glycofurol determinate was 8% (w/w). SEM images show that all samples have a heterogeneous pore distribution influenced by depressurization rate and glycofurol presence: higher depressurization rate produces smaller pores and additive presence generates larger pores at the same depressurization rate.

Largest pores obtained were for SFM10. There is a small difference in pore size between SFM20 and SFM30 which suggests that, on a small scale, like the ocular implants one, this variation in the depressurization rate may not be relevant. At same depressurization rate, additive presence generates larger pores. DSC analysis shows that PCL thermal properties were not significantly altered after processing.

Dexamethasone implants revealed controlled release for more than 500 hours.

Comparing DXMT implants produced by hot melting and processed with supercritical carbon dioxide, it is observed that until 200h of release, SFM implants release is more stable and higher than HM implants. After that time, HM appears to become stable but still lower. This is due to higher porosity in SFM implants. Implants produced by hot melting have smaller surface area and less porosity.

Despite a similar release trend, due to porosity effect, 2-Cl-IB-MECA SFM20-G implants have higher drug release than 2-Cl-IB-MECA SFM30-G. 2-Cl-IB-MECA implants showed sustained release over 100 h. Release is greater in the first 60% of release profile and faster without additive addition due to less polymer-drug compatibilization.

The amount of drug released in the first hour was eight times higher than the required on an intravitreal injection. Preliminary *in vivo* tests with PCL implants drug free didn't reveal adverse side effects.

Resumo

Apesar dos avanços na farmacologia oftalmológica, o tratamento das patologias do olho apresenta limitações uma vez que os métodos convencionais de administração de fármacos não são completamente eficazes. Uma possível solução para a manutenção de níveis terapêuticos no interior do olho inclui os sistemas de libertação controlada na forma de implantes, cápsulas, lipossomas e iontoforese.

A metodologia de foaming/ mistura com dióxido de carbono supercrítico é um método de processamento alternativo que tem vantagens únicas relativamente às técnicas padrão, incluindo o controlo da porosidade, a possibilidade de ausência de solventes orgânicos e a redução da temperatura de fusão e de transição vítrea, que é uma condição importante no que se refere à incorporação de fármacos térmica e quimicamente sensíveis. Neste tipo de processamento ocorre fusão da fase polimérica, devido à dissolução da fase do fluido, e formam-se poros aquando da libertação do fluido.

Assim, o principal objectivo deste trabalho é o desenvolvimento de implantes oculares de poly(ε-caprolactona) (PCL) carregados com fármacos de interesse terapêutico a nível oftalmológico (a dexametasona (DXMT), utilizada para provar o conceito e devido ao seu baixo preço e à sua utilização frequente no tratamento de patologias oftalmológicas, e 2-Cloro-N^6-(3-iodobenzil)-adenosina-5′-N-metiluronamida (2-Cl-IB-MECA), um fármaco promissor para futura aplicação *in vivo*) utilizando foaming/ mistura com dióxido de carbono supercrítico. Obter implantes de dimensões viáveis e avaliar o efeito da porosidade do material na libertação do fármaco foram também objectivos definidos. PCL pura, PCL com glicofurol e PCL com glicofurol e os fármacos seleccionados, foram processados por dióxido de carbono supercrítico, a 45ºC, 200 bar, durante duas horas. As taxas de despressurização testadas foram 10, 20 and 30 bar/min. Os implantes produzidos têm dimensões semelhantes aos implantes de aplicação oftalmológica actualmente comercializados (comprimento de 2 mm e diâmetro ≤ 0.464 mm).

O glicofurol mostrou ser importante para garantir a compatibilização da mistura polímero/ fármaco porque aquando da sua utilização houve maior incorporação de fármaco. A percentagem ideal de glicofurol a utilizar foi de 8% (m/m). As imagens de SEM mostram que todas as amostras apresentam uma distribuição de poros heterogénea influenciada pela taxa de despressurização e presença de glicofurol: maior taxa de despressurização gera poros mais pequenos e a presença de aditivo gera poros maiores para uma mesma taxa de despressurização. Os maiores poros obtidos foram para SFM10. Existe pouca diferença no tamanho de poro entre SFM20 e SFM30 o que sugere que, numa escala pequena, esta variação da taxa de

despressurização poderá não ser relevante. A presença de aditivo para uma mesma taxa de despressurização gera poros de maiores dimensões. A análise de DSC mostra que as propriedades térmicas da PCL não foram significativamente alteradas depois do processamento. Os implantes de Dexametasona revelaram libertação controlada durante mais de 500h. Comparando os implantes de DXMT produzidos por *hot melting* e processados com dióxido de carbono supercrítico, constata-se que até às 200h de libertação, esta é mais estável e elevada no caso do *foaming* do que no caso do *hot melting*. Depois deste tempo a libertação dos implantes de HM estabiliza mas continua mais baixa. Isto fica a dever-se a uma porosidade superior nos implantes processados por tecnologia supercrítica. Os implantes produzidos por HM têm uma área de superfície e porosidade menores.

Apesar de uma tendência de libertação semelhante, nos implantes de 2-Cl-IB-MECA com uma taxa de despressurização de 20 bar/min a libertação de fármaco é maior do que nos implantes com uma taxa de despressurização de 30 bar/min devido ao efeito da porosidade. Os implantes de 2-Cl-IB-MECA apresentaram libertação sustentada mais de 100 h. A libertação foi muito superior nos primeiros 60% do perfil de libertação e foi mais rápida na ausência de aditivo devido a uma menor compatibilização entre o polímero e o fármaco.

A quantidade de fármaco libertada foi, na primeira hora de libertação, oito vezes superior ao requerido numa injecção intra-vítrea. Os testes preliminares *in vivo* com implantes de PCL sem fármaco não revelaram efeitos secundários adversos.

List of Abbreviations and Nomenclature

AIBILI – Association for Innovation and Biomedical Research on Light

ARMD – Age-related macular degeneration

CAS – Chemical Abstracts Service

CMV - Cytomegalovirus

CO_2 – Carbon Dioxide

DDS – Drug delivery systems

DME – Diabetic Macular Edema

DSC – Differential scanning calorimetry

DXMT - Dexamethasone

FDA – Food and drug administration

HPLC – High performance liquid chromatography

HM – Implant produced by hot-melting

HM-G – Implant produced by hot-melting using Glycofurol as additive

IOP – Intraocular pressure

I.V. – Intravenous

min – Minute

mL – Milliliter

mm – Millimeter

Mn – Number average molecular weight

PCL – Poly(ε-caprolactone)

PLA – Poly(lactic acid)

PLGA – Poly(lactic-co-glycolic acid)

PVA – Poly(vinyl alcohol)

scCO 2 – Supercritical carbon dioxide

SCF – Supercritical fluids

SEM – Scanning electron microscopy

SFM – Supercritical CO_2 foaming/ mixing process

SFM10 – Implant processed by Supercritical CO_2 foaming/ mixing processed with 10 bar/min depressurization rate

SFM20 – Implant processed by Supercritical CO_2 foaming/ mixing processed with 20 bar/min depressurization rate

SFM20-G – Implant processed by Supercritical CO_2 foaming/ mixing, using Glycofurol as additive, processed with 20 bar/min depressurization rate

SFM30 – Implant processed by Supercritical CO_2 foaming/ mixing process with 30 bar/min depressurization rate

SFM30-G – Implant processed by Supercritical CO_2 foaming/ mixing, using Glycofurol as additive, processed with 30 bar/min depressurization rate

Tg – Glass transition temperature

Td – Degradation temperature

TGA – thermogravimetric analysis

Tm – Melting temperature

UCST / LCST – Upper critical solution temperature/lower critical solution temperature

USA – United States of America

2-Cl-IB-MECA – 2-Chloro-N^6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide

µm – micrometer

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Goals and Motivation

Ophthalmic disorders are majorly caused by eye posterior segments diseases and they are a limiting factor in life's quality. Topical ocular drugs are insufficient to achieve therapeutic levels and multiple intravitreal injections have several risks associated. Such a premise is enough to stimulate and assign the development of ocular implants that provide a controlled drug delivery in eye tissues. Controlled drug delivery systems allow the inclusion of a therapeutic substance in the body, on its target release site and with a desirable release rate. These features make these systems safer and more effective than traditional approaches.

The synthesis of such implants can be performed based on polymers with proven biocompatibility that may be or not biodegradable. Melting, extrusion and hot molding are traditional methods of implants processing that have limitations regarding the incorporation of heat sensitive drugs and the need of solvents addition. In many cases, the use of such solvents requires intermediate processing steps to guarantee their removal or neutralization in order to avoid potential toxicity. Non-biodegradable implants involve multiple invasive techniques that have several risks associated.

The main goal of this work is the development of PCL ocular implants for drug delivery using a supercritical carbon dioxide ($scCO_2$) assisted fluid foaming/mixing methodology. PCL is a polymer biodegradable and biocompatible, known for its slow degradation rate. Supercritical carbon dioxide is an advantageous solvent for processing polymers with desirable shape and porosity. Porosity is especially important when we consider drug release systems.

SFM does not require the use of solvents – except the CO_2 that volatilize out of the matrix – so it may assume as a safe alternative.

Besides the mentioned main goal, this work also does a morphologic characterization and thermal analysis of samples. Drug release assays were performed to evaluate release amount and kinetics.

The results obtained so far in other biomedical applications, such as PCL ibuprofen impregnation and preparation of imprinted contact-lenses for drug delivery, demonstrated the feasibility of using $scCO_2$ methods.

Potential targeted diseases treatable with these implants include uveitis, cytomegalovirus retinitis, AMD and macular edema.

1. Introduction

1.1 Intraocular Drug Delivery Systems

Vision is a primary sense. Nutheti *et al.* and Aspinall *et al.* studies proved that visual impairment is associated with a significant decrease in quality of life among population.
The eye is typically the representation of sight (Figure 1).

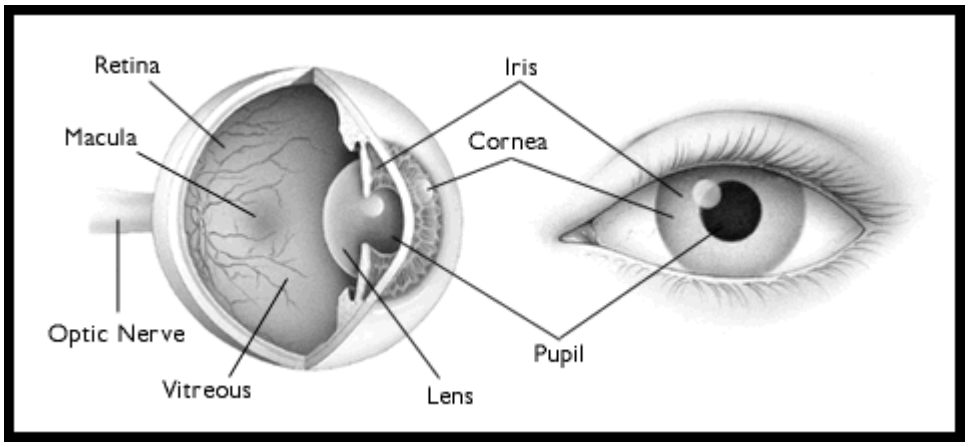


Figure 1 - Basic Eye Anatomy (www.eyesightresearch.org)

The treatment of eyes sicknesses back to Cleopatra times when atropine eye drops (known as *Belladonna*) was used as a mydriatic. In the late 1800s, in Europe, United Kingdom, gelatin cocaine inserts were the first polymeric system of continuous drug release; they were used for local anesthesia. In the 1900s, several topical ocular delivery methods were developed: soluble ophthalmic drug inserts (Amo and Urtti, 2008), liquid state delivery systems (Shedden *et al.*, 2001), microspheres (Yarangumeli and Kural, 2004), drug immersed hydrophilic contact lenses and topical ocular liposomes (Amo and Urtti, 2008). None of those approaches could treat totally capably posterior ocular tissues.
Despite drug delivery to the anterior portions of the eye is well established in the literature, in what concern the posterior segment it remains a challenge due to its particular anatomy and physiology. The obstacles for the adequate drug delivery are static barriers, such as different layers of cornea, sclera and retina (blood aqueous and blood-retinal barriers) and dynamic barriers as conjunctival and choroidal, blood flow, lymphatic clearance, and tear dilution (Hughes *et al.*, 2005; Gaudana *et al.*, 2010).

The most frequent diseases that affect the posterior segment are age-related macular degeneration (ARMD), diabetic macular edema (DME), endophthalmitis and proliferative vitreoretinopathy. All this pathologies can lead to vision impairment and blindness (Hughes *et al.*, 2005).

In recent years there has been a marked development in scientific understanding of the mechanisms associated with these pathologies and new treatment strategies. Nevertheless, the success of new therapeutic approaches is dependent upon strategies which allow the drug reaching the intended site of action. While topical treatments can achieve drug therapeutic levels in the anterior segment, the natural barriers of the eye referred previously, make then must less effectively to the posterior segment. This is easily confirmed if we consider that in the case of topical instillation of ophthalmic drops – which is the most usual therapeutic method in ocular diseases – less than 5% of the applied dose reaches the aqueous humor (Gaudana *et al.*, 2010).

Due to these limitations, the treatment of eye posterior segment diseases is done by subconjunctival injections, systemic administration of drugs or intravitreal injections. All these treatment strategies have limitations.

Subconjunctival injection is less invasive than intravitreal injection but the main problem is the lack of control in the drug concentration that reaches the vitreous (Yasukawa *et al.*, 2001; Hughes *et al.*, 2005; Amo and Urtti, 2008). Several authors refer that low bioavailability is determinate by rapid drug elimination into systemic circulation, following subconjunctival administration (Weijtens *et al.*, 1999; Hosseini *et al.*, 2008; Kim *et al.*, 2008).

In systemic administration, parenteral and oral administration may be considered. Parenteral route is conditioned by the blood-retinal barrier which regulates strictly the entry of therapeutic agents from blood circulation into the retina. Hydrophobic drugs cross poorly the blood-retinal barrier which is a significant aspect in what concern drug delivery systems. As only a small amount of medications overcomes this barrier, several systemic doses are required to maintain the concentration of drugs in effective therapeutic levels (Gaudana *et al.*, 2010; Haghjou *et al.*, 2011).

Therapeutic efficacy is also a problem in oral administration because there is, as well, limited accessibility to ocular tissues; it requires high dosage to observe therapeutic efficacy. After gastrointestinal absorption, molecules in systemic circulation must also cross the blood-aqueous and blood-retinal barriers (Gaudana *et al.*, 2010).

In both cases – parenteral and oral administration – high drug among can lead to systemic side effects and toxicity to the patient (Yasukawa *et al.*, 2001; Hughes *et al.*, 2005; Amo and Urtti, 2008).

Direct intraocular drug injection into the vitreous cavity provides efficient drug delivery for several drugs, including low molecular mass drugs and macromolecules, achieving higher concentrations in the retina and vitreous. This route of administration reduces systemic side-effects because it is not carried in the bloodstream, acting *in loco*. The therapeutic effect of that drugs depends on the retention time of the injected drug at the administration site. However, the half-life of drugs in the vitreous is relatively short which request repeated injections to maintain drug concentrations in operative therapeutic levels. To keep drugs in therapeutic range, repeated injections are requested at regular intervals. Repeated injections may cause discomfort to the patient and lead to therapy discontinuation; technically, the risk of vitreous hemorrhage, infection, globe perforation, orbital fibrosis, ptosis, cataract and retinal detachment are increased by repeating the procedure (Ogura *et al.*, 2001; Yasukawa *et al.*, 2001; Choonara *et al.*, 2007; Haghjou *et al.*, 2011).

Intraocular implants are a good alternative and possible solution to the problem because they can be controlled release systems, prepared from different biocompatible polymers, biodegradable or non-biodegradable, that are placed after the blood-retinal barrier, releasing the drug directly to the site of action in a controlled manner and for a long time. This mechanism, especially important in chronic eye diseases, reduces the possibility of adverse effects that are often associated with administration of drugs by systemic route (Yasukawa *et al.*, 2005; Bourges *et al.*, 2006; Manickavasagam and Oyewumi, 2013), reduces the need of multiple injections for *in situ* application, allow drug release at therapeutic levels and decrease the amount of drug required for the treatment (Amo and Urtti, 2008; Cunha *et al.*, 2009; Manickavasagam and Oyewumi, 2013).

Intraocular insertion of implants must be performed by surgical procedure and, although it is invasive, the several implants advantages cited above outweigh the inconvenience caused by the surgical procedure.

Intraocular implants may be non-biodegradable or biodegradable according to the different kind of biocompatible polymer used in its formulation (Dash and Cudworth II, 1998; Manickavasagam and Oyewumi, 2013).

Non-biodegradable intraocular implants can be monolithic systems (matrix) or reservoirs. In the matrix system, the drug is dispersed homogeneously in the polymeric matrix or absorbed on the surface. The slow diffusion of drug through the polymer matrix provides a controlled release. Otherwise, in the reservoir system the drug is surrounded by a non-degradable permeable membrane. In this system, water diffuses through the membrane by dissolving the drug and creating a saturated solution within the reservoir. Since saturation occurs, the drug diffuses outwards into a release rate – based on Fick's Law – conditioned by polymer coating

thickness, implant shape, diffusibility of the drug from the polymer coating and release area (Yasukawa *et al.*, 2005; Bourges *et al.*, 2006).

Several non-biodegradable polymers have been used in implants with a long-term controlled release of drugs. Silicones, poly(vinyl alcohol) (PVA) and poly(ethylene-co-vinyl acetate) (EVA) are the polymers commonly used to produce this implants. Silicones and PVA have hydrophobic character thus are permeable to various lipophilic drugs; EVA is used to cover around the reservoir to decrease drug diffusion rate because it is impermeable to most medications (Okabe *et al.*, 2003; Yasukawa *et al.*, 2005; Bourges *et al.*, 2006; Manickavasagam and Oyewumi, 2013).

The non-biodegradable polymeric intraocular implants allow controlling the release of the drug in a predictable kinetics for long periods of time and they are less likely to produce burst drug release when compared with biodegradable implants. They have the disadvantage of need to be surgically removed from the eye after the complete release of the drug, which poses a risk to the patient. Otherwise, prolonged intraocular location could potentially trigger immunity responses (Bourges *et al.*, 2006; Manickavasagam and Oyewumi, 2013).

Therefore, natural and synthetic biodegradable polymers have been widely investigated for the development of intraocular implantation devices (Jain, 2000; Amo and Urtti, 2008). Bovine and human albumin, collagen, gelatin and hemoglobin are types of natural protein-based polymers. However, their utilization is limited due to the high cost and questionable purity. Polymers often used are polyamides, polyaminoacids, polyesters, polyurethanes, polyacrylamides, poly(glycolic acid) (PGA) and poly (D, L-lactic-glycolic acid) (PLGA) (Jain, 2000; Manickavasagam and Oyewumi, 2013). These polymers are biocompatible, well tolerated, consider safe for clinical use and allow the possibility of modifying their time degradation. For instance, the PLGA degradation rate is predisposed by lactide and glycolide monomers ratio; with more glycolide units, degradation will be faster. Extent of crystallization and polymer molecular weight are other factors that affect drug release mechanism.

One of the most promising polymers in biomedical applications is PCL (Table 1).

Poly (ε-caprolactone) (PLC) is a hydrophobic and semi-crystalline aliphatic polyester synthesized from e-caprolactone monomers polymerization.

Table 1 - Poly (ε-caprolactone) Chemical and Mechanical Properties

Melting Temperature (Tm)	≈ 60ºC (Jenkins *et al.*, 2006; Bassi *et al.*, 2011 using Differential Scanning Calorimetry)
Glass Transition Temperature (Tg)	-60ºC (Jenkins *et al.*, 2006; Bassi *et al.*, 2011 using Differential Scanning Calorimetry)
Crystallization Temperature (Tc)	≈ 27ºC (Bassi *et al.*, 2011 using Differential Scanning Calorimetry)
Thermal Stability (Td)	≈ 350ºC (Jenkins *et al.*, 2006 using Differential Scanning Calorimetry)
Compressive Strengh	14MPa (Bassi *et al.*, 2011 using the Instron 1122 mechanical tester)
Young's Modulus	0.9 GPa (Bassi *et al.*, 2011 using the Instron 1122 mechanical tester)

As PCL can be degraded by hydrolytic mechanisms in a physiological environment, it is consider to be biodegradable and that is very important in what concern biomedical applications (Jenkins, 2006; Cunha *et al.*, 2009; Bernards *et al.*, 2013). The hydrolytic degradation of PCL is due to the presence of ester bonds. The hydrolysis of the PCL aliphatic ester group does not generate toxic degradation products (Fernandez and Richa, 2011). The degradation rate of this polymer is approximately 2 to 3 years which is considerably slow and consequently attractive for biomedical long-term applications (Boruges *et al.*, 2006; Nair and Laurencin, 2007; Cunha *et al.*, 2009; Bernards *et al.*, 2013).

The poly-α-ester degradation occurs by random hydrolytic cleavage and enzymatic fragmentation. The random hydrolytic cleavage it's accelerated by carbonyl ends of polymeric chains and starts in amorphous areas. The fragments resulted of bulk fragmentation are taken up by macrophages and degraded inside the cells. When the remains are small enough to diffuse through matrix, mass loss occurs (Dash and Cudworth II, 1998; Bernards *et al.*, 2013).

The enzymatic fragmentation is based on the fact that although enzymes are programmed for highly specific interactions with particular biological substrates, some of them are able to recognize "non-natural" such as polymers like PCL (Labow *et al.*, 2002). Several studies, such as Aktusu *et al.*, (1998), Labow *et al.*, (2002), Dash *et al.*, (2011), proved the importance of enzyme hydrophobic domain to polymer surface adsorption and catalytic domain for the hydrolysis of the ester bond.

Multiple biochemical and cellular parameters can be directly involved in biodegradation because the *in vivo* environment complexity allows activation of other degradation mechanisms furthermore to hydrolytic, enzymatic and oxidative.

The slow degradation, high permeability to hydrophobic drugs and high biocompatibility are characteristics that make PCL and excellence polymer for the development of controlled eye drug delivery systems (Dong *et al.*, 2006; Fialho *et al.* 2003; Cunha *et al.*, 2009) and Cunha *et*

al., 2009 and Bernards *et al.*, 2013 studies confirmed the feasibility of PCL usage in the production of intraocular devices.

Implants made from biodegradable polymers can also be monolithic (matrix) or reservoirs as mentioned in the case of non-biodegradable polymers. In the matrix system, the polymer degrades slowly in physiological conditions and the drug is released as the degradation occurs. The drug can also be released by diffusion through the pores of the matrix. In the reservoir system, and depending on the polymer, the membrane degrades in a slower rate than the drug diffusion (Dash and Cudworth II, 1998; Fialho *et al.*, 2003). Polyanhydrides degrades faster by surface erosion than PLA and PLGA and this can lead to excess drug release (burst). Discontinuity of the matrix is also associated to irregular drug release and final burst release. In literature there are reported alterations that leaded to maintaining controlled polymer erosion and drug release (Yasukawa *et al.*, 2005; Kunou *et al.*, 2000). Balancing polymer mass loss and drug release is challenging for most biodegradable systems.

Ophthalmic implants have been commercialized for more than 20 years.

Vitrasert® (Bausch &Lomb, USA), the first polymeric (EVA and PVA) ganciclovir non-biodegradable implant, was approved by FDA in 1996. It was used on the treatment of cytomegalovirus retinitis, releasing the 4.5 mg of ganciclovir for 4 to 5 months. Complications associated with prolonged use of the implant, such as vitreous hemorrhages, led to withdrawal from the European market in 2002.

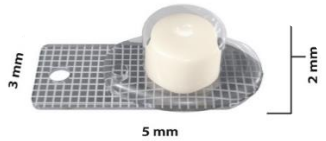
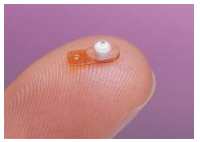
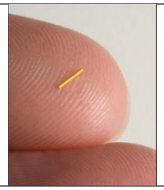
Retiser® (PVA and silicone) and Medidur® (Polyimine, PVA and silicone) are fluocinolone acetonide non-biodegradable implants commercialized used for uveitis treatment. For Retisert®, drug release is for approximately 30 months. The development of cataracts, the need for surgery in 100% of cases and the increase in intraocular pressure were some of the adverse effects resulting from the use of this implant (Jaffe *et al.*, 2006). Medidur® has the advantage of could be intravitreally inserted as an injection, instead of a surgery due its dimensions (3mm long and 0.37 mm of diameter) (Amo and Urtti, 2008).

Ozurdex® is a PLGA and dexamethasone biodegradable implant used on the treatment of macular edema. Drug release occurs for about 6 months and it is refered to be well tolerated by patients (Kuppermann *et al.*, 2007).

Surodex® is other of dexamethasone biodegradable implants. Structurally it is a 60 µg dexamethasone pellet coated in PLGA that provides sustained release for 7-10 days after insertion into eye anterior chamber. Its primarily pretended application is the treatment of postcataract surgery inflammation. The use of this implant has no reported adverse events and it showed to be safe and effective (Tan *et al.*, 1999).

Implants further promoted and used in ophthalmic applications are specified in Table 2. All of them have limitations as drug loading capacity, drug release profiles, degradation time, biodegradability and solvents request in their manufacture.

Table 2 - Common ocular implants commercialized.

Registered Name	Manufacter	Active Substance	Therapeutic Application	Dosage	Polymeric Matrix	Biodegradability	Implant size	
Vitrasert®	Bausch & Lomb	Ganciclovir	CMV retinitis	4.5 mg	EVA and PVA	Nonbiodegradable	2.5 mm diameter by 1 mm thick tablet	
Retiser®	Bausch & Lomb	Fluocinolone acetonide	Uveitis	0.59 mg	PVA and silicone			
Medidur® (Iluvien)	Alimera Sciences		Uveitis	0.19 mg	Polyimine, PVA and silicone		3.5 × 0.37 mm ∅	
Surodex®	Ocular Pharmaceuticals	Dexamethasone	Postoperative inflammation	60 µg	PLGA	Biodegradable	1.0×0.5 mm	Not available
Ozurdex®	Allergan		Macular Edema	700 µg			6×0.46 mm ∅	

Beyond solid implants, there are micro and nano particles that can be colloidal carriers. The main goal of this approach is the development of injectable formulations long term actives and with selective actuation in specific tissues and cells (Choonara *et al.*, 2007).

These systems have the advantage of the administration to be made by injections, as present themselves as colloidal solutions, which avoids the potential complications of surgical processes associated with implants application (Behar-Cohen, 2002). This colloidal may be prepared based on synthetic polymers such as PLA, PLGA and PCL. The micro and nano particles are polymeric structures classified by size: the micro particles have diameter over 1 µm and the nano particles below 1 µm. As the implants, these systems are also classified structurally in monolithic (matrix) or reservoirs (Kimura and Ogura, 2001; Fialho *et al.*, 2003; Choonara *et al.*, 2007). Several studies have been performed with microspheres for release of drugs such as ganciclovir, retinoic acid and fluorescein. The release time obtained as about two to eight weeks. Low amount of encapsulated drug, the need of repeated injections in short period of time and the visual limitations caused by the presence of suspended particles in vitreous cavity are reported limitations of this methodology (Fialho *et al.*, 2003).

Liposomes are biocompatible and biodegradable layer, which surround an aqueous medium, and can be also used in colloidal systems for intraocular applications. They may be positively, neutral or negatively charged depending on its composition. Their size is between 0.025 µm and 2.5 µm. According to the size and number of layers, the liposomes can be classified as multilamellar vesicles, large unilamellar or small unilamellar. Drugs may be incorporated into phospholipid layers or the aqueous solution which is an advantage because it allows the simultaneous transport of hydrophilic and hydrophobic agents. The distribution of the drug is controlled by the size of the vesicles and lipid bilayers of the liposomes. The limitations of this approach are related to complex preparation methods, difficult due to low storage stability and induction of constraints in vision due to its suspension in the vitreous cavity (Behar-Cohen, 2002; Fialho *et al.*, 2003; Choonara *et al.*, 2007).

Iontophoresis is another possibility under investigation as drug transport system in the eye. In this process, ions are conducted in a tissue by a low intensity electrical current that modifies cells permeability and facilitates drugs penetration. The transport depends on an electromotive force that repels ions of an electrode of the same charge and makes them migrate to the oppositely charged. It is a non-invasive procedure and can be repeated several times with few adverse effects. However, drug transports by iontophoresis disadvantages are the risk of shocks and burns caused by contact of the electrodes with the eye, overdose and damage of the application site. Iontophoresis can enhance the intraocular administration and reach the target

tissues but as this technique is not a sustained-release system it is necessary to repeat the treatment several times (Fialho *et al.*, 2003).

Therefore, intravitreal route minimizes the occurrence of adverse systemic effects and offers advantages as the drug is directly introduced into the vitreous. However, drug distribution in the vitreous is heterogeneous and the blood flow in the choroid and retina promotes the half-life reduction of the drug which causes a reduction of its concentration to sub therapeutic levels in a short period of time. On the other hand, the increase of drug dose could prolong the effectiveness of single injections but could also induce peak drug concentration within the eye, exposing the patient to undesirable toxic effects.

To overcome the abovementioned limitations, several sustained-release drug delivery devices are being developed in order to obtain a drug therapeutic level inside the eye with the smallest possible complications associated. That will be discussed following.

1.2 Implants Manufacturing Techniques

Some of the techniques most used in the preparation of biodegradable intraocular implants polymer based are films, molding and extrusion. The technique and technical parameters to be used are conditioned by the type of polymer to be processed, the drug to be loaded and the properties of the final drug/polymer mixture (Rothen-Weinhold *et al.*, 1999; Kimura and Ogura, 2001; Breitenbach, 2002; Choonara *et al.*, 2007).

Films preparation may be performed in two ways: adding the solution, with the solubilized components, in a suitable solvent that is then evaporated or melting and pressure the polymer and drug. The dry film is then removed from the surface. To dissolve the polymer, solvent-casting using organic solvents might be involved and this could be a problem in biomolecules loading (Kimura and Ogura, 2001; Choonara *et al.*, 2007).

In the molding technique the polymer and drug are heated and compressed in molds of the desired configuration. In extrusion, the polymer and drug are continuously propelled under pressure through high temperature areas, causing melting and compacting the powder mixture into the shape of the implant (Rothen-Weinhold *et al.*, 1999).

Melt extrusion has an important role on pharmaceutical manufacturing granules, pellets, tablets, stents, implants, suppositories and ophthalmic inserts. It is considered to be an efficient

technique with advantages over solvent procedures like co-precipitation however the influence of heat stress and shear forces on the drug active are disadvantages related (Breitenbach, 2002). These two processes, molding and extrusion, are temperature aided and, for that reason, might be inadequate for drugs that are thermolabile. (Kimura and Ogura, 2001; Choonara *et al.*, 2007). The biological degradation kinetics of intraocular systems is affected by chemical nature composition and molecular weight of the polymer, morphology and structure of the device and thermal processes influence. Polymer processing techniques regulate the structure of the device and impact its morphology, specifically its microporous structure, polymeric chain orientation and crystallinity. Rothen-Weinhold *et al.* analyzed extrusion and injection-molding in the preparation of biodegradable implants and proved that molecular weight and polydispersity decreased after extrusion or injection-molding and this decrease was higher in injection-molding. The crystallinity analysis verified that the crystalline structure was not destroyed in both manufacturing approaches and, in what concern *in vitro* degradation, the extruded implants degraded more rapidly than the injection-molded ones.

Fialho and Silva-Cunha studied polymeric sustained-drug release systems comparing the impact of implant manufacturing techniques, compression and hot molding, on the *in vitro* degradation of the polymeric matrices and on the release of dexamethasone acetate. Their outcomes revealed that the manufacturing technique decidedly influences degradation and drug release progressions. The degradation was faster in compressed systems which also allowed one faster release of the drug.

Development of polymer-based drug controlled release systems may be performed in several ways, however, traditional approaches have limitations such as difficulties in the incorporation and heterogeneous distribution of the drug, use of potentially toxic solvents, drug dissolution and photochemical and thermal degradation. The use of supercritical technology is a viable alternative for the development of polymeric materials with loaded bioactive agents for biomedical application because it overcomes much of these limitations.

Polymeric foams involve a solid–polymer matrix and gaseous voids derived from a blowing agent. This materials have excellent thermal properties, flexibility to template to desired morphologies, high strength–to–weight ratio and good energy/mass absorption (Lee *et al.*, 2005; Brun *et al.*, 2011).

Due to its characteristics, there are several different applications for polymeric foams: constrution and aerospace industry, coating, acoustic insulation controlled release systems and scaffolding, among others (Zeng *et al.*, 2003; Zhai *et al.*, 2006, Bao *et al*., 2011).

The foaming method can be physical or chemical according to the nature of the gas formation. Physical blowing agents are inert elements, usually liquids with low boiling points, which

gasify under foaming circumstances. Chemical blowing agents are substances that produce gases due to chemical reactions, as water, sodium bicarbonate and citric acid (Ashida, 2007).
Several methods may be used to produce foams (casting and leaching, thermally induced phase separation, extrusion with chemical blowing agent) however all of them use solvents that contaminate the final polymeric foam. The purification of the foam is not always possible because it often require temperature rise that may degrade thermal sensitive compounds such as drugs (Jacobs *et al*., 2008).
Supercritical fluids are an alternative to traditional solvents in order to avoid this complications (Jacobs *et al.*, 2008; Duarte *et al.*, 2012).
A certain substance is considered to be in a supercritical state when its temperature and pressure are higher than its critical temperature and pressure. Supercritical fluids (SCF) have higher density than that of a gas and higher diffusivity than that of a liquid so it may be consider that they have intermediate properties to those of liquids and gases. Due this behavior, SCF are useful solvents for some compounds (Knox, 2005). The solvent choice depends on several aspects such as toxicity, cost, environmental issues and, obviously, pressures and temperatures in the desired processing supercritical region. Supercritical carbon dioxide ($scCO_2$) is a good possibility for medical applications because it is non-toxic, recyclable, environmentally-friendly, with acceptable cost, chemically inert and has reasonably low critical temperature (304K) and pressure (7.4MPa), which allows processing of thermolabile and biologically-active compounds (Kazarian, 2004; Woods *et al.*, 2004; Xu *et al.*, 2004; Jenkins *et al.*, 2006; Tai *et al.*, 2007; Shieh *et al.*, 2009; Morèrea *et al.*, 2011).
In the $scCO_2$ foaming process, the polymer saturation (with constant temperature and pressure) with $scCO_2$ lead to a homogenous mixture because the $scCO_2$ can plasticize amorphous and semi-crystalline polymers (Karimi *et al.*, 2012; Liao *et al.*, 2012; White *et al.*, 2012). In semi-crystalline polymers, $scCO_2$ penetrates favorably the amorphous phase because the gas dissolution is increased in those parts. The homogeneity occurs because the polymer glass transition and melting temperature are reduced – caused by the interactions between polymer and gas molecules (Gualandi *et al.*, 2010) – so the polymer is less viscous and polymer chains are more mobile and can rearrange themselves into an ordered configuration (Kiran, 2009; Liao *et al.*, 2012). This is mostly important in the case of high molecular weight polymers in which viscosity of the bulk polymer is relatively high. The high viscosity would require huge temperatures to processing and those high temperatures could lead to drugs thermal degradation. The $scCO_2$ reduce the intermolecular interactions and increase the chain separation facilitating the process (Jenkins, 2006). The cell nucleation happens inducing a thermodynamic instability factor in the equilibrium system. The instability may be caused by temperature

increase or pressure decrease. In the case of pressure decrease and in systems using CO_2, as the gas depart from the polymer, occurs nucleation of gas bubbles that lead to foams formation. The nucleation can be homogeneous or heterogeneous, but heterogeneous nucleation is energetically preferred since it requires a lower activation energy barrier (Jenkins *et al.*, 2006; Léonard *et al.*, 2008; Tsimpliaraki *et al.*, 2011). The number and size of pores depends on the growth and bubble rates. With high depressurization rates, more bubbles nucleate thus the gas available for growth is separated into more cells and foams will have more pores but with smaller diameters. The inverse occurs with low depressurization rates; as the energy barrier for nucleation increases, the nucleation rate decreases leading to less nucleation rate and bigger bubbles (Jenkins, 2006; Léonard *et al.*, 2008).

In the end, the polymeric foam results from the growth of the cells, coalescence and expansion (Karimi *et al.*, 2012; White *et al.*, 2012) and its morphology and structure are determinate by foaming conditions (temperature, pressure, saturation time and depressurization rate (Tsimpliaraki *et al.*, 2011; Liao *et al.*, 2012).

Though the advantages, CO_2 has difficulty to dissolve compounds with high molecular weight. Besides that, its lack of some specific solvent-solute interaction and non-polarity could lead to deposition yields. Adding small amounts of co-solvents improve $scCO_2$ solvent power and increase the solubility (Natu *et al.*, 2008; Braga *et al.*, 2008). Glycofurol is a greener and safer additive that acts as porogenic agent, plasticizer and polymer compatibilizer. It is water-miscible and frequently used on pharmaceutical formulations so it's a good option as co-solvent for the development of implants (Boongird *et al.*, 2011; Allhenn and Lamprech, 2011).

The $scCO_2$ processed materials have no residual solvent presence except when co-solvents are used. This gains over other solvents made that $scCO_2$ has been analyzed not only as a solvent but also as an anti-solvent or plasticizer for polymerization, modification and extraction (Tai *et al.*, 2007, Kiran 2009).

This technology has proven to be effective in biomedical applications including PCL ibuprofen impregnation (Yoganathan *et al.*, 2010) and preparation of imprinted contact-lenses for drug delivery (Yañez *et al.*, 2011).

2. Materials and Methods

2.1 Chemicals

The PCL pellets (CAS [24980-41-4], Mw of 45000 $g \cdot mol^{-1}$), glycofurol (tetraglycol, CAS [31692-85-0]), methanol (purity ≥ 99.9%, CAS [67-56-1]), acetone (purity ≥ 99.5%, CAS [67-64-1]), acetonitrile HPLC (purity ≥ 99.9%, CAS [75-05-8]) and dexamethasone (purity ≥ 98%) were supplied by Sigma-Aldrich.

Carbon dioxide was obtained from Praxair (purity (v/v) ≥ 99.998%).

The 2-Chloro-N^6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (2-Cl-IB-MECA, Mw of 544.74 $g \cdot mol^{-1}$, CAS [163042-96-4]) was from Tocris.

Except for the PCL, all chemicals were used without other processing.

2.2. Methods

PCL preparation: from pellets to powder form

In order to allow the introduction of the PCL in the catheter used in the processing with $scCO_2$, the polymer was powderized and sifted. Only the particles with a diameter less than 250 μm were used. The small diameter of the PCL power also allow an increase of the superficial area, which favors the interaction with $scCO_2$ molecules, and optimize the physical mixing between the drugs and the polymer.

For powderization, 12 g of PCL were dissolved in 200 mL of acetone at environmental temperature. This solution was precipitated adding 20 mL of methanol followed by 20 mL of water, both dropwise, and then it was centrifuged at 5000 rpm for 10 minutes. The supernatant was removed and the PCL powder dried at room temperature, in petri dishes, for one week. After that, the powder was sieved to rich the desired diameter (≤ 250 μm) and stored in suitable vials.

Implants preparation

Before the foaming process, a mixture of PCL power, glycofurol and drug – Dexamethasone or 2-Cl-IB-MECA – was made with a proportion of 8% (w/w) of glycofurol and 26% (w/w) of

drug. This combination was physically mixed in a glass vessel until homogenization. Some tests were performed with pure PCL and others without glycofurol in order to compare the glycofurol effect as compatibilizer.

Several containers with different shapes and materials were tested to reach implants with viable dimensions and structure for biomedical application.

To achieve a realistic and sustainable ocular implant size, I.V. Optiva® catheters have been chosen to use as a mold. They were purchased from Smiths Medical and are composed of a needle inserted in a polyurethane tube with a maximum diameter inside of 0.464 mm.

The mixture to be processed was introduced into two thirds of the catheter polyurethane tube and compressed with the needle therein. The tube was then removed from the remaining catheter to be processed. This time-consuming and meticulous process was completely manual. Detailed description of the filling procedure is in Appendix A.

Implants Processing

The filled polyurethane tubes were placed horizontally, in a supporting metallic mesh, inside the pressure cell for foaming processing. The maximum number of simultaneously processed tubes was 40.

Implants were set with the batch foaming technique, using $scCO_2$ as foaming agent, in a supercritical solvent unit (Appendix B).

The samples were placed in a high pressure cell (23 cm^3) which was immersed in water with controlled temperature (45º C; Thermoscientific, Haake AC 150).

Thereafter, CO_2 was slowly introduced (nearly 0.6 bar *per* second) in the high pressure cell until reaching a pressure of 200 bar (controlled with manometer – Lab DMM, REP transducer). For two hours the system was maintained in constant conditions of temperature and pressure under magnetic stirring (average 740 rpm) to facilitate the mixture homogenization and CO_2 diffusion. After that time, the high pressure cell was depressurized at a pre-established rate of 10, 20 or 30 bar/min. All process conditions are presented in table 3.

The processed material was recovered and stored in petry dishes. The storage of dexamethasone implants was done at room temperature and 2-Cl-IB-MECA implants in the refrigerator; both storage containers had silica gel to ensure humidity control.

After several tests, it was concluded that the removal of the implants from polyurethane tubing should only be done at least 24 hours after processing to ensure complete CO_2 output. Prior to that time, the implants were more adherent to the tube wall. Removal of the implants within the tube walls was made by taking out the polyurethane structure with a scalpel (*peel the implant*).

After their removal, implants were optimized in the standard defined dimensions (2 mm length and maximum diameter of 0.464 mm).

The operating temperature (45º) and pressure (200 bar) conditions were that defined because, according to Leeke *et al.*, 2006, $scCO_2$ has the highest solubility in PCL at these operating parameters. With high solubility, PCL swelling degree would increase drug loading.

PCL used was Mw of 45000 $g \cdot mol^{-1}$ because low molecular weight polymer disrupt after processing (Matos *et al.*, 2013) and high molecular weight would increase the melting point.

With the purpose of investigate porosity effect and compare methodologies of processing, some implants were processed placing filled polyurethane catheters tubes in an oven at 62.5ºC to ensure PCL melting.

Glycofurol presence, supercritical processing and variation of the depressurization rate influences were tested in order to obtain proper pores, in number and size, on a resistant and manageable implant (Table 3).

Table 3 - Experimental conditions

P (bar)	T (ºC)	System stability (min)	Co-solvent (Glycofurol)	Depressurization rate (bar/min)
200	45	120	0	20
			8% (w/w)	20
			8% (w/w)	10
			8% (w/w)	30
~ 1	62.5		0	-
			8% (w/w)	-

Implants Characterization

Implants were evaluated according to their morphology, porosity and thickness.

Samples morphology was evaluated macroscopically (using digital photographs with an enlargement of 4x), microscopically (using Olympus BH-2 optical microscope) and with a scanning electron microscope (Zeiss Merlin 61.50, operating at 0.1 kV and 2.0kV and 80 pA).

Implants microporosity was measured by N_2 adsorption (ASAP 2000 V2.04). For each microporosity analysis, implants from 80 processed catheters were used, for itch variation parameter study, so that ensure reliability of results.

To calculate the surface area, Brunauer, Emmet and Teller (BET) method was used. According to BET theory, surface area is estimate by physical adsorption of a gas on a solid surface and by calculating the quantity of adsorbate gas matching to a monomolecular layer on the surface (Teixeira *et al.*, 2001). The total porosity was calculated according to equation (1).

$$P = 1 - \rho_{apparent}/\rho_{solid} \qquad (1)$$

where $\rho_{apparent}$ is the calculated as the ratio between the mass and volume of the samples and ρ_{solid} is the real density (Bueno *et al.*, 2014).

In a porous material, real density relates to the volume of material not including the porous spaces (Lowell *et al.*, 2004). Helium picnometry (Quanta-Chrome, MPY-2) was the method used to evaluate samples real density.

The mean pore size was determinate based on the horizontal Feret diameter of the pores and using ImageJ software.

Implants thickness was determined with a digital micrometer (Electronic Outside Micrometer IP 54, 0-25 mm, 0.001 mm) and it referred as an average of 5 measurements.

Drug quantification

High-Performance Liquid Chromatography (HPLC - Prominence UFLC Shimadzu coupled to a photo diode array detector SPDM20A) was used for implants drug quantification and drug delivery. The operation was made with appropriate schedules for Dexamethasone and 2-Cl-IB-MECA.

In Dexamethasone analysis, the column was Eurospher 100-5C18 RP (250 × 4 mm i.d., 5 mm, Germany) and the chromatographic conditions were based on Chim *et al.*, 2012: mobile phase constituted by methanol/water in a proportion of 9:1 (v/v), isocratic elution (15 min) and flow rate of 1 $mL \cdot min^{-1}$, at 35 °C. To clean the column, runs with acetonitrile have been made. Injected sample chromatographic profile was measured at 239 nm at least in triplicate.

The chromatographic conditions for 2-Cl-IB-MECA were defined based on Kim *et al.*, 1996: mobile phase with water (A) and acetonitrile (B) in a proportion of initial A=20%, increasing to 30%, from 0 to 9 minutes, and reducing to 20%, from 9 to 12 minutes, at 35ºC. The column used was also Eurospher 100-5C18 RP (250 × 4 mm i.d., 5 mm, Germany).

To quantify the release of Dexamethasone and 2-Cl-IB-MECA off the implants, standard curves have been elaborated, for both drugs, in methanol and water from solutions of known concentration (Appendix C and D). Analysis of total drug release was made using methanol as solvent to facilitate the process since the drugs are more soluble in this solvent. Mass-known implants were placed into glass vials with a defined volume of methanol (250 µL) and made up HPLC readings until the drug was undetectable.

Between analyzes the samples remained stored in thermoshaker at 25° C, 100 rpm.

In view of forward applications, for the 2-Cl-IB-MECA, a saline solution similar to *in vivo* environment, cell culture medium and explants culture medium were previously tested but the HPLC detection peak was coincident with the drug one (Appendix E) so it was not feasible for analysis. In drug releasing study, water was used as solvent since the vitreous humor is essentially constituted by water. The implants were emerged in water and measurements were made hourly during the first 6 hours and then increasing the time intervals daily. Due to PCL hydrophobicity, some implants floated which reduced the interaction with water. This may have affected some results. At the end of delivery process, the dry implants were weighed for comparison of masses. All analysis were made at least in triplicate.

2-Cl-IB-MECA degradation analysis

In several tests carried out, 2-Cl-IB-MECA degradation appeared to occur. In order to confirm this degradation and better understand this process, drug degradation analysis was carried out using HPLC. A solution of 2-Cl-IB-MECA was exposed to thermal shocks between 25ºC and 80°C and subsequently analyzed by HPLC to detect degradation and the way it was visually manifested on the graphs.

Modulated Differential Scanning Calorimetry (MDSC)

Implants, without glycofurol, and with different depressurization rates (SFM10 and SFM20), implants with glycofurol addition with a depressurization rate of 20 and 30 bar/min (SFM20-G and SFM30-G) and HM processed implants were analyzed in what concern their glass transition temperature by MDSC (Q100, TA instruments) with nitrogen purge gas (50 $mL.min^{-1}$). The calibration process was executed with indium and measurements were made in alumina pans equilibrated at -80ºC for 5 min, modulate ± 0.50ºC every 40s, and heating at 2ºC min^{-1} until 200ºC. The glass transition temperatures were determined with duplicate analysis performed to ensure results reliability.

Diffusion coefficients

Mass transfer through porous polymeric membranes depends on several factors such as solubility and diffusivity of the drug into the polymer, morphology and plasticization (Karimi, 2011). According to the second Fick's law, to describe the drug release, a zero-order kinetics equation (2) was used. In the equation, M_t represent the cumulative amount of drug released at time t and M_∞ the cumulative amount of drug released at infinite time, k is a kinetic constant that incorporates geometric and structural characteristics of the implant and n is the release

exponent (Natu *et al.*, 2008, Yañez *et al.*, 2011). Below 60% of the amount drug released, the drug release profiles were fitted to equation 2:

$$\frac{M_t}{M_\infty} = kt^n \qquad (2)$$

Other equations used were the following:

$$\frac{M_t}{M_\infty} = 4\left(\frac{Dt}{\pi l^2}\right)^{\frac{1}{2}}$$

$$\frac{M_t}{M_\infty} = 1 - \left(\frac{8}{\pi^2}\right)\exp\left(-\frac{\pi^2 Dt}{l^2}\right) \qquad (3) \text{ and } (4)$$

where l represents the thickness (~ 2 mm) of the sample and *D* is the diffusion coefficient, which is presumed to be constant. Eq. (3) is only useable for the first 60% of the total release (M_t/M_∞) while Eq. (4) is valid for the last 40% of the total release (M_t/M_∞) and has been commonly used to obtain diffusion coefficients from drug release investigational data. The drug diffusion coefficients *D* can be determined from the resulting slopes, after linearization and regression analysis of these equations (Yañez *et al.*, 2011).

In Vivo analysis

In vivo PCL implants tolerance were performed in AIBILI by Professor Raquel Santiago team. The implants were implanted into the posterior chamber of one of rat's eye under general anesthesia and sterile conditions. The incision was made using a 24G needle. The other eye was left untreated for control.

The evaluation of safety and tolerability were made by monitoring adverse events such as inflammation, edema, cataract formation and polymer appearance and location (Myers *et al.*, 2016).

3. Results and Discussion

3.1 Morphological characterization

Glass tubes have been used in several sizes as a support and mold to produce ocular implants however, it was found that this was not the best option since the dimensions of the obtained implants were not suitable for *in vivo* applications and the removal process of the implant outside the glass – *breaking the glass* – caused damage therein and some glass fragments could remain in the implant (Figure 2).

Polyurethane tubes of 24G catheters were found to be a good option due to its size (inside diameter equal to or less than 0.464 mm), biocompatibility, bio stability and mechanical properties (Wang and Wang, 2012). The removal of the inner implant from polyurethane tubes was made by cutting the walls with a scalpel, and by microscopic analysis, it was found that this procedure do not damage significantly the implants structure (Figure 3).

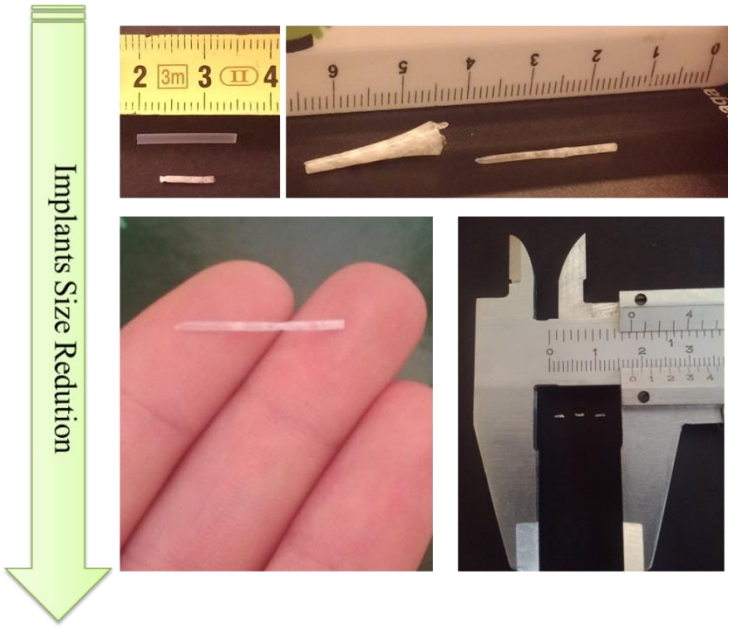


Figure 2 - Evolution of Implants Size Reduction during investigation.

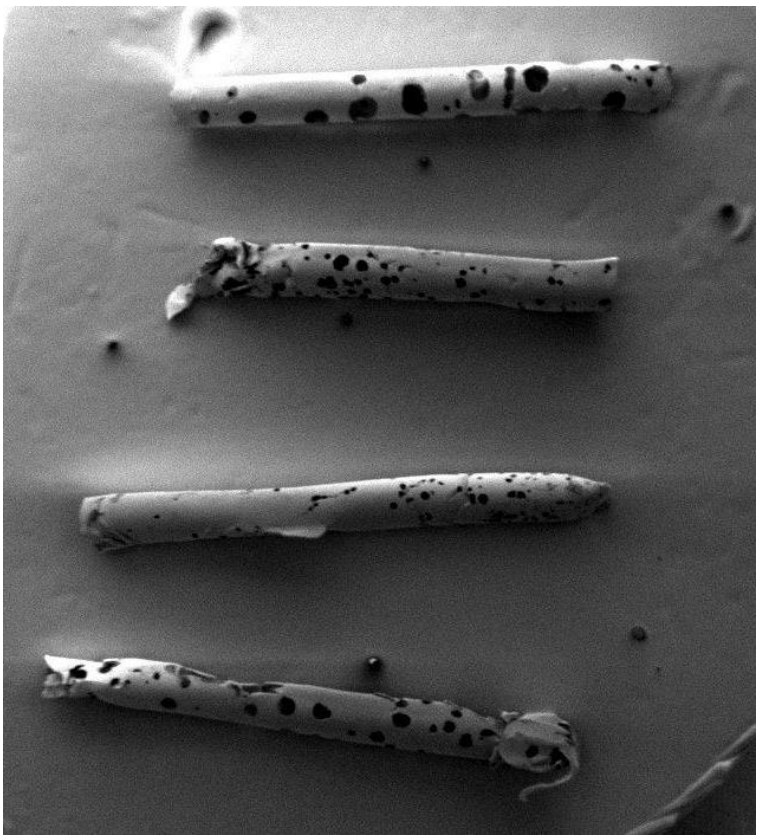


Figure 3 – Implants macroscopic view.

The average mass of PCL introduced into a catheter for processing was 1.16 ± 0.01 mg and the average mass of PCL implants was 0.19 ± 0.01 mg. During processing in $scCO_2$ unit, average PCL mass lost was 0.068 mg ± 0.003. This mass loss is mainly caused by drag with CO_2 during the depressurizing process.

The produced implants have similar dimensions to implants currently available into market (table 4). Implants size has a substantial role in the viability of securing implants with minimal invasiveness.

Implants with dimensions higher than those can trigger foreign body reaction with formation of fibrous capsule by the deposition of fibroblasts, foreign body giant cells and macrophages around the implant. This capsule prolongs degradation rate (Kuno and Fujii, 2010; Manickavasagam and Oyewumi, 2013).

Choonara *et al.* (2009) and Manickavasagam and Oyewumi (2013) refer the importance of the development of ocular devices that are geometrically small because they are less aggressive and well tolerated; the produced implants follow that requirements.

Table 4 - Commercial and produced implants dimensions.

Implant	**Diameter (mm)**	**Length (mm)**
Iluvien®	0.37	3.5
Ozurdex®	0.46	6
PCL implants	≤ 0.464	2

The operating conditions (temperature, depressurization rate and additive presence) were defined based on studies already carried out in PCL processing with supercritical technology (Yoganathan *et al.*, 2010; Takahashi *et al.*, 2012; Matos *et al.*, 2013). Although there are studies in this area, it was important to analyze the effect of the parameters on a scale as small as that of intraocular implants.

Use of glycofurol is important to ensure homogeneity of the polymer/drug mixture and porosity. Tests with varying amounts of glycofurol were made: 22% (w/w), 12% (w/w) and 8% (w/w). A proportion of 22% (w/w) glycofurol is not viable because cause excessive increase of porosity and adhesiveness of the implant to the walls of the catheter which does not allowed removal (Figure 4). The implants pores were so large that the implant not present a tube defined structure. Reducing the amount of glycofurol to 12% (w/w) the implant removal was still difficult because it appears not to have a resistant structure. Desired results were achieved at 8% (w/w) glycofurol: homogenous physical mixture between PCL and the drug and porous structured implants easily removable from catheters (Figure 5).

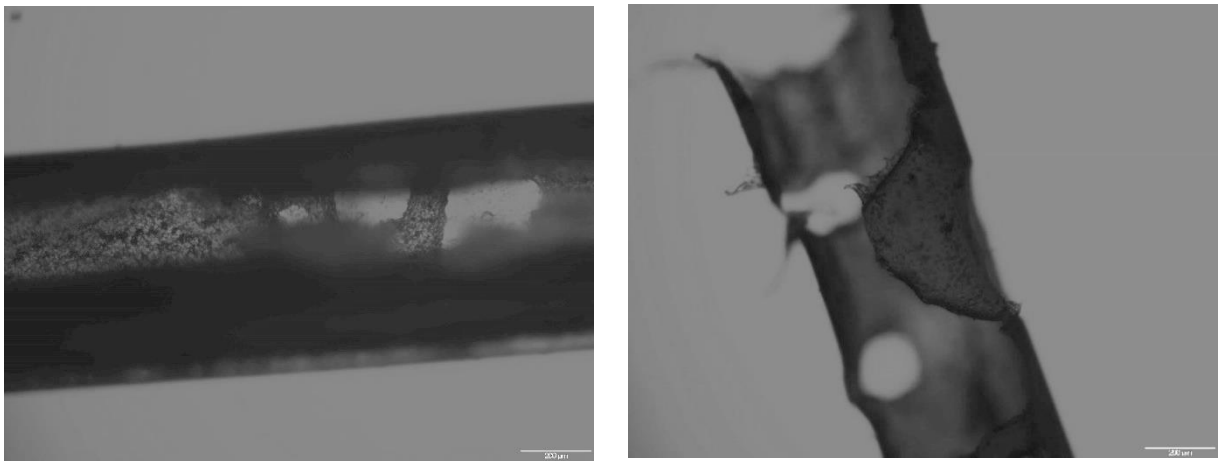


Figure 4 - Optical Microscope images of 22% (w/w) glycofurol in PCL implant: catheter walls that could not be removed in its entirety and large pores of the implant are visible.

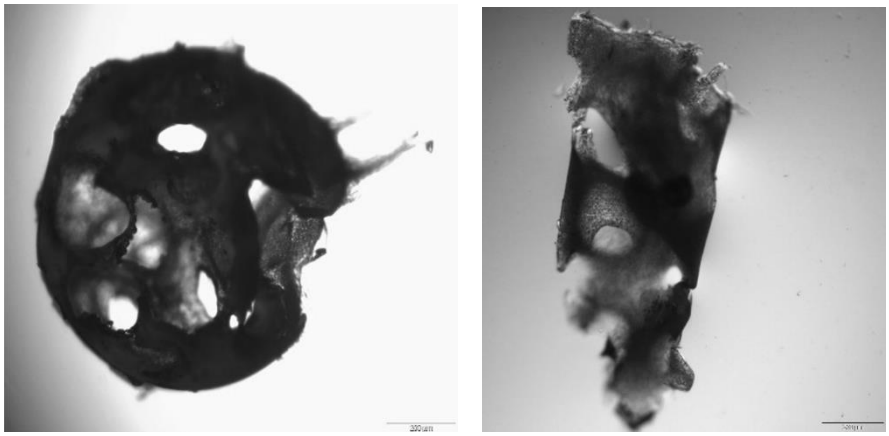


Figure 5 - Optical Microscope image of 8% (w/w) glycofurol in PCL implant: initial tests. Implants were totally removed from catheter tube and have porosity.

The effects of glycofurol presence and depressurization rate on porosity were analyzed. According to the SEM images (Figure 6), it is evident that heterogeneous pores distribution were obtain in all samples and implants porosity is different depending on the depressurization rate and additive presence.

As the depressurization rate increases, the pore size decreases and the pores number increases. This differences may be caused by higher cell density and smaller pore size are achieved when more CO_2 is dissolved into a polymer (Karimi *et al.*, 2011). As the depressurization rate increases, the $scCO_2$ has more contact time with the polymer and may be more dissolved on it leading to small pores. Otherwise, glycofurol presence seems to restring the $scCO_2$ interaction with the polymer, reducing the contact between them and consequently increasing the pore size. Glycofurol additive appears to act generate nucleating spots in witch contact between polymer and gas particles is facilitated which leads to the lowering of energy barrier for cell nucleation; this fact increases nucleation rate and origin pores with large diameter (Zhai *et al.*, 2006; Jacobs *et al.*, 2008).

The SEM images shows macro and nano structures on the implants surface with morphologies similar to spherical indentations or cavities.

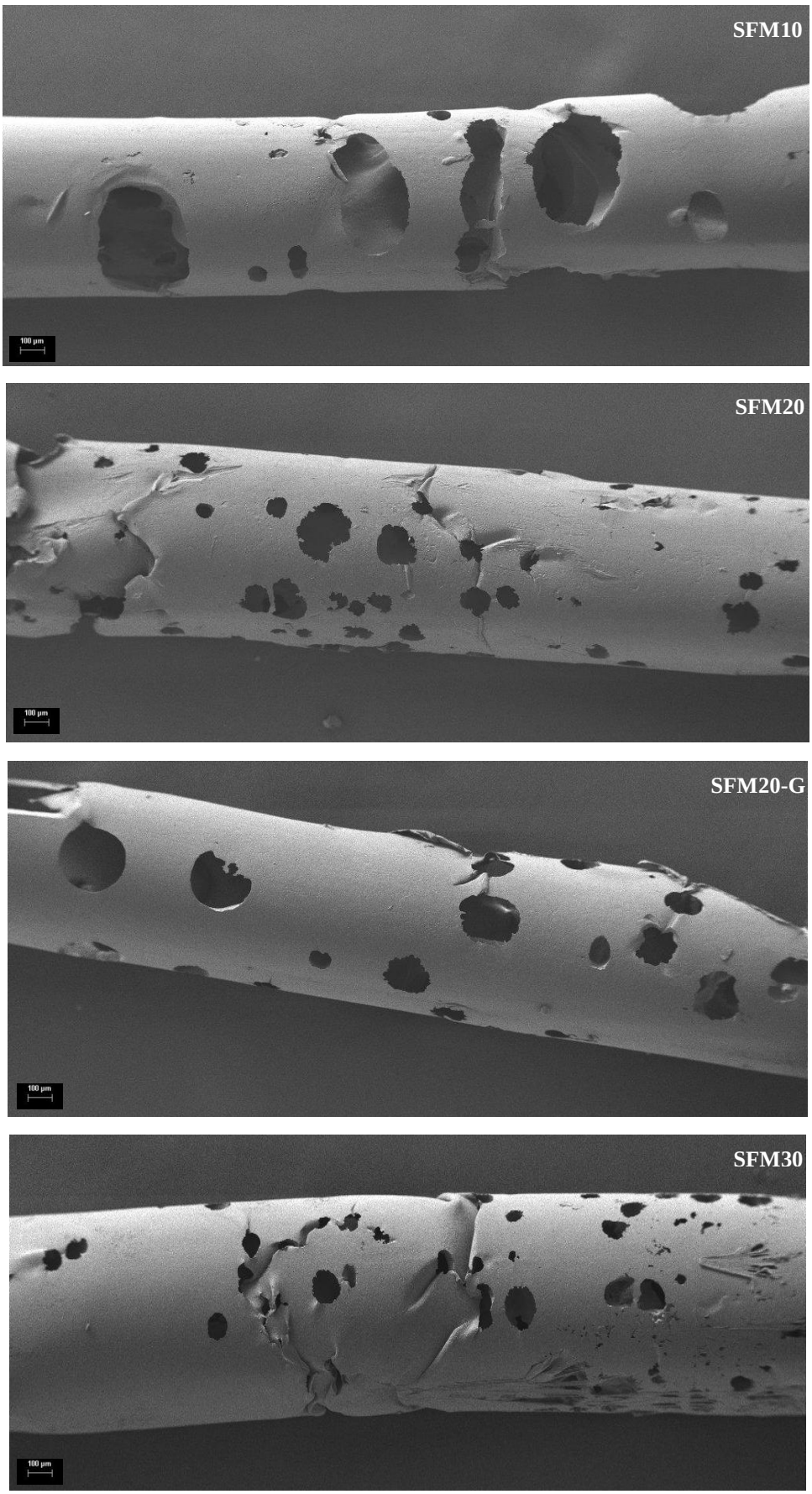


Figure 6 - SEM: implants prepared by $scCO_2$ foaming process for 2h, 20 MPa, 45°C. Depressurization rates were variable. At depressurization rate of 20 bar/min, samples with and without Glycofurol were analyzed.

Comparing $scCO_2$ foaming processed and hot melting produced implants, it is seen that hot melting produced implants has significantly lower pores number due to the fact that no nucleation process occurs (Figure 7).

Implants porosity is important for gradual drug release, so $scCO_2$ appears to be a better alternative. Moreover, temperature required for hot melting would degrade most of the drugs.

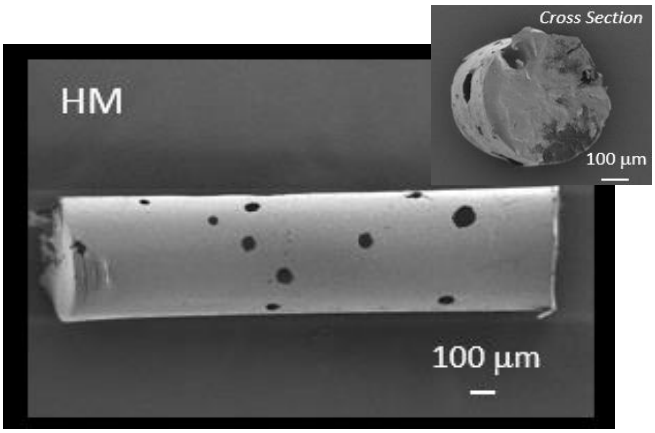


Figure 7 - SEM: implant prepared by hot melting.

Implant physical analysis confirm the influence of depressurization rate and glycofurol in porosity.

BET surface area, pore volume and average pore diameter were determinate by nitrogen adsorption (Figure 8 and Table 5).

Regarding surface area, it was verified that this parameter is superior for higher depressurization rates (20 and 30 bar/min) with an unexpected no relevant difference between them. SFM20-G showed a reduction of the surface area compared to the sample without glycofurol (SFM20).

In theory, samples subjected to fast depressurization typically show lower average pore diameters (Jenkins *et al.*, 2006; Tai *et al.*, 2007; Kiran, 2010). Adding glycofurol, CO_2 solubility in the polymer is increased and more $scCO_2$ is accessible for pore nucleation leading to the formation of smaller pores. Pore volume results and average pore diameter are similar to those of BET surface area: higher pore volume and pore diameter in SFM20 and SFM30 for samples without additive and lower values for SFM20-G relatively to SFM20 sample. Nucleation theories were expound on section 1.2.

HM implants appear to have similar pore size to SFM20 and SFM30 but surface area is much lower. In all analyzed cases, surface area was higher in SFM processed implants than HM.

Besides temperature degradation, smallest surface area may limit drug diffusion.

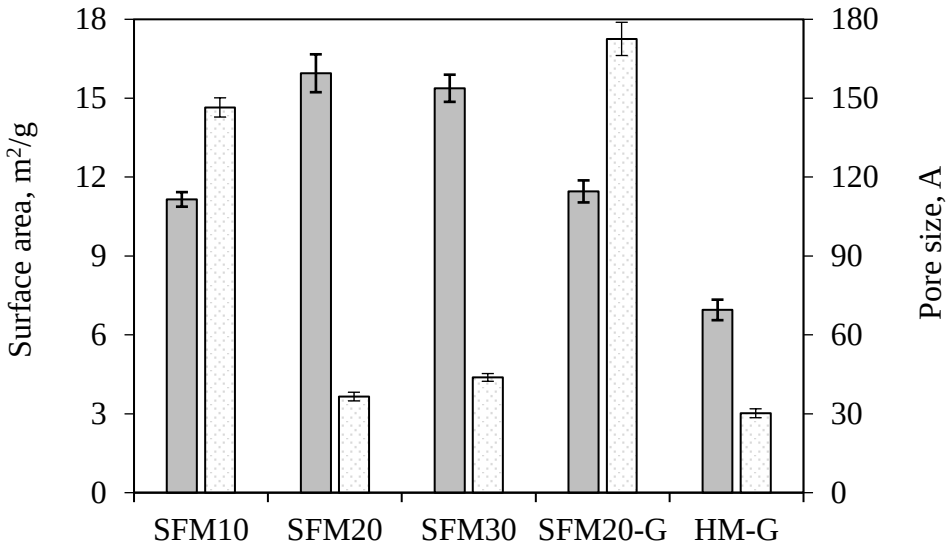


Figure 8 - Implants surface area and pore size in different processing conditions:

■ surface area □ pore size

Analyzing porosity (Figure 9), it may be consider that SFM processed implants have similar porosity (c.84%) which is higher than HM implants porosity (c.52%).

Depressurization rate and glycofurol seems not to affect significantly the implants density (Table 5) since all the analyzed values are within error intervals. This fact as also been reported by other authors that analyzed the effect of this parameters on the density of pure PCL porous biomaterials (Matos, 2012; Rosa, 2013; Churro, 2016).

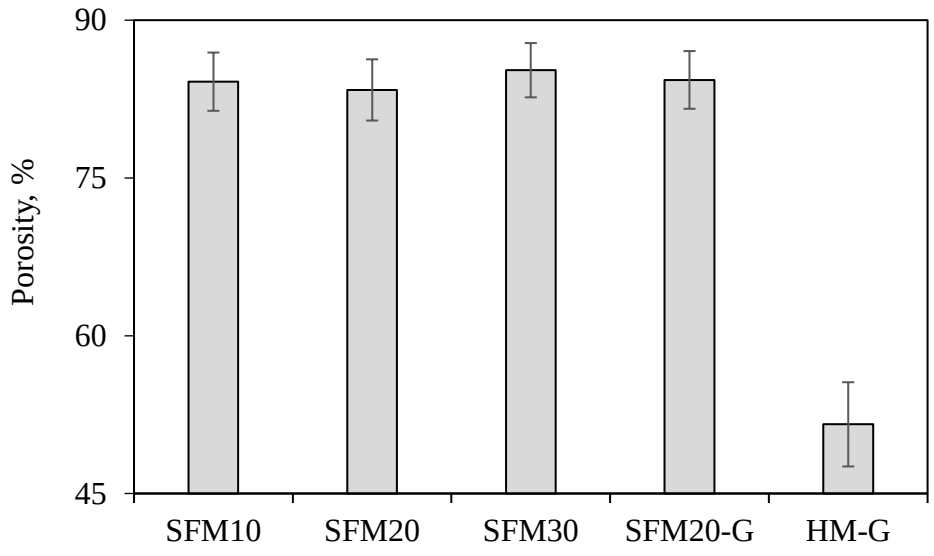


Figure 9 - Implants porosity under different processing conditions.

In what concern average pore diameter calculated with ImageJ software, the results almost underwrite the aforementioned. SFM10 presents bigger diameter pores from 120 µm – 250 µm; SFM20 from 120 µm – 170µm; SFM30 from 90 µm – 100 µm and SFM20-G from 110 µm – 120 µm. The difference in SFM20-G may be justified by the fact that the SEM image would not have captured the larger pores.

Table 5 – Morphological and Thermal characterization.

Samples	Helium Pycnometry	Nitrogen Adsorption			SEM	DSC	
	Real density (g cm^{-3})	BET surface area (m^2 g^{-1})	Pore volume (cm^3. g^{-1})	Average pore diameter (Å)	Average pore diameter (μm)	T_m (ºC)	Enthalpy (J/g)
SFM10	1.04 ± 0.03	11.15 ± 0.28	0.04 ± 0.01	146.50 ± 5.0	185 ± 65	62.03 ± 0.25	1227 ± 65.05
SFM20	0.99 ± 0.09	15.95 ± 0.72	0.01 ± 0.01	36.54 ± 2.2	145 ± 25	62.19 ± 0.31	1260 ± 59.40
SFM30	1.12 ± 0.07	15.38 ± 0.52	0.02 ± 0.01	43.82 ± 3.6	95 ± 5.0	-	-
SFM20-G	1.06 ± 0.12	11.46 ± 0.42	0.05 ± 0.01	172.55 ± 6.0	115 ± 5.0	61.82 ± 0.26	1353 ± 71.42
SFM30-G	-	-	-	-	-	61.71 ± 0.35	1360 ± 0.71
HM	-	-	-	-	-	56.60 ± 0.20	-

3.2. Thermal Analysis of Samples: DSC

Influence of processing method, depressurization rate (in SFM) and additive presence on T_m were obtained using DSC analysis.

PCL powder melting temperature obtained was 61.00 ± 0.20 ºC which is in accordance with the literature (Lebourg *et al.*, 2008 and Salerno *et al*., 2010).

Pure processed PCL melting temperature is reported in the literature (Matos *et al.*, 2013 and Rosa, 2013) to decrease but this was not verified on this analysis (T_m: 61.04 ± 0.20). This reduction on T_m value was observed on HM (56.60 ± 0.20).

Obtained values for SFM didn't reveal important differences so it is possible to assume samples homogeneity (Table 5).

At ordinary conditions, PCL melts around 60º C (Jenkins, 2007; Bassi *et al.*, 2011). According to Charoenchaitrakool *et al.* (2000), the T_m of PCL in $scCO_2$ presence is around 36.6º C at 147 bar; 36.2º C at 163 bar and 34.2º C at 276 bar. T_m at 200 bar is approximately 36.6º C (Matos *et al.*, 2013 and Rosa, 2013). All samples were produced at 45º C and 200 bar so the PCL was melted and $scCO_2$ was penetrating profoundly within polymer chains.

All samples exhibited a melting temperature between 61.71º C and 62.19º C. Considering the error range it is possible to achieve that the depressurization rate and the presence of glycofurol do not distress PCL (Figure 10). Considering that depressurization rate determines material porosity, it can be conclude that porosity does not affect the thermal properties of the PCL.

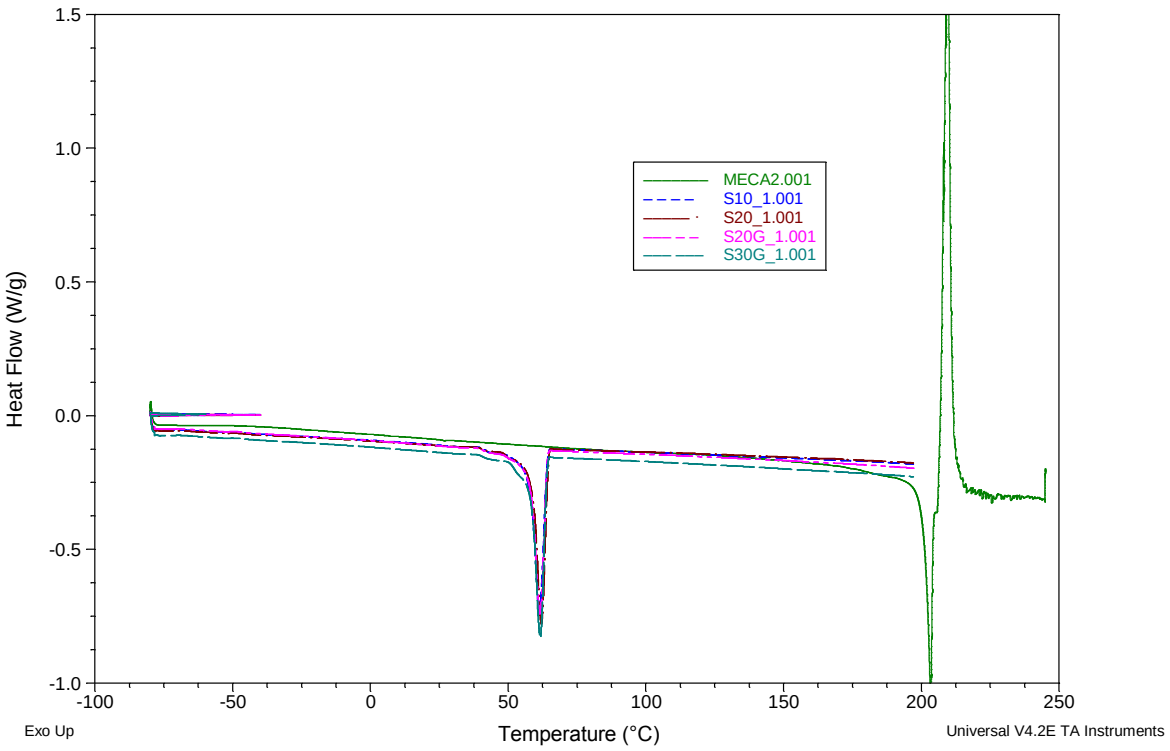


Figure 10 - Samples thermal analysis: DSC thermograms.

For enthalpy (Table 5), obtained values exhibit some differences. In samples without additive, it appears that the enthalpy tends to increase with decreasing pore size. Although the differences are small this may be due to the fact that the presence of larger pores become the polymer chain links weaker and so that reduce the amount of energy required for fusion.

Otherwise, when comparing implants with the same depressurization rate but with the presence of variable glycofurol, it appears that, despite the glycofurol increase the pore size, the enthalpy increases. Apparently, glycofurol tends to increase the energy required for fusion. However, the analyzed data are limited and do not allow to safely inferred that so this situation should be investigated in the future research.

From thermal analysis it is concluded that, although the implants exhibited different porous structures dependent on the experimental conditions, the thermal degradation of PCL is not a concern on processing. This conclusion is similar to the study by Takahashi *et al.* (2012).

All melting temperatures are above body temperature which make the PCL implants suitable for human biomedical applications.

Crystallinity was not calculated because several previous studies concluded that it does not change in the foaming process (Matos, 2012; Rosa, 2013).

3.3. Dexamethasone Implants: quantification and release

The influence of additive and processing technique were analyzed by comparing total dexamethasone release in implants produced by foaming (with and without glycofurol) and implants produced by temperature raising until melting (Figure 11). In the melting procedure the system drug-polymer were exposed to 62.5ºC to ensure PCL melting.

According to the graph, there is less variability in SFM sample results. It occurs because the technique allows a more homogenous drug distribution throughout implant body.

Analyzing the influence of additive, in samples with glycofurol, total dexamethasone release was bigger which suggests a higher drug incorporation. Glycofurol seems to act as a polymer-drug compatibilizer.

Considering these results the most desirable option was the obtained with SFM20-G: greater drug incorporation and lower associated error.

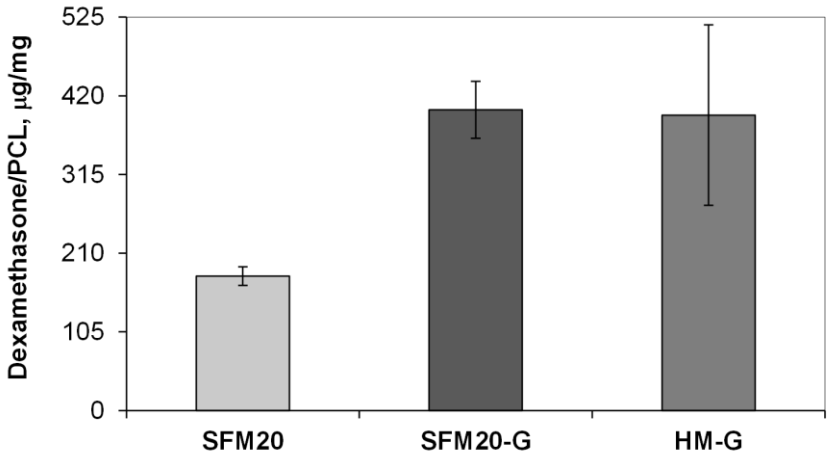


Figure 11 - Total Dexamethasone implants release comparing SFM with HG and additive.

Sustained drug release is affected by factors that influence polymer degradation and drug release such as the type of polymer and is molecular weight (which is the same in this analysis), hydrolysis mechanism, erosion properties, shape and porosity (Manickavasagam and Oyewumi, 2013). Dexamethasone Implants revealed sustained release over 500 h.

The foaming implants release profile is more uniform and constant because drug distribution in the polymeric matrix is pretend to be uniform and the porosity induced by depressurization allows a drug release phased and regular. The contact area with the environment is increased by pores. Otherwise the implants produced by melting, are not expected to have pores in their structure therefore the drug release occurs only by diffusion and erosion of the external surface

of the implant. In Figure 12 it is possible to see that until 200h of release, SFM release is more stable and higher than HM. After that time, HM behavior release become more stable but still lower than SFM.

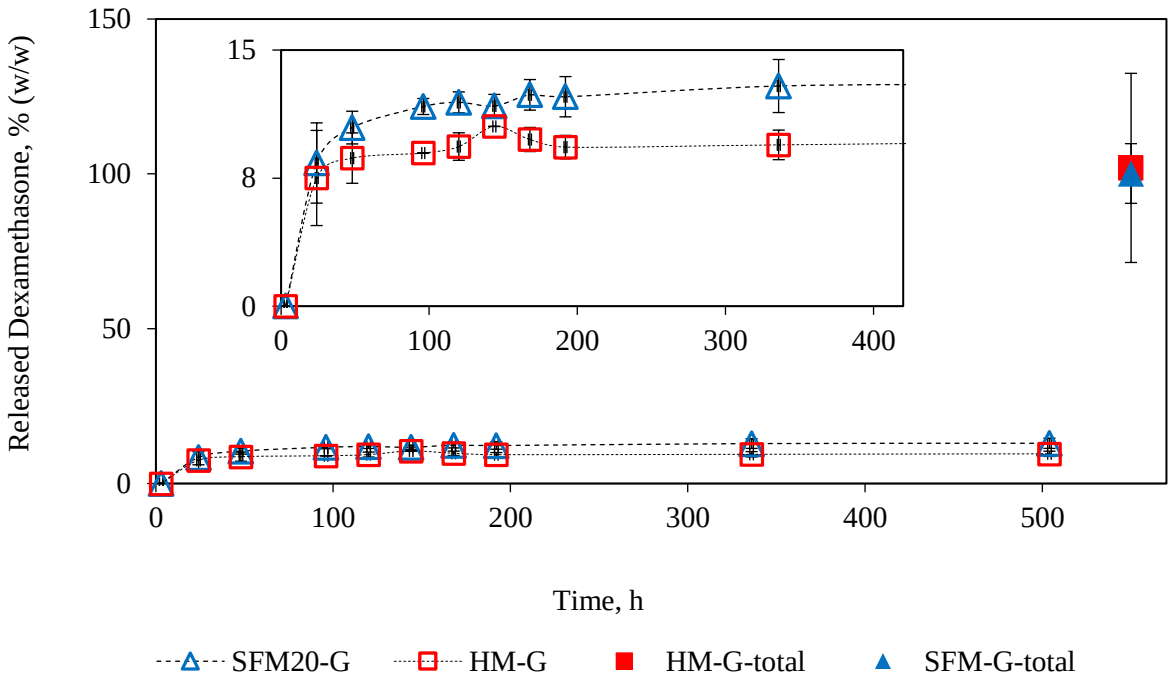


Figure 12 - Dexamethasone release profiles in water.

3.4. 2-Cl-IB-MECA Implants: quantification and release

At the time this investigation was performed, there were no theoretical solubility data of 2-Cl-IB-MECA in water or methanol.

The values obtained in the first assays were sub-quantified due to the lack of solubility data. It was only known that release was greater in methanol than in water. Solutions where the implants were placed, saturated and, therefore, no further release occurred. The releases obtained at this time were around 3% of total drug amount. In later studies, 100 hours of release was subsequently achieved by increasing the volume of solvent in order to prevent the

saturation of the medium. In analyzes after this investigation, release profiles were obtained up to 30 days.

It was found that the 2-Cl-IB-MECA was very difficult to dissolve in water to form a homogeneous solution in order to make a standard curve of drug release in water. In several tests performed, there was degradation of the drug after the first 24 hours with stirring (100 rpm) and thermal effect (37° C). On *Galal et al.* research, the preparation of 2-Cl-IB-MECA solutions were refer to be made with an organic solvent and further dilutions with distillated water so, based on that, the dissolution of the drug was made in Glycofurol adding subsequently water.

With this step, it was possible to achieve the 2-Cl-IB-MECA standard curve in water (Appendix D).

It was found that the drug and polymer particles interact in order to move away from each other not achieving a cohesive and homogeneous mixture. This behavior difficult the delicate methodology of to fill catheters with the mash and the yield of the products used was 1/3 lower compared to using Glycofurol. The Glycofurol is a determining factor in the mixture and homogeneity between the 2-Cl-IB-MECA and PCL.

As in the case of dexamethasone, the total drug amount was measured in methanol because this drugs are very soluble in this solvent which facilitates full release process. SFM20-G and SFM30-G implants were analyzed (Figure 13). Values obtained for the same processing conditions are similar which allows to infer the homogeneity of samples. The release of 2-Cl-IB-MECA in methanol occurs in a fast and sudden early stage. About 84% of drug released is released in the first 2 hours which validates the feasibility of using methanol for this purpose.

Total drug release was found to be very close to theoretical: 78.7% in SFM20-G and 95.4% in SFM30-G.

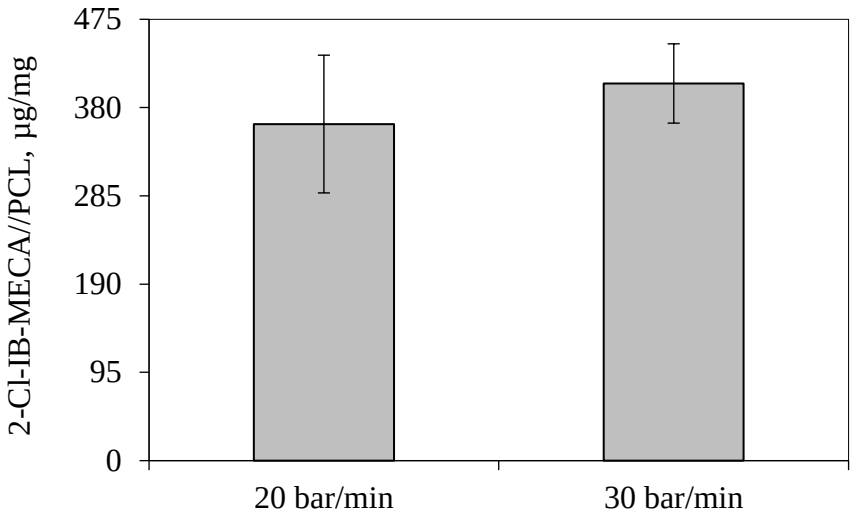


Figure 13 - 2-Cl-IB-MECA implants total release.

In what concern sustained release, the effect of glycofurol was also analyzed (Figure 14). 2-Cl-IB-MECA SFM20 implants, with and without additive were tested. The associated errors from the release without glycofurol are very high and it is more difficult to reduce them than in the other samples.

This means that there is great variability when glycofurol is not used, although the release is greater. This release is superior because as glycofurol will standardize the matrix probably take longer to release the same amount. The glycofurol dispersed the drug inside the polymer matrix as it was already refer.

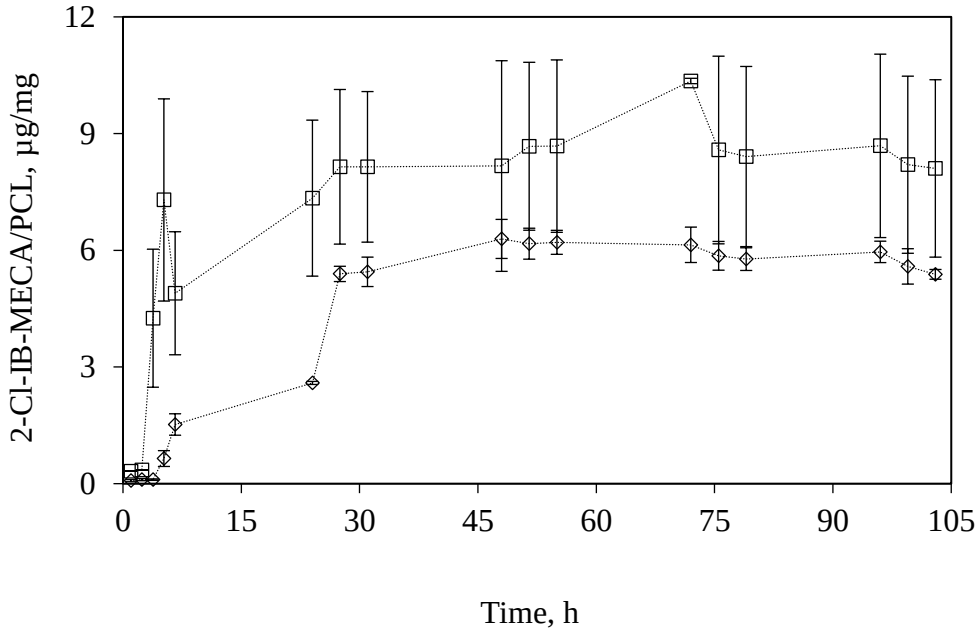


Figure 14 - Glycofurol effect on 2-Cl-IB-MECA implants profile release:

◊ with Glycofurol □ without Glycofurol

In the first hours of release, in free glycofurol implants seems to happen release of a large amount of drug (Table 6) due to surface erosion. This effect may not be desirable and may be toxic in biological environment. Otherwise, implants with glycofurol present a more controlled drug release mainly regulated by surface degradation including inside pores.

Burst release is more marked having a hydrophilic drug in a polymer hydrophobic matrice due to their poor drug-polymer interaction (Manickavasagam and Oyewumi, 2013). Dexamethasone and 2-Cl-IB-MECA are hydrophobic drugs and PCL a hydrophobic polymer and that is favorable to prevent evident burst.

Analyzing the release profile of 2-Cl-IB-MECA implants SFM20-G and SFM30-G (Figure 15 and Table 6), is seen that despite the similar tendency release, 20 bar/min implants drug release is higher than the 30 bar/min. In implants, the drug is dispersed on the polymeric matrix and also deposited on the surface. Thus, as stated before, SFM20-G implants have, theoretically, larger pores than SFM30-G which facilitate contact with the surrounding environment, in particular the phenomena of erosion and diffusion, thereby increasing the amount of released drug.

If the study were prolong in time, SFM30-G implants release would probably be longer due to the liberation is slower and the amount of drug per implant is similar.

2-Cl-IB-MECA implants shown sustained release over 100h.

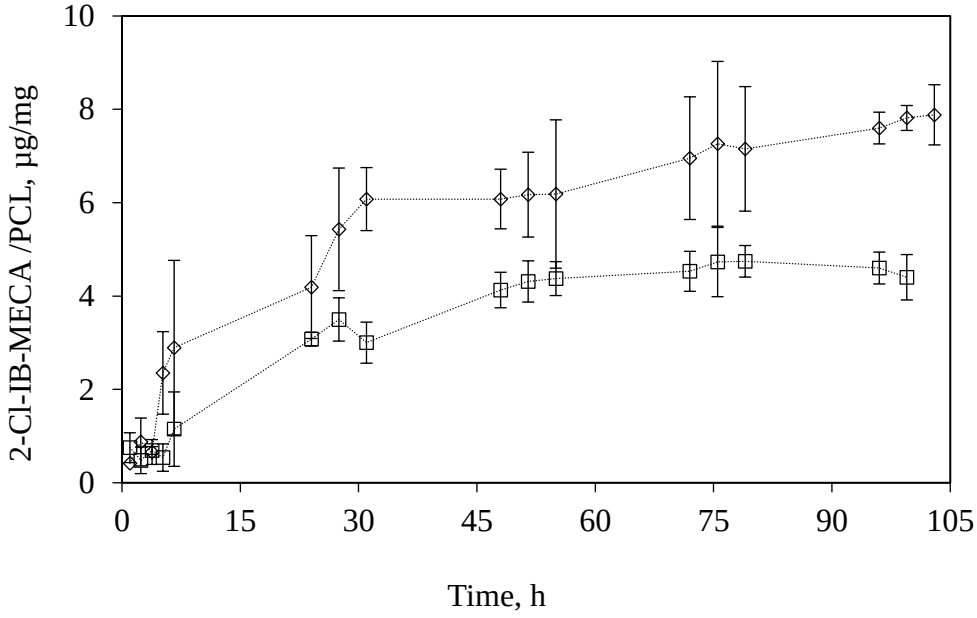


Figure 15 - Profile release of 2-Cl-IB-MECA implants with different depressurization rates: ◊ 20 bar/min □ 30 bar/min

Investigation show that release profile is made in two steps. The initial phase is resultant from the release of a higher dose of the drug present on the surface of the polymeric matrix, and depends on the total surface area of the implant, the percentage of drug added to the implant, and aqueous solubility of the drug. Therefore, the larger the surface area of the system, the greater the hydrophilicity and concentration of drug added; if this release is biologically exacerbated it can lead to happen toxic effects (*"burst"*) (Yasukawa *et al.*, 2002).

The second step is the diffusion phase characterized by gradual release of the drug due to its dissolution on the surface of the polymeric matrix. This phase is regulated by the polymer degradation rate, the total surface area of the implant, the percentage of drug added and the aqueous solubility of the drug.

In our study a potential third phase, also referred to by Yasukawa and Manickavasagam *et al.*, was not achieved. This final stage is characterized by the hydrolysis into the matrix and release of a sudden high dose of drug. The release of a high drug dose may represents a possibly problematic drawback of biodegradable systems. The produced implants seems to have a stable and controlled release profile over time. The similar release profile suggest that the impact of porosity affects the amount of drug release and the differences in that amount may be caused by the time 2-Cl-IB-MECA takes to reach equilibrium across the implant structure.

Table 6 - Diffusion Coefficients for 2-Cl-IB-MECA.

Samples	**Diffusion Coefficients (mm^2/h)**			
	60% release		40% release	
	D_1	R^2	D_2	R^2
SFM20	0,0046	0,99	1,52E-07	0,82
SFM20-G	1,04E-04	0,99	6,08E-08	0,94
SFM30-G	6.46E-05	0,99	2,07E-08	0,90

Understanding that porosity influence polymer degradation and drug release is important to achieve sustained drug release.

Implants with large surface areas tend to degrade faster than those with small areas because the area under the effect of external factors (ocular fluids, for example) is bigger (Manickavasagam and Oyewumi, 2013).

In achieved results, total drug quantification was higher in the SFM30-G implants but release over analyzed time was superior in SFM20-G implants.

According to the AIBILI team, the amount of 2-Cl-IB_MECA provided in which intravitreal injection is about 3 ng. In studies performed, the amount of drug released in the first hour is eight times higher than the amount administered monthly in intra-vitreous injections. This value reflects the potential of biomedical applicability but also the need to adjust the therapeutic doses of the implant.

Implants mass difference before and after the release was 0.04±0.005 mg for SFM20-G and 0.07±0.039 mg for SFM30-G. After release, SEM images reveal no surface morphological changes in the implants probably due to the fact that PCL has a degradation time much longer than the time under review (Figure 16 and 17).

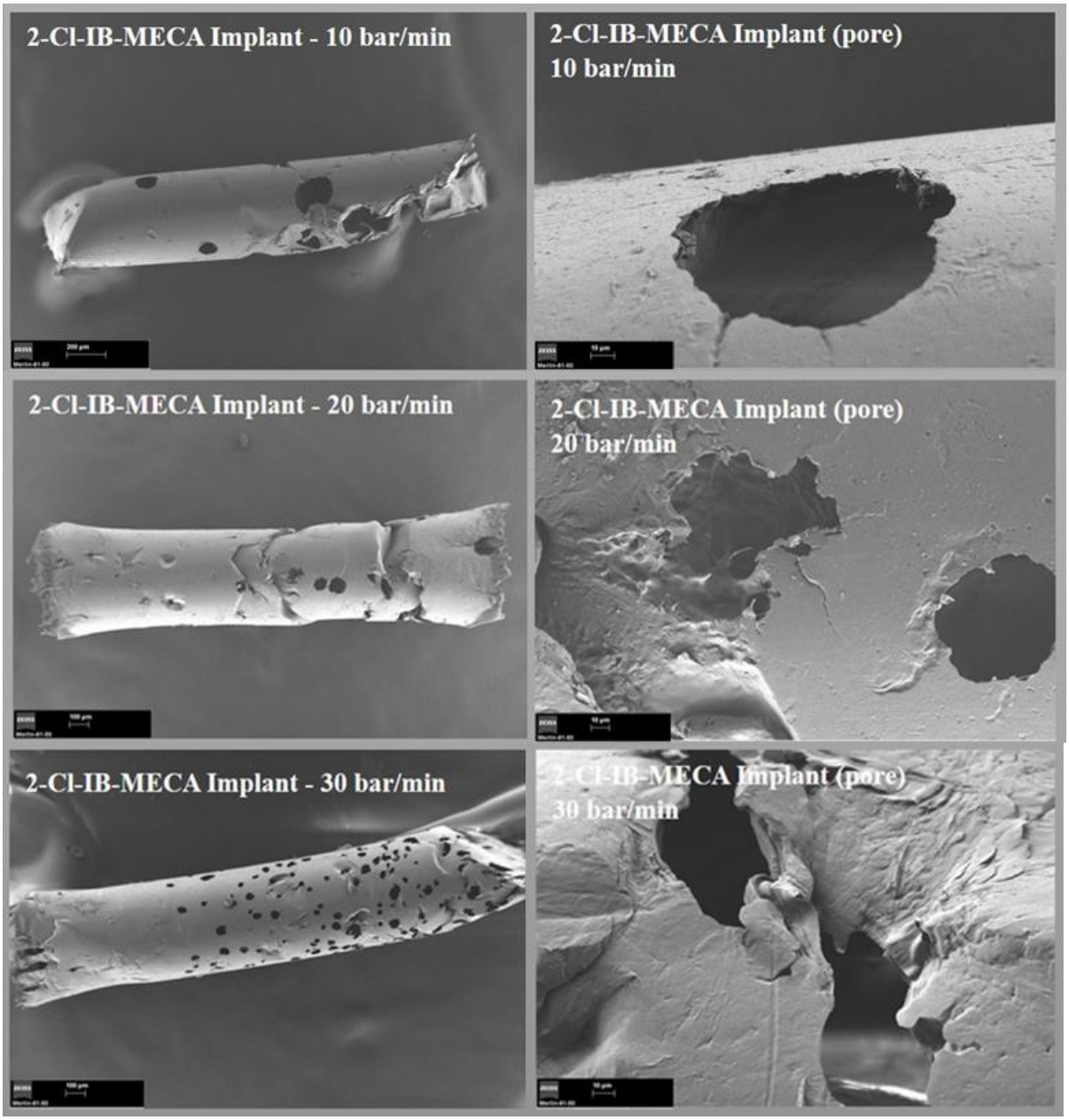


Figure 16 - After release SEM images of 2-Cl-IB-MECA implants.

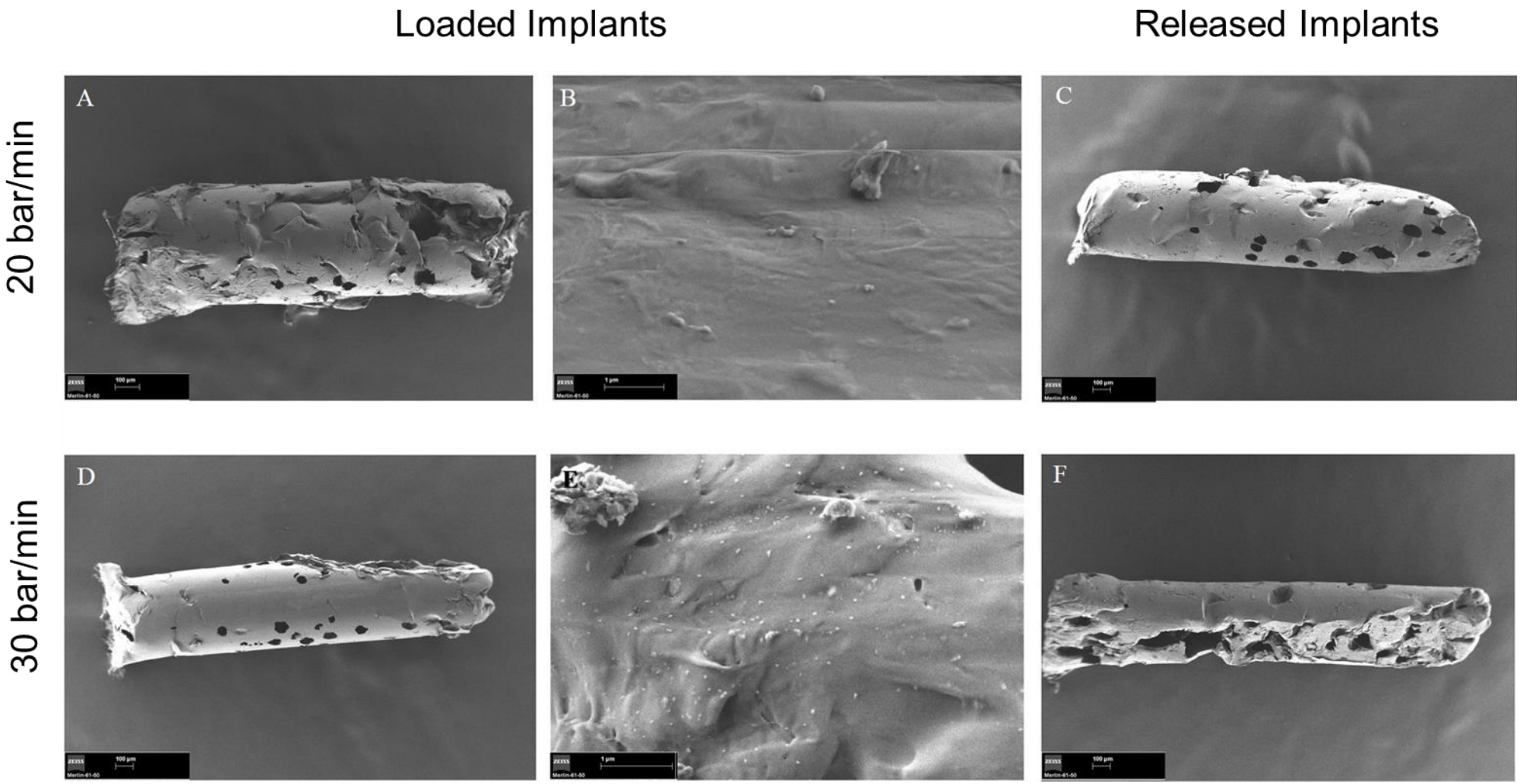


Figure 17 - 2-Cl-IB-MECA implants SEM images before and after release with body and surface details.

3.5. 2-Cl-IB-MECA degradation analysis

2-Cl-IB-MECA degradation was one challenging problem during this investigation. For better understanding the phenomena, drug's degradation was analyzed in HPLC using a drug solution in three different media: water, methanol and saline solution (Figure 18).

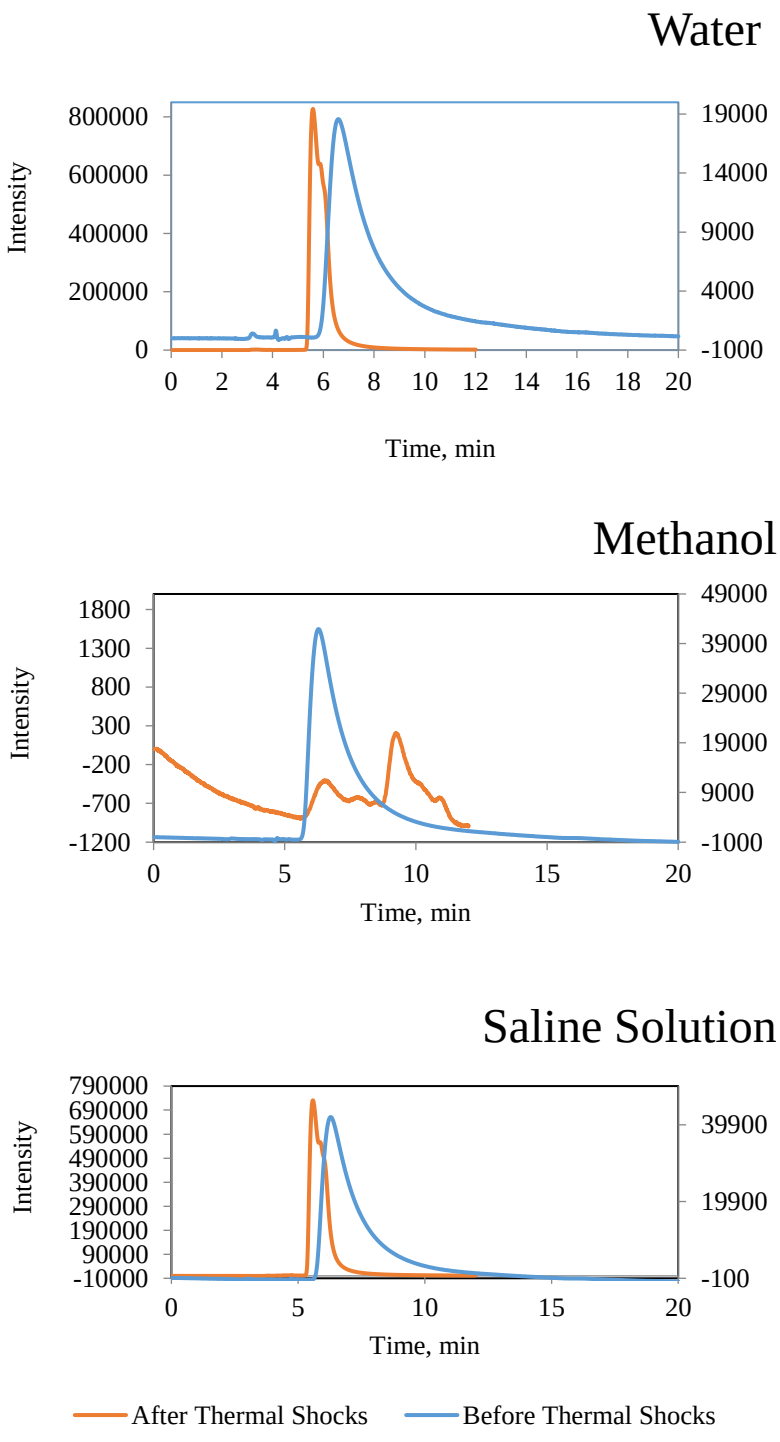


Figure 18 - 2-Cl-IB-MECA degradation analysis in different medium.

Aqueous solution analysis was relevant since the release was made in water. Methanol test was made because it was the solvent used in drug total quantification. Drug saline solution was analyzed because it was the most similar *in vivo* environmental media.

As can be verified by graphs analysis, drug degradation occurred in the three situations studied. Degradation behavior is very similar in water and in saline solution since the solvents are chemically more similar. Peaks increased after thermal shocks are related to increased drug solubility in the media.

In methanol profile, no peak is recorded after thermal shocks, which leads to the possibility that the combination of the drug, solvent and sudden increases in temperature led to a more pronounced degradation.

In the analyzes performed in this investigation and in future *in vivo* applications, thermal shock is not accentuated and it is considered that drug degradation will not be as intense. Biomedical application are made in a medium similar to the saline solution which has also appear to be more resistant to drug degradation.

3.6. In Vivo Experiments

As a medical device, implants biocompatibility is a major requirement. Several studies confirmed the high biocompatibility of PCL when used in intraocular applications (Dong *et al.*, 2006; Fialho *et al.* 2008; Cunha *et al.*, 2009; Natu *et al.*, 2011; Takahashi *et al.*, 2012; Bernards *et al.*, 2013). It is a FDA approved biodegradable polyester used in biomedical engineering applications.

In vivo tolerance experiments were performed using PCL implants drug free. Seven days after implantation there were no side effects reported such as inflammation, edema and cataract formation (Figure 19). The implants seems to remain at the implantation site (Figure 20).

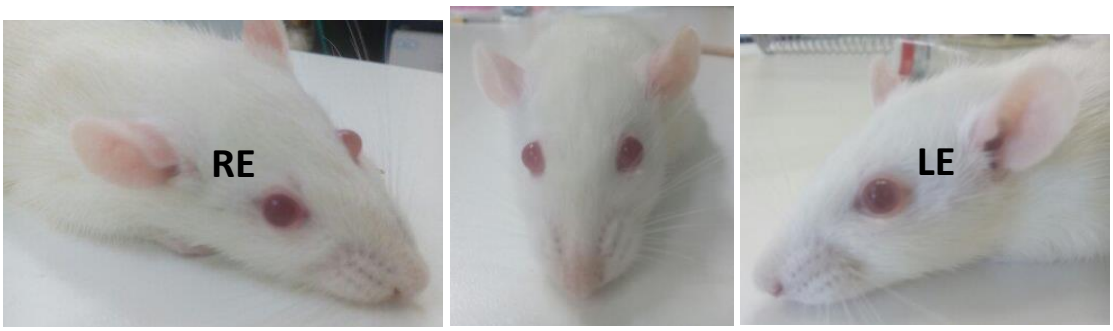


Figure 19 - Macroscopic view of the rat's eyes after 7 days implantation on the left eye. RE – Right eye; LE – Left eye. (Pictures from Raquel Bóia – AIBILI).

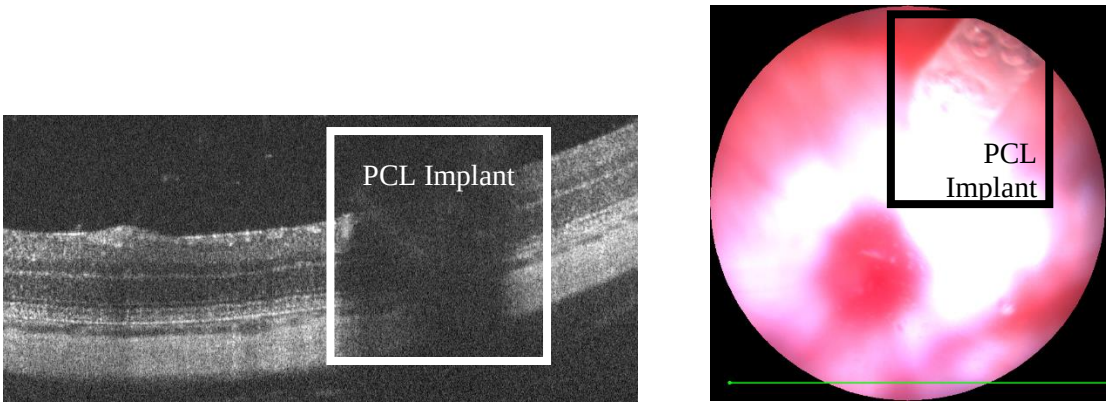


Figure 20 - Left eye OCT 7 days after implantation (Pictures from Raquel Bóia – AIBILI).

The absence of side effects or signs of rejection confirm that PCL implants are biocompatible.

4. Conclusion and future remarks

The main goal of this work – development of PCL ocular implants for drug delivery using supercritical carbon dioxide assisted fluid foaming/mixing methodology – was attained. PCL Dexamethasone and 2-Cl-IB-MECA implants were successfully produced. Different working parameters were used and diverse morphologies and profile release were obtained.

PCL thermal properties were not significantly altered after processing and are adequate for biomedical use.

Porosity is important for controlled drug release. From SEM images it is concluded that the implants produced have variable porosity according to the processing conditions (additive presence and depressurization rate). Low depressurization rates and additive presence seems to increase pore size. At a same depressurization rate, glycofurol presence increase porosity. In SFM 2-Cl-IB-MECA implants release profiles, additive leads to greater releases. Otherwise, hot melting produced implants have expressively less porosity and high variability. Drug release express that differences: DXMT implants release was more stable over time in SFM methodology.

The effect of depressurization rate on produced implants porosity was not always consistent with the results of earlier investigations undertaken with bigger materials such as bone substitutes. SFM20 and SFM30 implants had similar pore size: analysis repetition is suggested because the prosperous advance in porous implants for biomedical applications depends on the effective control of their morphology.

As in previous several works, we may also conclude that supercritical fluid technology is an efficient approach on drug delivery devices development. The use of supercritical fluids, particularly CO_2, provides alternative pathways for processing polymers.

In the future it is recommended to optimize drug amount to incorporate in each implant considering potential *in vivo* application.

Another request to new investigations are meticulous studies of 2-Cl-IB-MECA degradation and solubility in different media because there is scarce literature on this.

Minimize losses during the process and examine the long-term behavior of the implants near exhaustion are suggested as future work. *In vitro* implants degradation studies and *in vivo* ocular drug bioavailability and systemic side effects at administration site must also be analyzed.

The development of an extruder applicable to the project should be a priority given the physical and mental requirements of the manual process carried out in this research.

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Supplementary Data

Appendix A – Catheters Filling Process

Figure A1 – I.V. Optiva® catheters from Smiths Medical.

A small amount of the mixture (PCL, glycofurol and drug) or pure PCL was placed in the yellow portion of the catheter (Figure A1) and it was pushed and compressed therein with the aid of two needles (one at each end). The filling was done in about two thirds of the total area of the catheter. After filling, the yellow part was removed with a scalpel (Figure A2).

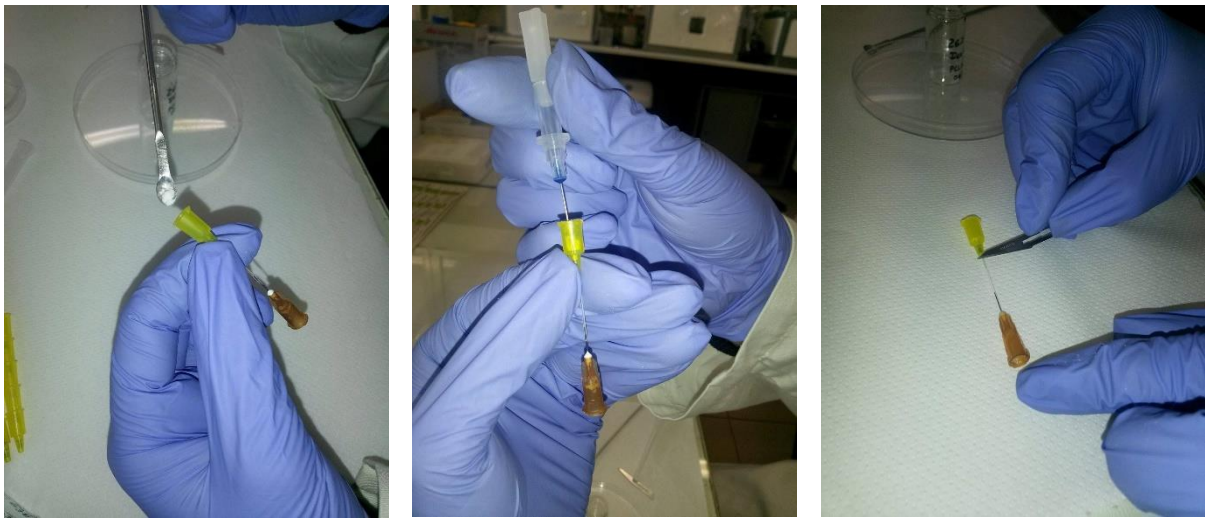

Figure A2 – Catheter completion.

Appendix B – Supercritical Solvent Unit

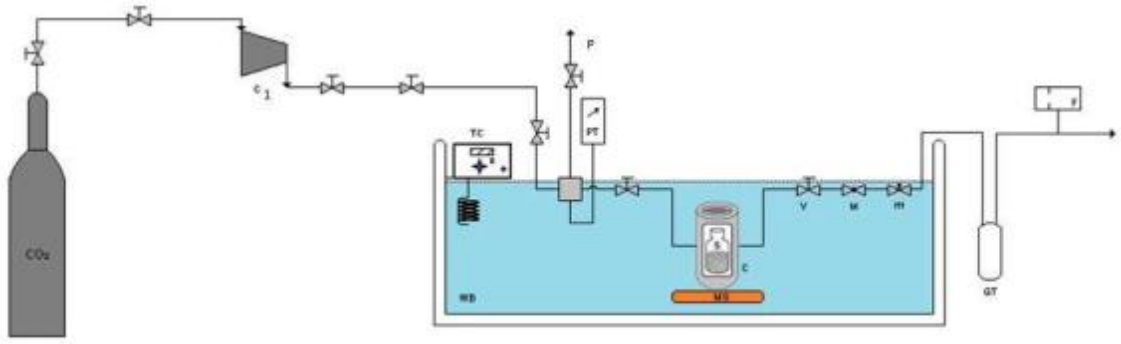

Figure B1 – Schematic diagram of the supercritical solvent unit. C1: compressor; TC: temperature controller; WB: water bath; P: purge; PT: pressure transducer; S: sample; MS: magnetic stirrer; C: high pressure vessel; V: screw down valve; M: macrometric valve; m: micrometric valve; GT: glass trap; F: mass flow meter (Rosa, 2013).

Appendix C – Dexamethasone Standard Curves

According to HPLC data obtained, the standard curves of dexamethasone release in methanol and in water are on figure C1 and C2:

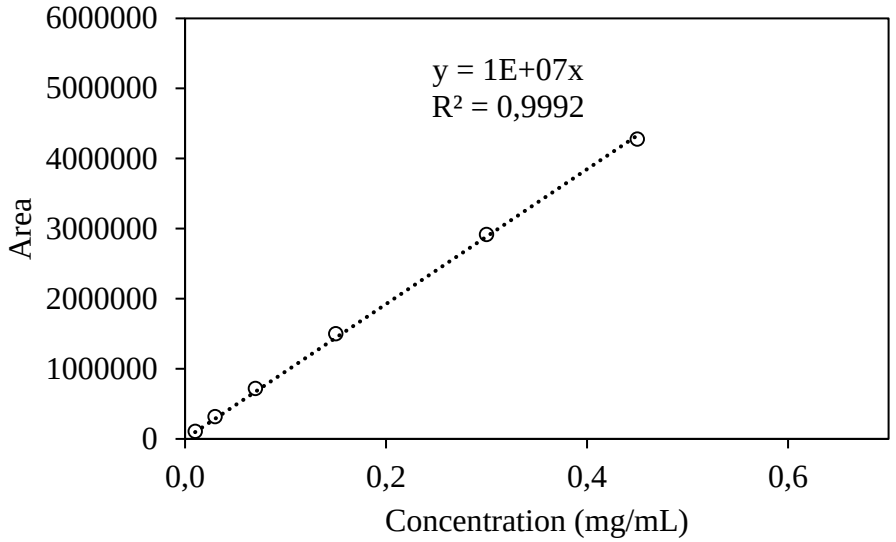

Figure C1 - Dexamethasone standard curve, in Methanol, used to determine the total amount of drug in the implants.

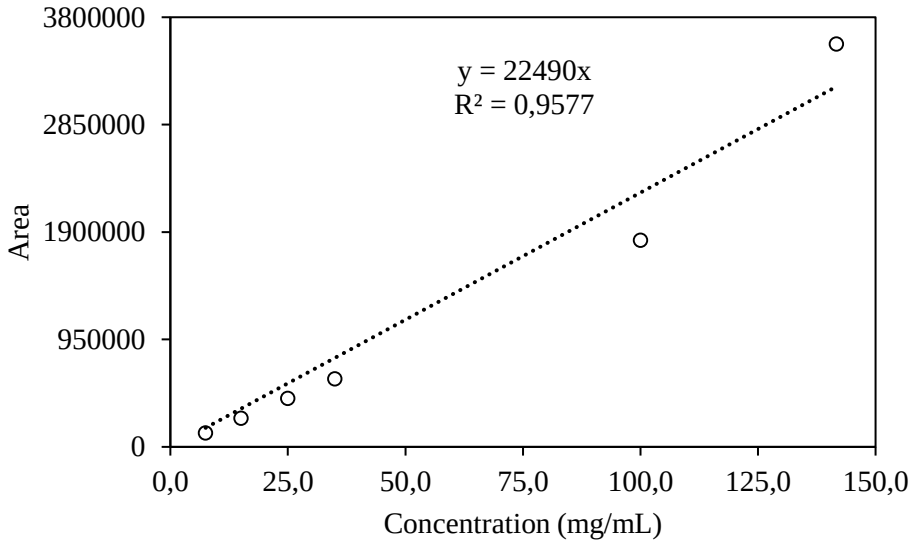

Figure C2 - Dexamethasone standard curve, in Milli-Q water, used to determine implants drug release.

Appendix D – 2-Cl-IB-MECA Standard Curves

According to HPLC data obtained, the standard curves of 2-Cl-IB-MECA release in methanol and in water are on figure D1 and D2:

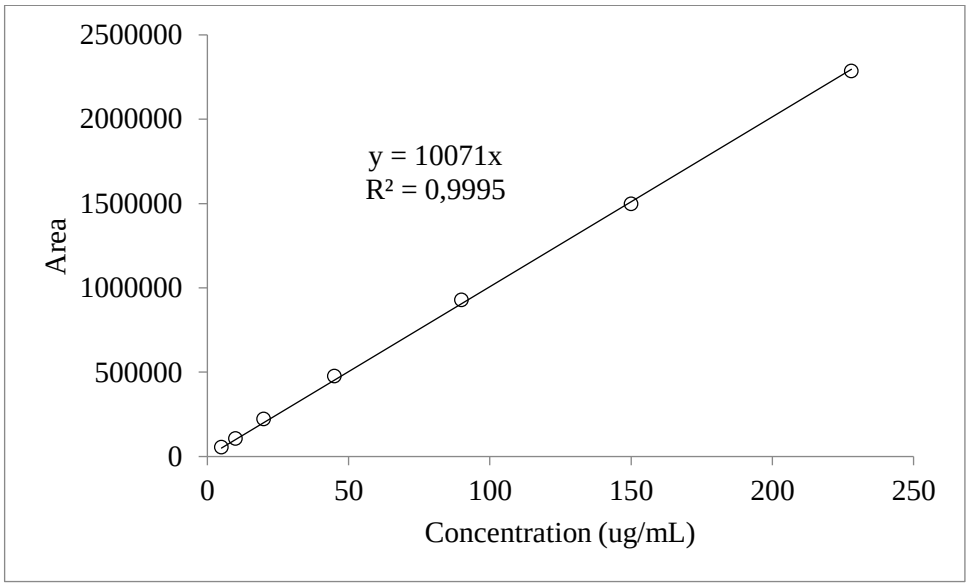

Figure D1 – 2-Cl-IB-MECA standard curve, in Methanol, used to determine the total amount of drug in the implants.

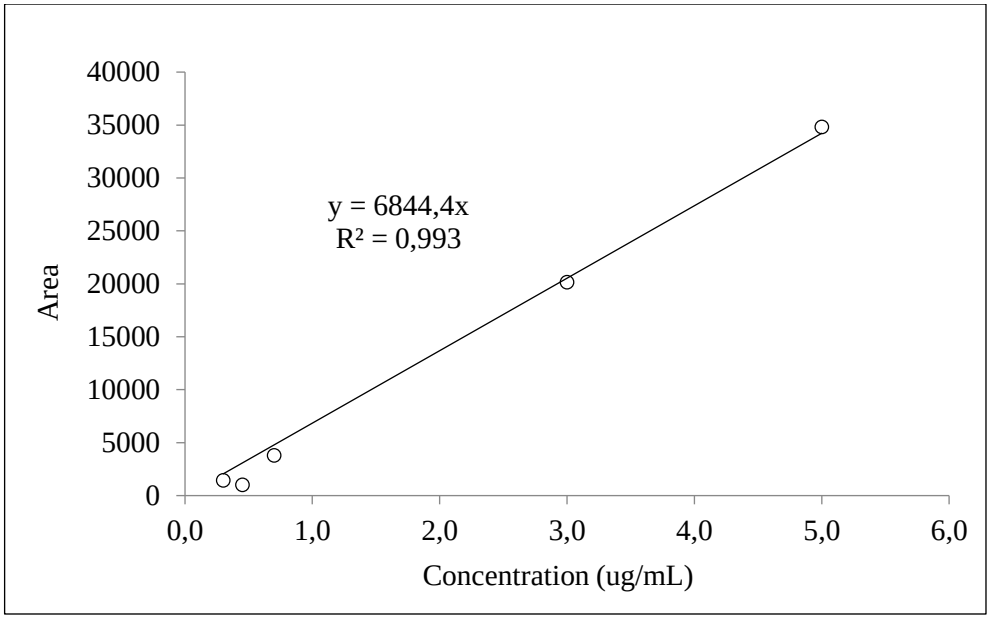

Figure D2 – 2-Cl-IB-MECA standard curve, in Milli-Q water, used to determine implants drug release.

Appendix E – Comparison of detection peaks, in HPLC, of 2-Cl-IB-MECA, saline solution, cell culture medium and explants culture medium.