UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA

TESIS DOCTORAL

Sistemas de liberación controlada de cannabidiol para optimizar el tratamiento de cáncer de mama triple negativo

Controlled-delivery formulations of cannabidiol to optimize the treatment of triple negative breast cancer

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Ana Isabel Fraguas Sánchez
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Madrid

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Without any distinction between the hereafter and the afterlife
To my mother, to my father
To my sister Carola
To my grandparents.

To believe in me more than I do with myself.

Senza alcuna distinzione tra al di qua e aldilà
A mia madre, a mio padre
A mia sorella Carola
Ai miei nonni.

per credere in me più di quanto io faccia con me stessa.

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RESUMEN

Introducción

El cáncer de mama triple negativo (TNBC, por sus siglas en inglés) es una forma agresiva de carcinoma mamario caracterizada por la ausencia de receptores de estrógeno, receptores de progesterona y de receptores HER2. Son tumores de crecimiento rápido que presentan una mayor tasa de recurrencia y un peor pronóstico que otros cánceres de mama. Cabe destacar que alrededor del 45% de los pacientes con TNBC desarrollan metástasis, con lo que la quimioterapia es la principal estrategia terapéutica en esta enfermedad. No obstante, los tratamientos quimioterápicos presentan importantes limitaciones debido a su alta toxicidad. En este sentido, resulta de gran interés el uso de compuestos adyuvantes con una baja toxicidad y que potencien la eficacia de los antineoplásicos permitiendo reducir la dosis administrada de estos fármacos, y, por tanto, sus efectos adversos.

Entre estos compuestos, se encuentran los cannabinoides, que han demostrado tener un prometedor efecto anticancerígeno en diversos tipos de cáncer, incluido el TNBC. El cannabidiol (CBD), que carece de efectos psicoactivos, es uno de los cannabinoides más prometedores. Diversos estudios preclínicos han demostrado que el CBD inhibe el crecimiento y la metástasis de TNBC. Además, este cannabinoide potencia el efecto del paclitaxel y la doxorrubicina (antineoplásicos habitualmente empleados en el tratamiento de TNBC) a la vez que disminuye sus efectos adversos. Sin embargo, la elevada liposolubilidad y baja y errática biodisponibilidad por vía oral del CBD limitan su administración y uso en terapéutica.

El objetivo de esta tesis doctoral es diseñar, desarrollar, caracterizar y evaluar en modelos in vitro e in ovo sistemas de liberación controlada que permitan administrar el CBD y optimizar su actividad antitumoral en TNBC. Concretamente, se han desarrollado dos tipos de formulaciones: i) implantes de formación in situ (ISFIs) y ii) niosomas cargados con CBD.

Resultados

Se han desarrollado ISFIs a base de ácido poliláctico (PLA), ácido poliláctico-co-glicólico (PLGA) y policaprolactona (PCL) como polímeros, y dimetilsulfóxido (DMSO) o N-metil-2-pirrolidona (NMP) como solvente. Estos implantes solidifican en el lugar de administración debido a la difusión del solvente en el entorno acuoso. Los implantes elaborados con PCL

y NMP con un ratio fármaco: polímero 10:100 mostraron la liberación de CBD más controlada (se obtuvo una liberación contralada durante dos meses) y el menor efecto burst. Esta formulación no mostró signos de toxicidad en el modelo HET-CAM. A concentraciones de 100μM presentó un mayor efecto antiangiogénico que el CBD en solución. Además, fue capaz de inhibir la migración y proliferación de células humanas (MDA-MB-231) y murinas (4T1) de TNBC, observándose un efecto antiproliferativo prolongado durante al menos 8 días. Por último, estos implantes frenaron el crecimiento y redujeron el tamaño de tumores derivados de las células MDA-MB-231 generados in ovo, en la membrana corioalantoidea de embriones de pollo.

Los niosomas de CBD (CBD-Nio) se prepararon mediante el método de "evaporación y rehidratación", utilizando polisorbato 85 (Tween 85) como tensioactivo no iónico y colesterol. Estos niosomas presentaron un tamaño de partícula de 137nm (tamaño adecuado para su internalización en las células tumorales), una baja polidispersión (PDI=0.2), un potencial zeta negativo de alrededor de -37mV, una carga de CBD de 0.92 ±0.025 mg/ml de formulación, una eficacia de encapsulación cercana al 92% y una liberación controlada del CBD durante 24 horas. Además, esta formulación fue estable durante al menos tres meses almacenada a 4 y 25ºC. Con respecto a la eficacia antitumoral, cabe destacar que los CBD-Nio presentaron un mayor efecto antiangiogénico in ovo que el CBD en solución a concentraciones de 100μM, así como un mayor efecto antiproliferativo en células MDA-MB-231 y 4T1, lo que puede atribuirse a una mayor internalización en las mismas. La formulación de niosomas también fue capaz de inhibir la migración de estas células. Por último, estudios in ovo demostraron la capacidad de esta formulación de frenar el crecimiento y reducir el tamaño de tumores derivados de células MDA-MB-231.

Conclusiones

Los resultados obtenidos en esta tesis doctoral indican que tanto los ISFIs como los niosomas desarrollados representan estrategias efectivas para la administración y liberación de CBD cun futura utilidad en el tratamiento del TNBC. Los ISFIs demostraron ser una excelente formulación para lograr una actividad anticancerígena prolongada con una sola administración, lo cual es especialmente útil dada la duración de los tratamientos quimioterápicos. Por otro lado, los niosomas de CBD representan una buena estrategia para la administración de este fármaco sin necesidad de usar solventes orgánicos buscando su liberación selectiva a nivel tumoral.

SUMMARY

Introduction

Triple-negative breast cancer (TNBC) is an aggressive form of breast carcinoma characterized by the absence of estrogen receptors, progesterone receptors, and HER2 receptors. These tumors are fast-growing and have a higher recurrence rate and worse prognosis compared to other breast cancer subtypes. It is noteworthy that around 45% of TNBC patients develop metastasis. Chemotherapy is the primary therapeutic strategy for this disease. However, it shows significant limitations due to their high toxicity. In this regard, the use of adjuvant compounds with low toxicity that enhance the efficacy of antineoplastics, allow the reduction of the doses of these drugs, and consequently their side effects, is of great interest.

Among these compounds are cannabinoids, which have shown a promising anticancer effect in various types of cancer, including TNBC. Cannabidiol (CBD), which lacks psychoactive effects, is one of the most promising cannabinoids. Several preclinical studies have demonstrated that CBD inhibits the growth and metastasis of TNBC. Furthermore, this cannabinoid enhances the effect of paclitaxel and doxorubicin (chemotherapeutic agents commonly used in TNBC treatment) while reducing their side effects. However, the high liposolubility and low and erratic oral bioavailability of CBD limit its administration and therapeutic use.

The aim of this doctoral thesis is to design, develop, characterize and evaluate using in vitro and in ovo models controlled-release systems that allow the administration of CBD and the optimization of its antitumor activity in TNBC. Specifically, two formulations have been developed: i) in situ forming implants (ISFIs) and ii) niosomes of CBD.

Results

ISFIs based on polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), and polycaprolactone (PCL) as polymers, and dimethyl sulfoxide (DMSO) or N-methyl-2-pyrrolidone (NMP) as solvents were developed. These implants solidify at the site of administration due to solvent diffusion into the aqueous environment. The implants made with PCL and NMP, with a drug-to-polymer ratio of 10:100, showed the most controlled CBD release (achieving controlled release for two months) and the lowest burst effect. This formulation showed no signs of toxicity in the HET-CAM model. At concentrations of 100 µM, it exhibited a stronger antiangiogenic effect than CBD in solution. Moreover, it was able

to inhibit the migration and proliferation of human (MDA-MB-231) and murine (4T1) TNBC cells. A prolonged antiproliferative effect was observed for at least 8 days. Finally, these implants slowed down the growth and reduced the size of tumors derived from MDA-MB-231 cells developed in ovo on the chorioallantoic membrane of chicken embryos.

On the other hand, CBD-loaded niosomes (CBD-Nio), made of polysorbate 85 (Tween 85) as a non-ionic surfactant and cholesterol, were prepared using the "thin-film hydration method". These niosomes exhibited a particle size of 137 nm (suitable for tumor cell internalization), a low polydispersity index (PDI = 0.2), a negative zeta potential of around -37 mV, a CBD loading of 0.92 ± 0.025 mg/ml of formulation, an encapsulation efficiency (EE) of ≈92%, and controlled CBD release over 24 hours. Furthermore, this formulation was stable when stored at 4 and 25ºC for at least three months. Regarding antitumor efficacy, it is noteworthy that CBD-Nio exhibited a greater antiangiogenic effect in ovo than CBD in solution at concentrations of 100 μM, as well as a greater antiproliferative effect on MDA-MB-231 and 4T1 cells, which may be attributed to a higher internalization in these cells. The niosome formulation was also able to inhibit the migration of these cells. Finally, in ovo studies demonstrated the ability of this formulation to slow down tumor growth and reduce the size of tumors derived from MDA-MB-231 cells.

Conclusions

The results obtained in this doctoral thesis indicate that both the development of ISFIs and niosomes represent effective strategies for the administration and release of this cannabinoid. ISFIs proved to be an excellent formulation for achieving prolonged anticancer activity with single administration, which is especially useful given the long-lasting nature of chemotherapy treatments. On the other hand, CBD-loaded niosomes represent a good strategy for the administration of this drug without the need for organic solvents with the aim of obtaining a selective delivery at the tumor site.

INTRODUCTION

Triple Negative Breast Cancer

Cancer is a major societal, health and economic problem, being responsible for one in four deaths of noncommunicable diseases worldwide. Breast cancer (BC) is the second most common carcinoma diagnosed worldwide, and a leading cause of cancer-related deaths, especially in the female population (Figure 1). In 2022, more than 2.3 million women were diagnosed with this disease, and around 665000 patients died [1].

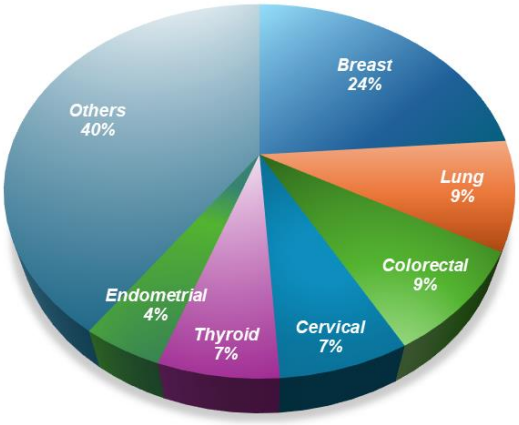


Figure 1: Statistics of carcinomas in the female population worldwide. Source: GLOBOCAN study 2022 [1].

Among all BC subtypes, triple-negative breast cancer (TNBC) is the most malignant and aggressive BC. It tends to spread in the lungs, brains and liver and shows a worse prognosis and a higher likelihood of relapse compared with other subtypes [2, 3]. TNBC accounts for approximately 15-20% of all breast cancers and is most often diagnosed in younger women, especially those under the age of 40 [4]. This carcinoma is characterized by a lack of expression of estrogen receptors, progesterone receptors, and human epidermal growth factor receptors-2 (HER-2). Consequently, TNBC patients do not benefit from conventional hormone and HER2-targeted therapies. The mainstay treatment currently used for TNBC includes surgery, in combination with chemotherapy, that is administered before surgery (neoadjuvant chemotherapy) to reduce tumor size or/and after surgery (adjuvant chemotherapy) to kill remaining tumor cells and prevent cancer recurrence [5]. Nevertheless, chemotherapy frequently leads to significant side effects and shows limited long-term success due to drug resistance. In this context, there is an urgent need for new, more effective treatments that can improve patient outcomes and reduce the toxicity of currently available antineoplastics.

Cannabinoids as anticancer agent

In the last decades, cannabinoids have attracted much interest in cancer research for their potential therapeutic benefits in cancer patients, including pain relief, appetite stimulation, and the ability to reduce nausea and vomiting caused by chemotherapy. Emerging studies also reported that cannabinoids possess anti-tumor properties due to their ability to inhibit tumoral growth and metastasis and potentiate the effectiveness of existing antineoplastics [6].

Cannabinoids are mainly divided into three groups: i) endogenous cannabinoids or endocannabinoids that are naturally produced by the body, ii) phytocannabinoids that are found in the cannabis plant and iii) their synthetic analogues. The most studied cannabinoids are Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) (Figure 2). Δ^9-THC is the main responsible for cannabis psychoactive effects and reduces nausea and vomiting related to chemotherapy and spasticity in multiple sclerosis patients, with different formulations commercialized for these purposes such as Cesamet®, Marinol® and Sativex®. Moreover, several studies have reported that Δ^9-THC shows promising anti-inflammatory, analgesic and anticancer activity. CBD is non-psychoactive and has garnered attention for its ability to relieve seizures. In fact, Epidiolex® is a formulation of CBD currently approved for the treatment of Lennox-Gastaut and Dravet syndromes. It also demonstrates utility in the treatment of anxiety and epilepsy and shows anti-inflammatory and anti-cancer effects [7, 8].

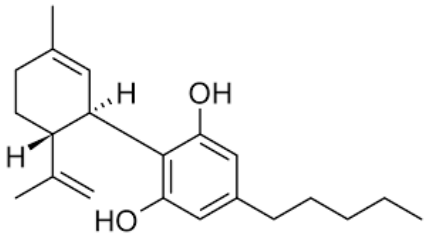


Figure 2: CBD chemical structure

CBD has demonstrated preclinical anticancer activity across various tumor types, including lung cancer, prostate cancer, and glioblastoma, among others [9]. One of the tumors where CBD has shown significant activity is breast cancer, including TNBC subtype.

The anticancer effects of CBD in TNBC can be attributed to several mechanisms. One key mechanism is the induction of apoptosis, a process that involves the activation of specific signalling pathways that dismantle the cellular machinery and lead to cell death. CBD

triggers programmed cell death in TNBC cells and can selectively induce apoptosis in these cells without affecting non-tumoral cells [10]. The apoptotic effects of CBD in TNBC cells have been attributed to the increased expression and activation of Peroxisome Proliferator-activated Receptor Gamma, which decreases the activity of the mTORC pathway and reduces the expression of Cyclin D1. Another key anticancer mechanism consists of the inhibition of cell proliferation. CBD can slow down or stop the proliferation of tumoral cells by inhibiting receptors and factors that promote the growth and survival of cancer cells, such as Epidermal Growth Factor Receptor and Cyclin-Dependent Kinases (CDKs). CBD particularly inhibits CDK4 and CDK6 [11, 12]. CBD also inhibits the metastasis of TNBC cells [13]. This effect has been mainly related to the downregulation of inhibitor differentiation protein-1, which is overexpressed in TNBC and associated with larger tumor size and higher invasiveness [14], but also to the suppression of matrix metalloproteinases via CDK4/CDK6 pathways [15].

Furthermore, it has been reported that CBD potentiates the effectiveness of conventional antineoplastics [16, 17]. For example, it has been showed that CBD increases the antiproliferative effect of paclitaxel and doxorubicin in TNBC cells. The combination of CBD and paclitaxel resulted in a synergistic activity [18, 19]. Interestingly, it has also been reported that CBD reduces some of the side effects associated with these drugs, such as the neuropathic pain of paclitaxel and the cardiotoxicity related to doxorubicin [20, 21]. Both drugs are commonly used in the treatment of TNBC.

All this proves the potential utility of CBD to be included in the chemotherapeutic regimens of TNBC. Combining CBD with conventional antineoplastics would allow the administration of lower doses of these antineoplastics, reducing their side effects without impairing their anticancer activity. Nevertheless, CBD shows several challenges that hamper its therapeutic use. CBD shows a low and erratic oral bioavailability of around 10% [22]. Moreover, it has very low aqueous solubility and high instability, hindering its administration [23]. In this context, drug delivery systems (DDS) such as implants, microparticles or nanoparticles may overcome the challenges related to the administration of this cannabinoid.

Drug delivery systems in cancer disease

DDS offer key advantages for the delivery of antineoplastics by enabling targeted drug delivery to the tumor area, reducing damage to healthy tissues and minimizing side effects. These systems facilitate the administration of poorly soluble drugs, enhance bioavailability, improve therapeutic efficacy, and allow for controlled release, ensuring a long-lasting effect with a single administration. As a result, DDS can increase treatment effectiveness, reduce drug resistance, and improve patient compliance and quality of life. There are a variety of controlled release systems that have been developed to enhance anti-tumour therapy such as implants, microparticles and nanoparticles.

Implants

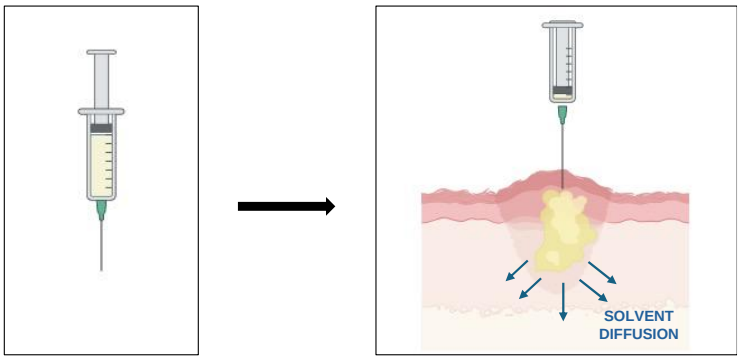
One of the main advantages of implant-based formulations relies on the possibility of obtaining a long-lasting effect with a single parenteral administration from weeks to months. These systems can be designed to release the drug in a sustained manner over time and to maintain therapeutical levels in the bloodstream. This prolongs the therapeutic effect; reduces the frequency of administration; and improves patient compliance [24–26]. This is especially important in the administration of biologics, which cannot be administered by the oral route and requires repeated parenteral administrations. Moreover, these systems can be administered in the tumor area where the drug is delivered, which is also beneficial. Nowadays, several implant-based formulations are currently approved for cancer disease, specifically for the treatment of prostate cancer and breast cancer that are positive for estrogen receptors (Table 1).

Trade Name	Drug substance	Administration route	Duration of the effect
Zoladex®	Goserelin Acetate	Subcutaneous	1-3 months
Suprefact Depot®	Buserelin Acetate	Subcutaneous	2-3 months
Vantas®	Histrelin Acetate	Subcutaneous	12 months
Leptoprol®	Leuproreline Acetate	Subcutaneous	3 months
Gliadel®	Carmustine	Intracranial	Over 3 weeks

Table1: Implant formulations that are currently approved for cancer therapy [27].

All the formulations listed in the table above are pre-formed solid implants. They are administered using large-diameter needles and sometimes a minor surgery (anaesthesia must be administered for this procedure) is also required, which is not comfortable for the patients. In the last decade, in situ-forming implants have gained attention in drug delivery as an alternative to pre-formed formulations. In situ-forming implants (ISFIs) consist of liquid formulations that form a solid deposit in the injected area upon contact with physiological conditions (Figure 2). In contrast to preformed implants, ISFIs can be administered easily by parenteral route using normal (low diameter) needles [18], reducing the patient's discomfort [28].

ISFIs show a huge potential for the administration of antineoplastics. In fact, an ISFI-based formulation of leuprorelin (Eligard®) is already approved for prostate cancer. It consists of a polymeric solution of PLGA dissolved in NMP [29]. The main advantage of this formulation over Leptoprol® (preformed implants) is its ease of administration.



Pre-filled syringe ***Depot formation***

Figure 3: ISFI formation after injection into the subcutaneous environment.

Nanoparticles

Nanoparticles have transformed oncology by improving the precision, efficacy, and safety of anticancer treatments. They offer several advantages over conventional treatments due to their unique properties at the nanoscale. One of the primary advantages of nanomedicines relies on the possibility of obtaining a targeted drug delivery. Due to the enhanced permeability and retention (EPR) effect nanoparticles tend to accumulate more in the tumors than in healthy tissues. This occurs due to the unique characteristics of tumor vasculature, which is often abnormal with enlarged, leaky blood vessels and poor lymphatic

drainage. This selective accumulation is known as passive targeting and minimizes the off-target toxicities that are commonly associated with conventional antineoplastics and may maximize their anticancer efficacy. Additionally, nanomedicines can improve the formulation and bioavailability of poorly water-soluble drugs, a common challenge in administering antineoplastics. Encapsulating these drugs within nanoparticles, eliminates the need for excipients such as ethanol or Cremophor EL® for their administration. These excipients are not completely safe (especially Cremophor EL®) and increase the overall toxicity of the formulation, limiting the dose that can be administered. For example, this happens with paclitaxel when it is formulated as Taxol®, it is dissolved in a mixture of ethanol and Cremophor EL (polyoxyethylated castor oil). This latter excipient has several side effects and toxicities that limit its use. For this reason, the maximum tolerated dose of Taxol, 175 mg/m^2 administered over 3 hours, is lower than paclitaxel nanoformulations that lack of this excipient. For example, the maximum tolerated dose of Abraxane® (albumin nanoparticles of paclitaxel) is $260mg/m^2$ administered over 30 minutes [30, 31].

Many types of nanocarriers, including polymeric nanoparticles, albumin nanoparticles, micelles, liposomes and metallic nanoparticles, among others have been investigated for the delivery of antineoplastics. Table 2 displays the nanoformulations that are currently approved by FDA and/or EMA for cancer disease.

Brand name	Active substance	Nanoformulation type	Cancer type	Year of approval
Doxil® (Caelyx®)	Doxorubicin	PEGylated liposomes	Ovarian cancer and AIDS-related Kaposi's sarcoma	1995 (FDA) 1996 (EMA)
Daunoxome®	Daunorubicin	Liposomes	AIDS-related Kaposi's sarcoma	1996 (FDA)
Depocyt®	Cytarabine	Liposomes	Lymphomatous malignant meningitis	1999 (FDA) 2001 (EMA)
Myocet®	Doxorubicin	Liposomes	Breast cancer	2000 (EMA)
Abraxane®	Paclitaxel	Albumin nanoparticles	Breast, Non-small Cell Lung Cancer (NSCLC), Pancreatic Cancer	2005 (FDA) 2008 (EMA)
Mepact ®	Mifamurtide	Liposomes	Osteosarcoma	2009 (EMA)
Marqibo®	Vincristine	Liposomes	Philadelphia chromosomenegative acute lymphoblastic leukemia	2012 (FDA)
Onivyde®	Irinotecan	Liposomes	Pancreatic cancer	2015 (FDA) 2016 (EMA)
Vyxeos ®	Daunorubicin Cytarabine	Liposomes	Acute myeloid leukemia	2017 (FDA) 2018 (EMA)
Apealea®	Paclitaxel	Polymeric micelles	Ovarian cancer	2018 (EMA)
NBTXR3 Hensify	Hafnium oxide nanoparticles that are stimulated with external radiation	Metallic nanoparticles	Suqamous cell carcinoma	2019 (CE Mark)

Table 2: Nanoformulations currently approved by FDA and/or EMA for cancer disease.

Liposomes are the most widely exploited nanocarriers. Most of the approved nanoformulations are based on these nanocarriers. This can be attributed to, among other aspects, their low toxicity and easier industrial manufacturing process compared with other nanosystems. However, they are not depicted as drawbacks. One of the main issues is their low stability, as the liposomal bilayers, which are made up of phospholipids, are prone to oxidation. This can limit their effectiveness. In the last few years, niosomes have gained a great deal of interest as an alternative to liposomes due to their higher stability [32].

Niosomes are lipid-based vesicles (similar to liposomes) composed of non-ionic surfactants, cholesterol, and sometimes other additives, forming lipophilic bilayers englobing an aqueous medium. They can encapsulate both hydrophilic drugs (localized in the inner aqueous phase) and hydrophobic compounds (placed in the lipid-surfactant bilayer) (Figure 4) [33]. Niosomes tend to be more stable than liposomes (even at room temperature), especially in terms of resistance to hydrolysis, due to the presence of non-ionic surfactants, and typically more cost-effective. They also show low immunogenicity. To the best of our knowledge, no niosome-based formulations have been approved by the FDA or EMA for clinical use. However, preclinical research has demonstrated their potential as carriers for the administration of a wide variety of drugs including antineoplastics [34-36].

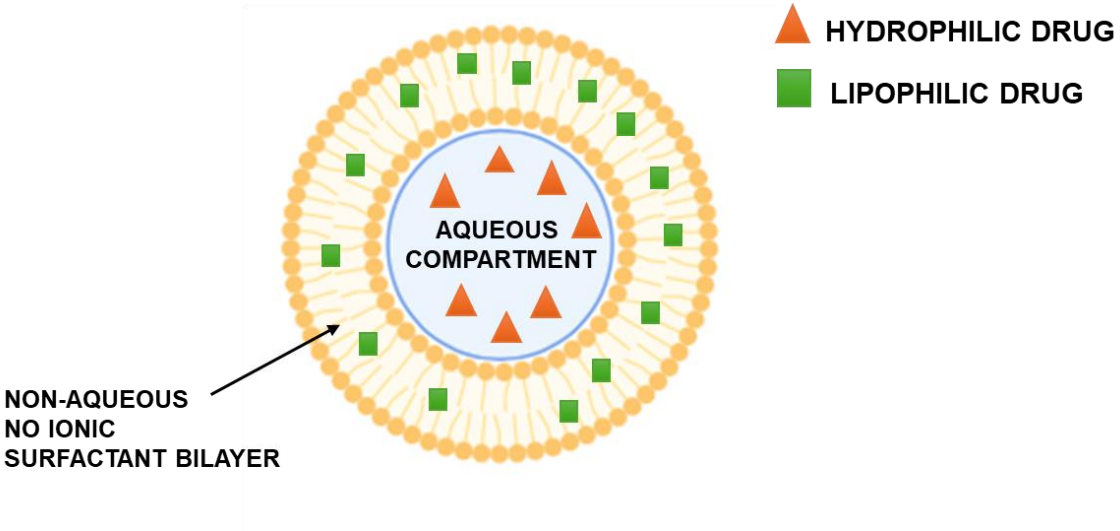


Figure 4: Scheme showing the structure of niosomes

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OBJETIVOS/AIMS

OBJETIVOS

El cáncer de mama es uno de los tumores más frecuentes a nivel global, constituyendo un importante problema de salud pública. De los diferentes subtipos de carcinomas mamarios, el cáncer de mama triple negativo (TNBC, por sus siglas en inglés) es especialmente problemático, pues es altamente invasivo y difícil de tratar. En los últimos años el cannabidiol (CBD), el principal fitocannabinoide no psicoactivo, ha despertado un gran interés en el tratamiento de esta patología, debido a su capacidad para inhibir el crecimiento y la formación de metástasis de estos tumores. En trabajos previos nuestro grupo de investigación han demostrado que este cannabinoide es capaz de potenciar el efecto de antineoplásicos convencionales comúnmente utilizados en el tratamiento de cáncer de mama triple negativo como el paclitaxel y la doxorrubicina. Sin embargo, a pesar del potencial terapéutico de este cannabinoide, su elevada liposolubilidad y baja y errática biodisponibilidad por vía oral limitan su administración y uso en clínica.

Por todo ello, el **principal objetivo** de esta tesis doctoral es diseñar, desarrollar, caracterizar y evaluar en modelos in vitro e in ovo sistemas de liberación controlada que permitan administrar el CBD y optimizar su actividad antitumoral en cáncer de mama triple negativo. Concretamente, se han desarrollado dos formulaciones diferentes de CBD: i) implantes de formación in situ (ISFIs) diseñados para ser administrados a nivel peritumoral y proporcionar una liberación controlada del CBD durante al menos 1 mes y por tanto un efecto prolongado con una sola administración y ii) niosomas cargados con CBD de administración intravenosa que se acumulen en el entorno tumoral y potencien la eficacia anticancerígena de este cannabinoide. Este objetivo global se puede desglosar en los siguientes **objetivos específicos**:

1. Desarrollo, optimización y caracterización de ISFIs cargados con CBD.
2. Diseño, desarrollo y caracterización fisicoquímica de niosomas cargados con CBD.
3. Evaluación en cultivos celulares de la capacidad de los ISFIs optimizados y niosomas de CBD para inhibir la proliferación y migración de células de TNBC.
4. Evaluación de la capacidad de los ISFIs optimizados y niosomas cargados con CBD para inhibir la formación de vasos sanguíneos en un modelo in ovo de angiogénesis.

5. Evaluación de la capacidad de ISFIs optimizados y niosomas cargados con CBD para inhibir el crecimiento de tumores de cáncer de mama triple negativo generados en la membrana coriolantoidea de embriones de pollo.

AIMS

Breast cancer is one of the most common cancers worldwide and represents a major health problem, especially triple negative breast cancer (TNBC) which is the most aggressive and difficult to treat breast cancer subtype. In recent years, cannabidiol (CBD), the main non-psychotropic phytocannabinoid, has gained attention as an anticancer agent in TNBC due to its ability to inhibit the growth and metastases of these tumors. Moreover, our research group has reported that it potentiates the activity of conventional antineoplastics like paclitaxel or doxorubicin, which are commonly used to treat this type of breast cancer. However, despite these benefits, its low aqueous solubility and low and erratic oral bioavailability hamper its clinical use.

On this basis, the **main aim** of this thesis was to design, develop, characterize and evaluate controlled drug delivery systems of CBD as a strategy to administer this drug and optimize its anticancer activity in TNBC. In particular, two different formulations were developed: i) in situ forming implants (ISFI) designed to be administered directly into the tumour area and to provide a sustained release of CBD for at least one month and consequently a long lasting effect after a single administration and ii) niosomes loaded with CBD developed to achieve a selective accumulation of this cannabinoid in the cancer cells after its intravenous administration and potentiates its anticancer activity. This overall objective can be broken down into the following **specific aims**:

1. Development, optimization and characterization of the CBD loaded ISFIs.
2. Design, development, and physicochemical characterization of CBD-loaded niosomes.
3. Evaluation of the antiproliferative and antimigration activity of the optimized ISFIs and niosomes of CBD in TNBC cell cultures.
4. Evaluation of the antiangiogenic activity of the optimized ISFIs and niosomes of CBD using an in ovo model of angiogenesis.
5. Evaluation of the ability of the optimized ISFIs and niosomes of CBD to inhibit the growth of TNBC tumors developed in the chorioallantoic membrane of chick embryos.

PART 1: IN SITU FORMING IMPLANTS

INTRODUCTION

In-situ-forming-implants (ISFIs), due to their versatility, simple manufacturing process, low invasiveness, and long-lasting effect, are among the most useful drug delivery systems in the clinic, having gained notable interest in recent years. They consist of parenteral liquid formulations that undergo a phase transition in the administration site, forming a solid or semisolid deposit in which the drug is entrapped and released in a controlled manner for days to months [1, 2].

ISFIs offer several significant advantages over other formulations. One of their benefits is their minimally invasive administration, as they can be injected using small-diameter needles without the need for a minor surgery as is sometimes the case with preformed implants. This minimizes patient discomfort and decreases healthcare costs. Moreover, they provide a sustained drug release and, consequently, a long-lasting effect with a single administration, which improves patient adherence compared to conventional formulations and avoids peaks in plasma levels and therefore their related adverse effects. In this context, ISFIs are useful for managing chronic conditions that require long-term treatments, such as cancer disease, addictions or mental disorders [3-5]. In these latter pathologies these systems also offer the advantage that continuous drug release does not depend on the patient, which is essential in the correct management of these diseases, becoming an alternative to oral medications. ISFIs are also useful to provide a localized release of therapeutic agents, maintaining high concentrations at the administration site and reducing their systemic exposure, which helps prevent their systemic side effects. For example, these systems are useful for the treatment of local conditions such as periodontitis [6]. Finally, the flexibility of ISFIs in terms of drug formulation and release profiles enables the customization of treatments to meet individual patient needs, supporting a more personalized treatment.

It should be mentioned that implant formation can be triggered by several environmental factors such as body temperature, pH, presence of specific ions or solvent exchange, among others. In fact, based on the mechanism that triggers the solidification of the implant, ISFIs can be grouped into i) in-situ solidifying organogel systems, that consist of amphiphilic lipids (water insoluble) dissolved in an organic solvent like ethanol that form a highly viscous solid or semisolid gel at the administration site [7]; ii) in-situ crosslinked systems that are made of polymers that solidify when the polymer crosslinks due to changes in the temperature (thermo-responsive polymers), the absorption of photons (photo-initiated crosslinking), the interaction of ionic polymers with small cations at the injection site (ion-responsive

crosslinking) or the presence of certain enzymes (enzyme-responsive crosslinking) [8, 9], and iii) in situ phase separation systems that are composed of biodegradable polymers whose solidification is triggered by a temperature, pH, or solvent change at the administration site.

Among all these systems, the in-situ phase separation systems due to solvent exchange are the most used. Several formulations based on this technology are currently approved by FDA and/or EMA for the treatment of periodontitis, prostate cancer, opioid addiction or schizophrenia. They are composed of Poly-lactic acid (PLA) or Poly-lactic-to-glycolic acid (PLGA) dissolved in dimethyl sulfoxide (DMSO) or N-methyl-2-pyrrolidone (NMP).

Chapter 1 of this doctoral thesis analyses the advantages and limitations of in-situ phase separation systems based on solvent exchange over conventional formulations and other drug delivery systems, as well as discusses the different formulations that are currently approved by FDA or/EMA and the formulations that are still under preclinical research.

Due to the potential of in situ forming implants in the management of chronic diseases such as cancer and the ability of CBD to reduce the growth and metastases of TNBC and to potentiate the activity of conventional antineoplastics [10-12], this doctoral thesis has developed in situ forming implants of CBD. These implants are intended to be administered in the tumor environment and provide controlled CBD release for at least one month, enhancing the effect of conventional chemotherapy. Formulations with different polymers (PLA, PLGA and polycaprolactone (PCL)) and solvents (DMSO and NMP) have been developed **(chapters 2 which has already been published and chapter 3).** The anticancer activity of the optimized formulation has also been evaluated using in vitro and in ovo models of TNBC **(Chapter 3)**.

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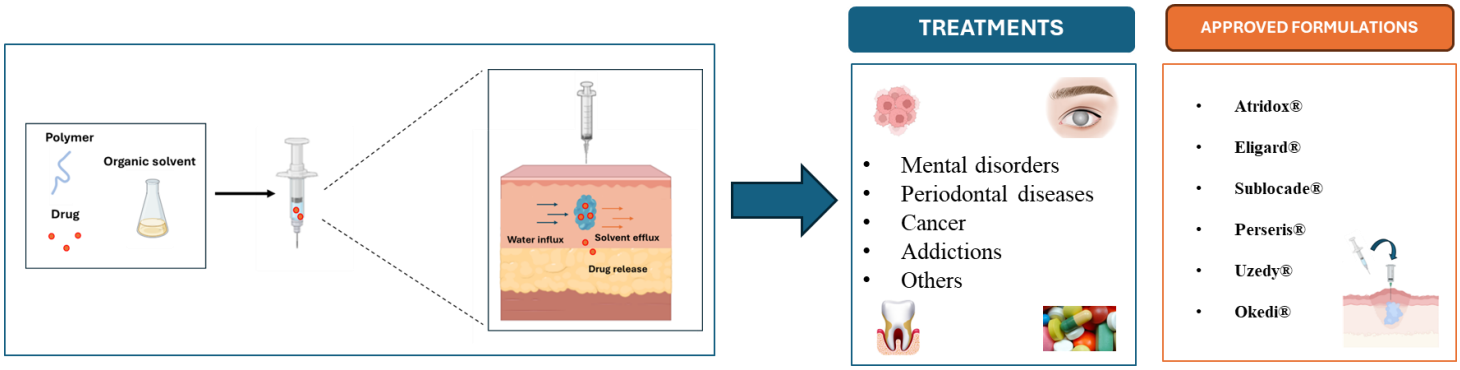
CHAPTER 1

Solvent-exchange-based in situ forming implants: advances and challenges as long-lasting formulations.



Abstract

In situ forming implants (ISFIs) are long-lasting drug delivery systems that transition from a liquid solution to a solid or semisolid deposit upon injection site. The drug is entrapped into this deposit and is released in a controlled manner for extended periods ranging from days to months. This extended drug release with a single administration reduces the frequency of dosing and improves therapeutic adherence, being especially useful for treating chronic conditions. Furthermore, this controlled drug release provides constant drug levels, avoiding the fluctuations related to conventional medications. In this context, ISFIs are highly valuable for the treatment of cancer, addictions and mental disorders like schizophrenia, among other conditions. Moreover, they are useful for achieving local therapeutic levels at the administration site while minimizing systemic plasma levels. In this regard, they have great potential for the treatment of periodontal diseases. There are several types of ISFIs classified based on the mechanism that triggers the formation of the deposit. Among all of them, the most studied and promising formulations, due to their high versatility and adaptability to the specific needs of therapeutical applications, are the implants whose formation is based on the diffusion of the solvent. They are commonly formulated with polymers like PLGA and PLA, and solvents such as NMP and DMSO are used. This review aims to analyze the in-situ forming implant formulations based on solvent change solidification that has been developed to date at both clinical and preclinical levels,

Keywords: Addictions, Cancer, Drug delivery, In situ-forming implants, Mental disorders, Polymeric implants.

1. Introduction

Parenteral prolonged drug delivery systems represent a widely accepted strategy to improve the treatment of chronic diseases. These systems offer a controlled drug release for extended periods and, consequently, a long-lasting effect with a single administration (months or even years), which reduces the frequency of administration, improves treatment adherence and avoids the risk of symptomatology impairment and relapse related to missed doses. Moreover, these systems provide stable therapeutic levels (the peaks and troughs associated with oral formulations are avoided), which also allow better management of the disease [1–5].

Implants are one of the most widely used parenteral controlled release systems in clinical practice. Preformed implants can be composed of biodegradable or non-biodegradable polymers and are usually administered using large needles (16 gauge with a 1.65 mm outer diameter) sometimes through a minor surgical procedure under local anaesthesia. This administration can cause discomfort and pain for patients. If surgery is needed, patients can also experience adverse effects associated with local anaesthesia [6, 7]. Moreover, non-biodegradable implants must be removed, which is an extra inconvenient for patients. Nexplanon®, a sub-dermal implant of etonogestrel that provides a contraceptive effect for 3 years [8], or Zoladex®, a cylindrical shape biodegradable implant loaded with goserelin acetate for the treatment of prostate and breast cancer, are examples of preformed implants in the market [9].

To overcome the limitations related to the administration of preformed implants, in-situ-forming-implants (ISFI) were developed. ISFIs consist of advanced controlled drug delivery systems that change from a liquid to a solid or semisolid state upon administration site. In the last decades, they have gained great interest as drug delivery systems. Compared to traditional preformed implants, ISFIs are minimally invasive, as they can be administered using small-diameter needles, 18 to 23G needles (1.27 mm-0.642mm of outer diameter), which reduces the pain and discomfort of the patients. Figure 1 displays the main advantages and disadvantages of ISFIs compared to preformed implants [10, 11].

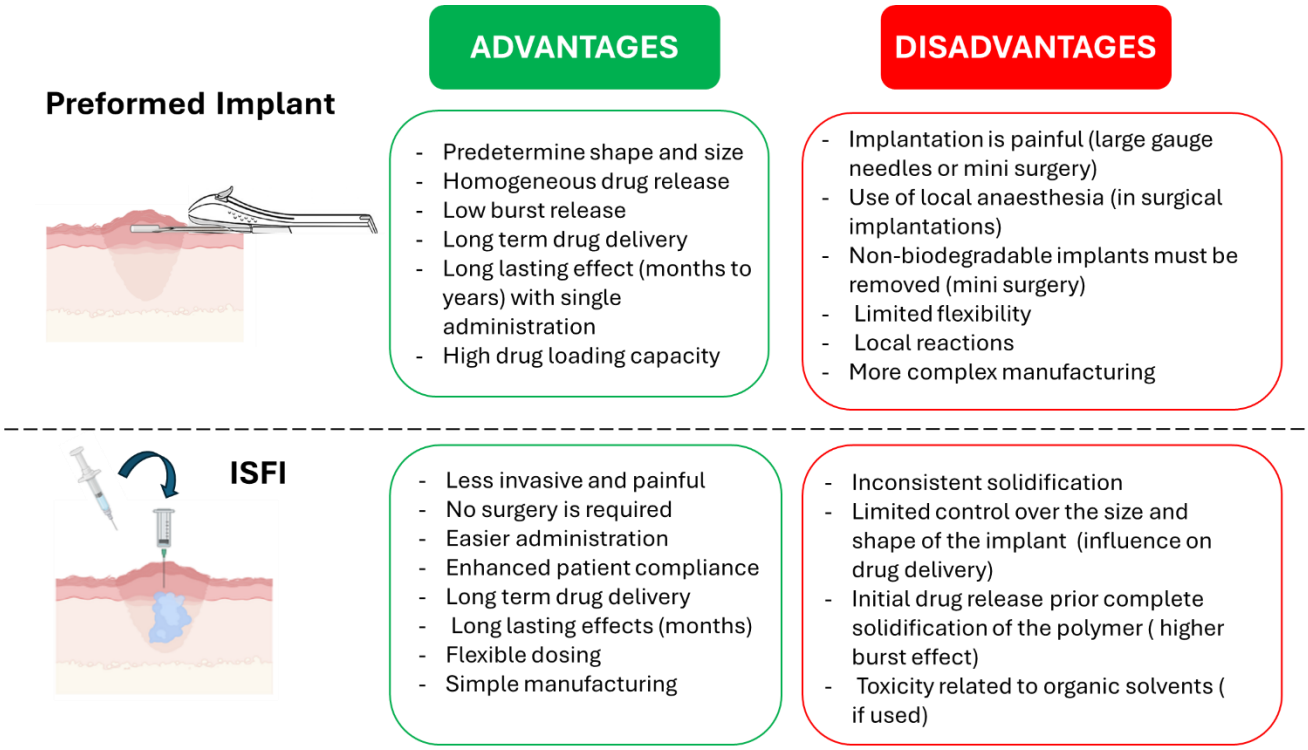


Figure 1: Advantages and disadvantages of preformed implants and ISFIs.

ISFIs can be classified into different types depending on the mechanism responsible for the solidification and formation of the implant at the administration site: i) in-situ crosslinked systems, ii) in-situ solidifying organogel systems, and iii) in-situ phase separation systems (Figure 2) [12].

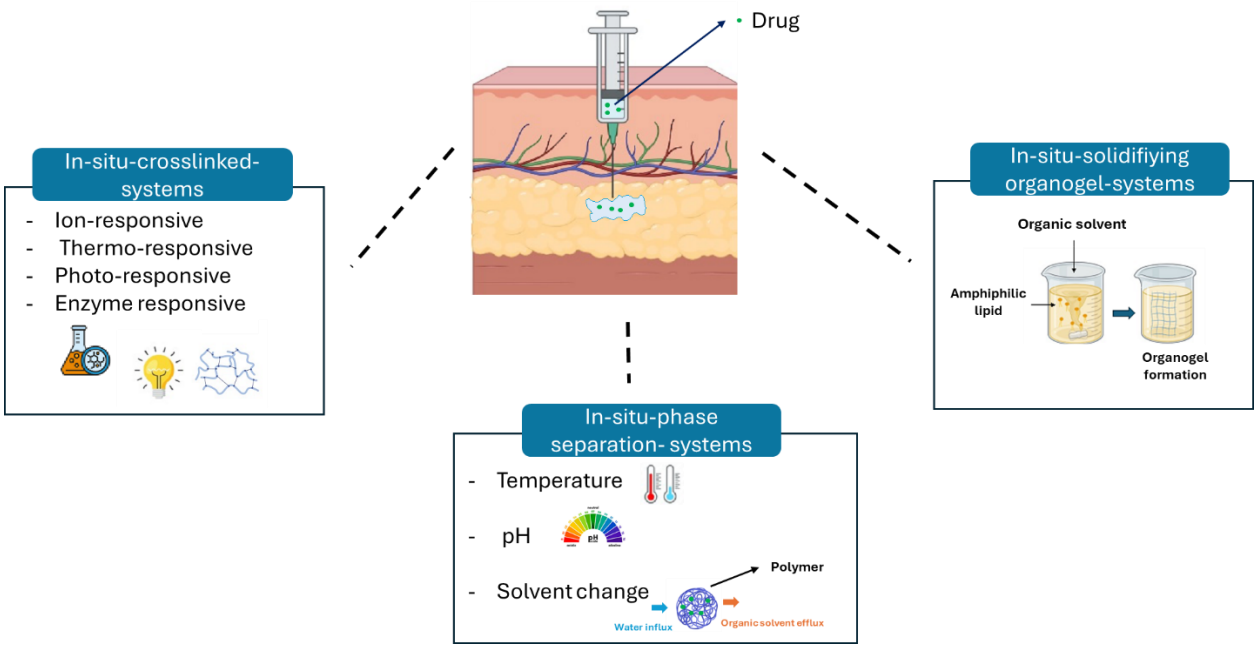


Figure 2: Scheme of types of ISFIs and their mechanisms of solidification

The solidification of in situ crosslinked implants is achieved when the polymer crosslinks due to changes in the temperature (thermo-responsive polymers), the absorption of photons (photo-initiated crosslinking), the interaction of ionic polymers with small cations at the injection site (ion-responsive crosslinking) or the presence of certain enzymes (enzyme-responsive crosslinking) [13, 14]. The in-situ solidifying organogel formulations consist of amphiphilic lipids (mainly fatty acid esters combined with glycerol) heated and dissolved in an organic solvent like ethanol. These lipids are insoluble in water, and when injected into an aqueous environment, they swell and turn into a highly viscous solid or semisolid gel that can prolong drug release. However, the possibility of incomplete gelation can limit their ability to control drug release and, therefore, its therapeutic outcomes [15, 16]. In-situ phase separation formulations are composed of biodegradable polymers whose solidification is triggered by a temperature, pH, or solvent change at the administration site [17]. The thermally induced phase separation systems can be divided into negatively or positively temperature-responsive formulations. Negatively temperature-responsive systems have lower critical solution temperatures (LCST) than body temperature. The polymer is soluble below the body temperature and solidifies when administered. On the contrary, positively temperature-responsive systems show upper critical solution temperature (UCST) than body temperature. These polymers are soluble above this critical temperature [18]. These formulations are typically heated (up to 65ºC) before being administered and solidified by cooling at body temperature [19]. Drugs that are unstable to temperature cannot be administered using positively temperature-responsive systems [20]. In pH-induced phase separation formulations, the polymers are pH-sensitive. Typically, polymeric solution exits at pH values below 5 and solidifies when injected due to the higher pH of the physiological environment (≈7.4) [17]. Finally, the solvent-induced phase separation ISFIs consist of a non-water-soluble but biodegradable polymer dissolved in an organic solvent that solidifies when administered into the body due to solvent removal or exchange.

Among the described ISFIs, those based on phase separation are the most widely used and preferred ISFIs compared to in-situ crosslinking and organogel systems. Solvent-exchange-based ISFIs have gained much interest as drug delivery systems, with several formulations in the market. This review aims to analyze the in-situ forming implant formulations based on solvent change solidification that has been developed to date at both clinical and preclinical levels, underlying their utility for drug delivery.

2. Mechanism of formation of solvent-exchange based ISFIs

As described above, the formation of these implants is triggered by the removal or exchange of the organic solvent. These formulations are composed of water-insoluble but biodegradable polymers dissolved in an organic solvent that is miscible or partially miscible with water. The drugs can be dissolved or dispersed into these polymeric solutions. When this solution is injected into the body, the solvent diffuses into the surrounding area and the polymer that is not soluble in water precipitates at the injection site and forms a deposit in which the drug is entrapped [21, 22]. Then, the drug is released from this deposit over an extended period (from weeks to months), obtaining a long-lasting effect after a single administration (Figure 3).

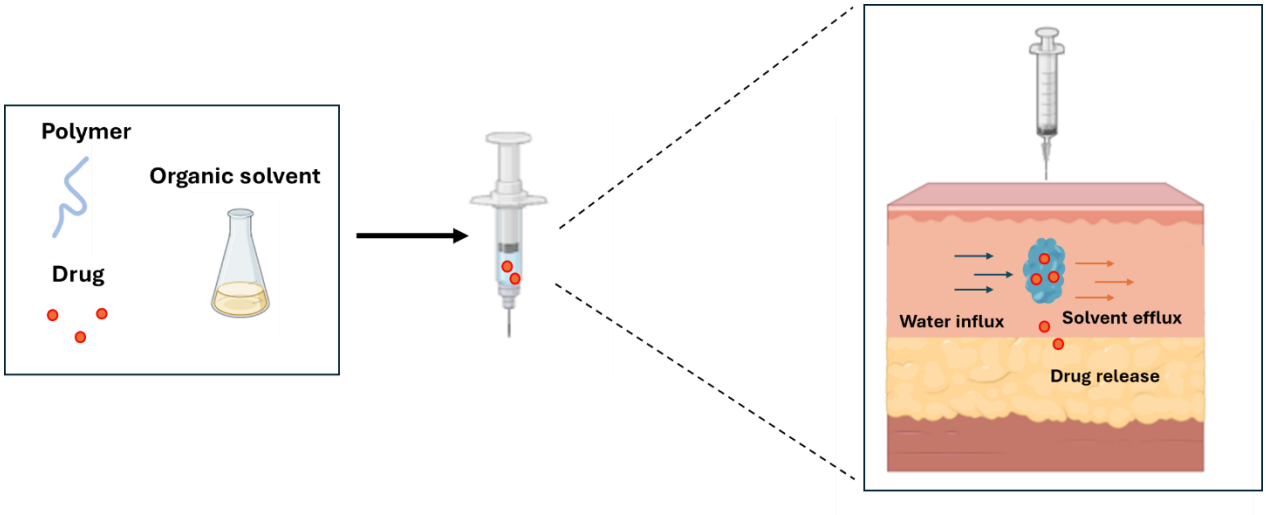


Figure 3: Mechanism of formation of solvent exchange-based ISFIs

It should be noted that the formation of the implant is a critical step that affects drug release, especially during the first 24 hours (burst effect) and, consequently, the therapeutic outcome of the formulation [23]. A high initial drug release can lead to drug levels beyond the therapeutic window and toxicity, especially in drugs with a narrow therapeutic index. On the one hand, the formation of the implant and its shape are also influenced by the speed of the injection and the specific characteristics of the injection tissue. These factors are difficult to control. For example, it has been reported that injections in normal epithelial subcutaneous tissue trigger the formation of flat disc-like-shaped implants and intramuscular injections the formation of fractionated micro-implants. When the polymeric solutions are injected in tumours globular and multi-lobed implants are formed. These differences in the shape of the implants can be attributed to the interstitial fluid pressure of the injection area that affects

the removal of the solvent from the implant and, consequently, drug release. On the other hand, the formation of the implant is strongly influenced by the qualitative and quantitative composition (polymer, drug and solvent) of the formulation. For example, the viscosity of the polymeric solution and the polymer molecular weight condition the solvent exchange and, consequently, the burst effect. These factors can be optimized to minimize this problem and will be extensively discussed in the following sections [24, 25].

3. Advantages and limitations of solvent-exchange based ISFIs

Solvent-exchange-based ISFIs show several advantages over the other separation (thermosensitive and pH-sensitive) systems, which can be summarized in Table 1. Firstly, solvent-exchange-based implants are often less influenced by slight fluctuations in physiological values of temperature and pH as their formation is completely independent of these factors. Consequently, their solidification is usually more predictable and controllable, which is essential to guarantee the reproducibility of the efficacy of the formulation [17]. Secondly, these systems are more versatile than temperature- or pH-change-triggered implants, which require specific conditions for polymer solubility and solidification [26]. Solvent-exchange ISFIs can incorporate a wider variety of polymers and drugs (both hydrophobic and hydrophilic, thermosensitive, ionic…). Moreover, their composition (polymer and solvent type and/or concentration) can be easily modified to customize drug release and, consequently, the duration of the therapeutic effect [27, 28]. Thirdly, they are easier to manufacture, store and administer as they do not require specific temperature or pH conditions. Finally, solvent-exchange-based ISFIs are, in general, better tolerated, as temperature and especially pH-triggered ISFIs are more likely to cause local irritation, although solvent-exchange-based ISFIs include organic solvents in their formulation, which may also cause irritation at the injection site [29].

Even if solvent exchange-based ISFIs are attractive drug delivery systems with several important advantages, they have some limitations to consider [30]. Their main challenge relies on the variability of implant formation in terms of the solidification process and shape. As aforementioned, they are strongly influenced by many factors, including the composition of the formulation (which can be easily controlled) but also by more difficult-to-manage parameters such as the specific characteristics of the injection site (e.g., fluid dynamics and tissue interactions) and injection speed. These factors affect the rate of solvent exchange and, therefore, drug release. Another aspect to consider is the solvent toxicity. The solvents

used in these systems must be safe and highly biocompatible. However, solvent currently used may produce irritation in the administered site, especially if the solvent diffusion is very quick, as high local concentrations can be achieved, causing tissue irritation and inflammation [31]. Finally, it should be mentioned that large molecules such as peptides or proteins may be denaturalized or destabilized due to solvent interaction, limiting the utility of these systems in the administration of biologics [32].

4. Formulation of solvent Exchange-based ISFIs

4.1. Polymers

The polymers used in ISFI formulations must be biocompatible by parenteral route, biodegradable and capable of forming a stable solid or semisolid deposit after their injection [33, 34]. Poly (lactic-co-glycolic acid) (PLGA) and poly (lactic acid) (PLA), meet all these requirements, being the most commonly used polymers to prepare solvent exchange-based ISFIs at preclinical and clinical levels. Both PLGA and PLA degrade through hydrolysis, leading to the formation of lactic and glycolic acid, which are subsequently incorporated into the Krebs cycle [35].

4.1.1. PLA

PLA is a biocompatible, biodegradable, and highly versatile aliphatic polyester derived from lactic acid (2-hydroxypropionic acid) (Figure 4A). This monomer can be obtained from the fermentation of renewable resources, plant-based materials such as cornstarch and sugarcane, or through chemical synthesis [36].

PLA is mainly synthesized by two methods: i) direct polycondensation of lactic acid molecules and ii) ring-opening polymerization (ROP) of lactide (cyclic dimer of lactic acid). This latter method is the most used as it allows better control over polymer molecular weight and polymer stereochemistry [37, 38]. Lactic acid exists in two stereochemical forms: L-lactic acid (L-PLA) and D-lactic acid (D-PLA), and can be polymerized to poly (L-lactic acid) (PLLA), poly (D-lactic acid) (PDLA) or racemic PLA (PDLLA) polymers [39].

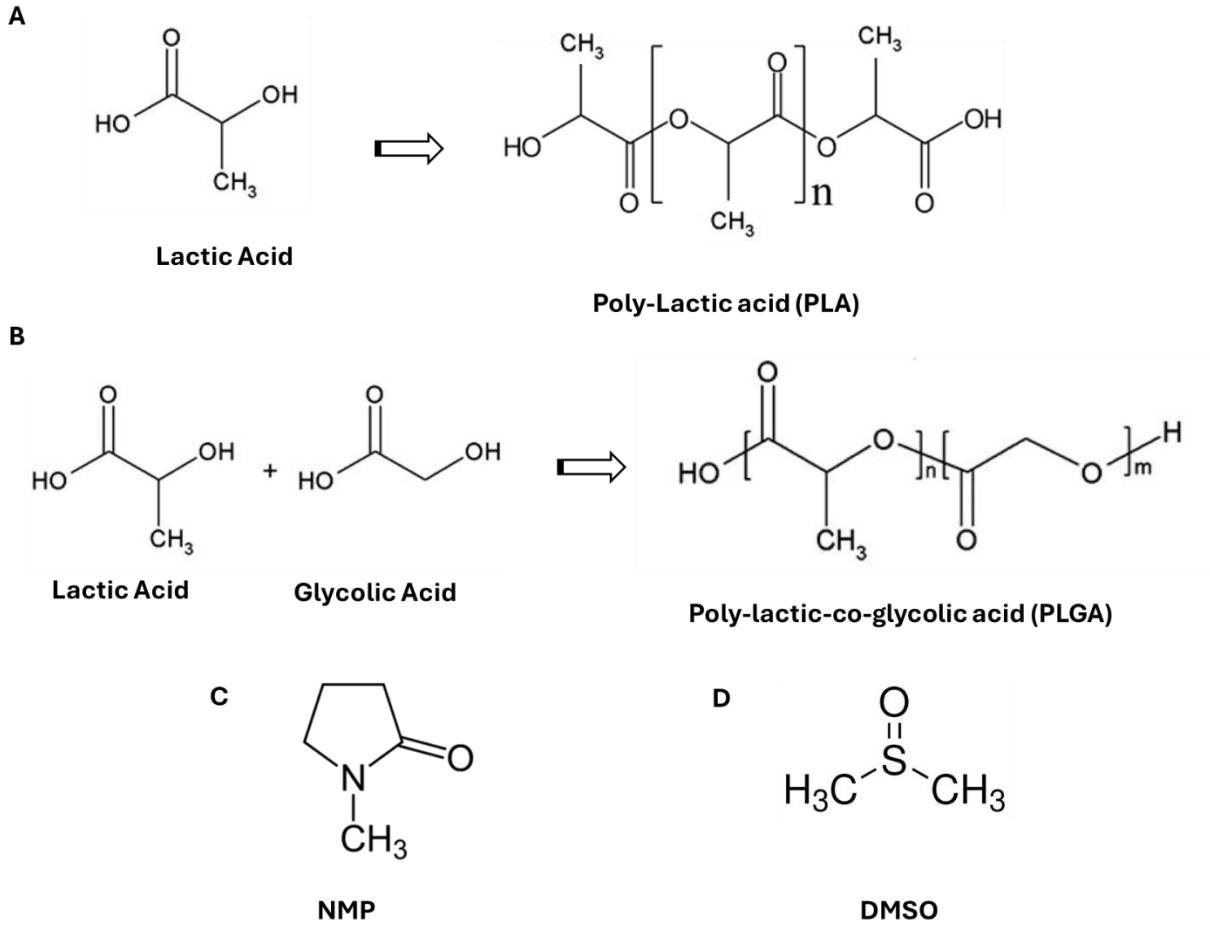


Figure 4: Chemical structure of the main polymers: PLA (A), and PLGA (B); and solvents: NMP (C) and DMSO (D), used in ISFI formulations.

It should be noted that the physicochemical properties of PLA (glass transition temperature (Tg), melting temperature (Tm) and crystallinity) depend on the molecular weight, stereochemistry and processing conditions of the polymer. The Tg of PLA increases slightly when the molecular weight is increased. With longer polymer chains, more thermal energy is required to move the chains to reach the glassy phase (the state in which the polymer changes from a rigid to a more flexible state) [40, 41]. A higher molecular weight can also increase the Tm of PLA, as longer molecules tend to form more stable structures and require more heat to melt. The molecular weight of PLA also conditions its degradation rate [42]. The higher the molecular weight of PLA, the slower the degradation of the polymer [43].

The crystallinity of PLA depends on several factors affecting the ability of PLA to organize its chains into an ordered, crystalline structure. Polymers with a lower molecular weight tend to crystallize more easily, as the chains are shorter and can be better aligned to form crystalline structures. However, the chains of the polymers with a high molecular weight tend

to become entangled, and crystallinity is reduced [44]. The ratio of L- and D-lactic acid significantly influences the crystallinity of PLA. Polymers with a high content in L-lactic acid have a greater tendency to crystallize [45]. In fact, PLLA polymers are considered semicrystalline and PDLLA polymers amorphous [46]. Finally, the processing conditions of PLA, such as the cooling rate, also affect the degree of crystallinity. Rapid cooling favours an amorphous (non-crystalline) structure, as the polymer chains do not have enough time to organize themselves. In contrast, slow cooling promotes crystallization, allowing the chains to align and form crystalline structures [47].

PLA has a broad range of applications in the chemical, biomedical, and food industries. In the biomedical field, it is approved by the FDA and EMA for the development of drug delivery systems, sutures, scaffolds for tissue engineering, prosthetics, and orthopaedic devices [48–50]. It should be mentioned that the physiochemical properties of PLA condition its applications. For example, low molecular weight PLA is often preferred for the development of sutures. These polymers provide flexibility and faster degradation rates, ensuring that the material is absorbed within a short timeframe. They are also preferable for the development of drug delivery systems that do not require to remain in the body long-term (years). On the contrary, polymers with a high molecular weight are used for systems that need to remain in the body for a long time, like pins, plates, and screws used in orthopaedic surgeries (devices that need to remain for at least 3 years). Apart from biomedical applications, PLA with moderate molecular weight has been widely used in food packaging due to its good physical properties and non-toxicity. It is employed in the manufacturing of bottles, jars and containers, as well as in fresh food packaging solutions (Table 1) [50–53].

Molecular weight (kDa)	**Degradation rate**	**Application**
Low molecular weight (7-63)	Fast (few months-1year)	Biomedical applications (drug delivery, sutures), cosmetics and short-term food packaging
Medium molecular weight (63-190)	Moderate (1-2 years)	Agriculture and food packaging.
High molecular weight (>190)	Slow (>2 years)	Biomedical applications (orthopaedics materials)

Table 1: Characteristics of the most commonly used PLA in each application

4.1.2. PLGA

PLGA is a biodegradable and highly biocompatible polymer formed by the copolymerization of lactic acid and glycolic acid (Figure 4B). It is a very versatile polymer in terms of degradation rates and molecular weight, allowing customization according to specific applications. It is widely used in the biomedical industry for the development of sutures, scaffolds in tissue engineering and, mainly, drug delivery systems [54].

PLGA is synthesized using ROP of lactide and glycolide (dimers of acid lactic and glycolic acid respectively) [51]. The ratio of lactic acid to glycolic acid can vary, with common ratios of 50:50, 65:35, 75:25, and 85:15. It should be considered that lactic to glycolic acid ratio conditions many physicochemical properties of PLGA, such as degradation rate, Tg, degree of crystallinity and polymer hydrophobicity, affecting their applications. When DLLA is used, the increase of the content of glycolic acid leads to a higher degree of crystallinity, which can increase the Tm value and reduce the Tg due to the more rigid structures of the crystalline regions [40, 55]. The ratio of lactic to glycolic acid also influences the degradation rate of PLGA. The higher the content of glycolic acid, the faster the degradation rate. Glycolic acid is more hydrophilic than lactic acid and favours the penetration of water and, consequently, the degradation of PLGA by hydrolysis. This is a critical aspect to consider when designing PLGA drug delivery systems, as faster degradation could lead to a faster drug release [55, 56]. Another aspect to be considered is that the end of polymeric chains of PLGA can be either free or esterified carboxylic groups. The polymers with ester terminal groups are more hydrophobic than the acid-terminated polymers and usually show a slower drug release [57].

Based on the different ratios of lactic acid to glycolic acid and molecular weight, PLGA can have different applications (Table 2). In the biomedical field, PLGA is widely used to develop drug delivery systems such as microparticles, nanoparticles, and implants. PLGA with a lactic-to glycolic-acid ratio of 50:50 (PLGA 50:50) is commonly used when a shorter therapeutic effect, from weeks to a few months (typically 1-2 months), is needed. However, when more extended effect is required (from a few months to 1 year), polymers with higher lactic-to-glycolic acid ratios (such as PLGA 75:25 and PLGA 85:15) are used [55, 58, 59].

Lactic-to-glycolic acid ratio	Molecular weight (kDa)	Degradation rate	Application
PLGA 50:50	7– 48	Fast (1-6 months)	Drug delivery systems (shorter effect from weeks to typically 1-2 months)
PLGA 65:35	40 – 75	Moderate (6 months-1year)	Drug delivery (longer effect from 3 to 6 months), tissue engineering, medical device
PLGA 75:25	38 – 115	Slow (1year)	Drug delivery (longer effects typically for 6 months) Plates, screws, tack
PLGA 85:15	76 – 240	Very Slow (Over 1 year)	Drug delivery (longer effects typically for more than 6 months), Screws, sutures anchors, reconstructions

Table 2: Characteristics and main applications of most used PLGA polymers

4.2. Solvents

The selection of the solvent is a critical step in the development of ISFI formulations, affecting the overall toxicity and performance of the system. Firstly, the solvent must be non-toxic, with minimal adverse effects at the injection site [60]. Secondly, they must be capable of dissolving the polymers (drugs can be either dissolved or dispersed in this solution), producing a solution with an adequate viscosity that allows easy injection through conventional needles [61]. Thirdly, the solvent must have adequate miscibility with water allowing rapid desolvation. When the solution is injected, the solvent diffuses upon contact with the aqueous environment and the polymer precipitates, forming the implant. The diffusion rate of the solvent significantly influences the speed at which the solid deposit in which the drug is entrapped is created. It should be considered that part of the drug can escape with the solvent before full solidification of the polymer. This leads to a high initial drug release which is the main limitation of ISFIs. The fast full solidification of the deposit prevents the premature escape of the drug [62].

N-methyl-2-pyrrolidone (NMP) and Dimethyl sulfoxide (DMSO) which are highly water-miscible solvents, are the most used solvents in the development of ISFIs. In fact, all the formulations that are currently approved contain one of these solvents.

4.2.1 NMP

NMP is a polar aprotic solvent commonly used in the pharmaceutical industry due to its favourable toxic and safety profile. It is cleared from the body primarily through renal elimination without significant accumulation [28] and is approved by the FDA and EMA for human use, even for parenteral formulations. The exposure to NMP should not exceed 5 mg/kg body weight per day for adults [63].

One of its main applications is precisely its use in the development of ISFIs due to its ability to dissolve a broad range of polymers, such as PLA and PLGA, and drugs (hydrophilic and hydrophobic) [64, 65]. NMP is highly miscible with water and, upon the injection site, rapidly diffuses out of the formulation, leading to the formation of the implant. Full solidification of the polymer deposit is not achieved immediately, and part of the drug escapes with the solvent. This initial fast drug release is more pronounced in hydrophilic drugs than in hydrophobic compounds, with low solubility in the aqueous tissular fluids and that trend to interact closely with the polymeric matrix, resulting in a more controlled drug release [28].

4.2.2. DMSO

DMSO is an aprotic organic solvent that is used as a pharmaceutical excipient with a variety of applications, such as permeability enhancer, cryoprotectant of biological materials, stabilizer in pharmaceutical preparations, and to solubilize drugs with low water solubility [66]. DMSO is generally considered safe for human use and is approved by the FDA and EMA as an excipient in topical and parenteral formulations. In fact, it is specifically approved for the treatment of interstitial cystitis [67]. A 50% DMSO solution administered via the intravesical route (instilled into the bladder) is typically used for the treatment of this pathology [68, 69].

The use of DMSO in the development of ISFIs is not as widespread as NMP. However, some formulations have already been approved with DMSO as a solvent [70]. In fact, the use of DMSO instead of NMP typically allows better control of the rapid initial drug release that occurs during the solidification of the implant, which is attributed to the more rapid diffusion of this solvent [71].

5. Technological resources to decrease initial drug release

As mentioned above, the main challenge of ISFIs is the premature loss of the drug until complete solidification of the polymeric matrix, resulting in a very rapid initial drug release and a shorter therapeutic effect. This is more intense in low molecular weight hydrophilic drugs, which are soluble in the interstitial fluid and tend to have less interaction with the polymeric matrix than hydrophobic actives [28]. Figure 5 displays the main technological strategies available to minimise the high initial drug release of ISFIs [12].

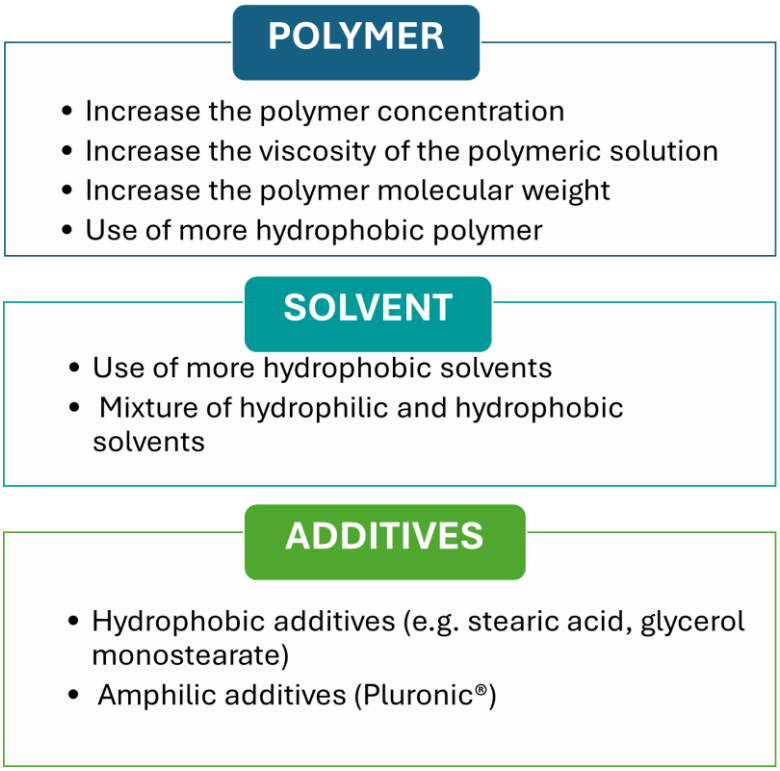


Figure 5: Scheme summarizing the technological resources available to minimise the loss of the drug during implant formation.

5.1. Polymer modification

One of the most important strategies is based on modifying the concentration or composition of the polymer. The increase in the polymer concentration results in an increase in the viscosity of the solution, which hampers the diffusion of the drug at the injection site during the formation of the implant [72]. However, it should be noted that solutions with a higher viscosity may not be easily administered [73, 74]. This is an aspect that must be optimized.

Another option is to increase the molecular weight of the polymer [75]. Polymers with a higher molecular weight produce a denser polymeric network that also difficult the diffusion of the drug during implant solidification. Moreover, it should be considered that increasing the molecular weight of the polymer leads to an increase in the viscosity of the formulation, which also may contribute to the reduction of drug escape [76]. For example, it has been reported that the higher the molecular weight of PLGA, the slower the initial release of leuprolide acetate from ISFIs prepared with NMP. While formulations prepared with PLGA of 34 KDa showed a drug release of around 36% during the first 24 hours, ISFIs made of PLGA with 48 KDa released around 14% of the drug within this time [77]. Similar results were also found in paclitaxel loaded ISFIs. These implants were prepared with PLGA and NMP at a polymer concentration of ≈34%. Polymers with a lactic-to-glycolic acid ratio 50:50, a free carboxylic end group and an average molecular weight of 12kDa (PLGA-502H), 31 kDa (PLGA-503H) or 46KDa (PLGA-504H) were used. All these systems showed a sustained paclitaxel release for around 28 days. However, while implants prepared with PLGA-502H and PLGA-503H exhibited a similar burst effect (around 35% of paclitaxel was released in 24 hours), PLGA-504H formulation showed a lower initial drug release of around 13% due to the higher molecular weight of the polymer [78].

The increase of polymer hydrophobicity is another technological resource available to reduce initial drug release. Gad and collaborators developed ISFIs loaded with both doxycycline and secnidazole. These formulations were prepared using PLA (medium to high molecular weight: 75-120 kDa) or PLGA (lactic-to-glycolic acid ratio of 50:50 and medium molecular weight of 40-75 kDa) and NMP at two different polymer concentrations: 25% and 35% (w/w). Implants prepared with PLGA at 25% exhibited faster doxycycline and secnidazole release (≈95% after 24 hours), followed by the formulations prepared with PLA at 25% (≈85% of both drugs were released after 24 hours) and the implants prepared with a polymer concentration of 35% (PLGA formulation showed a percentage release of 61-68% and PLA systems of approximately of 45% within 24 hours) [79]. As explained above, the higher the polymer concentration, the slower the solvent diffusion during implant formation and the slower and more controlled drug release. The higher molecular weight of PLA, along with its greater hydrophobicity compared to PLGA, results in a lower initial burst release for the same polymer concentration.

5.2. Solvent change

The solvent used in the formulation is one of the most important factors influencing the drug release during implant formation. The main characteristic of the solvent to take into account is its water miscibility [17]. The solvent used must have certain water miscibility so that it can diffuse freely at the injection site, triggering polymer precipitation. NMP and DMSO are highly miscible in water, diffusing rapidly at the injection site. However, the complete solidification of the polymer is not immediate, and part of the drug escapes from the formulation. This drug loss is more pronounced in hydrophilic drugs, which in general have a higher affinity for the solvent than for the polymer and tend to diffuse with this being released rapidly until the fully solidification of the implant. On the contrary, the lipophilic compounds have a higher affinity for the polymer and tend to diffuse less with the solvent and therefore are released in a more controlled manner. Joiner and collaborators evaluated the effect of drug hydrophilicity on release kinetics from ISFIs prepared with PLGA (27 KDa) and NMP. These authors found a negative correlation between the initial drug release and drug logP: the lower the logP the higher burst release [80]. Thus, while more hydrophobic drugs (e.g. Etravirine, Rilpivirine, Efavirenz and ritonavir) exhibited a low burst effect (<10% of drugs released in the first 24 hours), more hydrophilic drugs (e.g. Gemcitabine, 5-Fluorouracile, Zidovudine and idarubicin) showed a higher initial release (> 30% in the first 24 hours).

In general, NMP-based ISFIs show a faster initial drug release than DMSO-based formulations [81, 82]. DMSO shows higher hydrophilicity than NMP (with polarity indexes of 7.2 and 6.7 respectively) [83] and their diffusion to the aqueous environment is faster producing. This leads to a faster increase in the viscosity of the polymeric solution, thereby reducing the initial drug release, especially for hydrophobic drugs, which diffuse less into DMSO. For example, cannabidiol loaded ISFIs prepared with PLA-202 (molecular weight of 10-18 kDa) and PLGA-502 (lactic-to-glycolic acid 50:50 and molecular weight of 7-17KDa) exhibited a fastest drug release when prepared using NMP than DMSO as organic solvent. CBD is a highly hydrophobic drug, likely exhibiting greater solubility in NMP than in DMSO, and lower diffusion in this latter solvent.

One option to reduce the escape of hydrophilic drugs during implant formation is to use a mixture of highly water-miscible solvents with more hydrophobic solvents like triacetin or benzyl benzoate. In this case, the polarity of the solvent is reduced and consequently their diffusion is slowed down. For example, the use of a mixture of triacetin and NMP significantly

reduce the initial drug release of metoclopramide monohydrochloride, a very hydrophilic drug. While formulations prepared just with NMP exhibited a strong initial drug release of more than 30% in the first 24 hours, the implants prepared with a mixture of NMP and triacetin at ratios of 95:5, 90:10 and 80:20 showed a release below 15% by this time [84]. However, it should be taken into consideration that all the solvents used must be biocompatible and must be approved for human use by parenteral route. Triacetin is considered safe by FDA as food additive and is used as a solvent in cosmetic preparations. However, ISFIs containing this solvent have not yet been approved.

5.3. Incorporation of additives

Some researchers have demonstrated that the incorporation of hydrophobic additives such as stearic acid, glycerol monostearate, α-Tocopherol or benzyl benzoate in the formulation may help decrease the escape of the drug during implant formation, especially of hydrophobic drugs. The incorporation of these additives may slow down the diffusion of the solvent, leading to less porous matrices that result in a slower drug escape during implant formation [85]. For example, Lin and colleagues developed ISFIs of acetonide of triamcinolone and sodium diclofenac made of PLGA (lactic-to-glycolic acid ratio of 50:50 and molecular weight of 15 KDa) and NMP at polymer concentration of 30% and evaluated if the addition of α-Tocopherol or benzyl benzoate reduced initial drug release. In the case of triamcinolone acetonide implant (formulation with drug concentration of 10%), the incorporation of α-Tocopherol at 3% reduced the initial drug release during the first 24 hours from ≈20% to ≈13%. However, the incorporation of benzyl benzoate at 6% showed the opposite results, as by this time around 38% of acetonide of triamcinolone was released. In the case of sodium diclofenac, the incorporation of benzyl benzoate was effective and reduced drug release from more than 90% to ≈42%, as probably increased the interaction of this drug with the polymer. This study shows that the type of additive must be optimized and selected according to the characteristics of the drug[86].

The addition of amphiphilic additives is also useful. For example, this is the case of Pluronic® (poly(ethylene)oxide–poly(propylene)oxide–poly(ethylene)oxide) (PEO-PPO-PEO) that also limits the initial diffusion of some drugs. While the hydrophobic PPO chains are embedded into the polymeric matrix, the hydrophilic PEO chains are diffused into the aqueous medium, producing a diffusion barrier that limits drug escape [58].

Finally, it has been hypothesized that the addition of polyethene glycol (PEG) also reduces the initial drug release. This compound tends to increase the overall viscosity of the formulation, which, as explained above, contributes to reducing initial drug release. For example, Quin and collaborators demonstrated that the incorporation of PEG significantly decreased the initial drug release of tinidazole from PLA ISFIs. While formulations prepared with PLA (142.5 mg), NMP (318.25mg) and glycerol (14.25 mg) showed a drug release of around 45% within 24 hours, implants prepared with PLA (142.5 mg), NMP (285mg), glycerol (14.25 mg) and PEG (50 mg) exhibited an initial drug release around 25% [87]. Kamali and coworkers prepared formulations with PLGA or PLGA-PEG-PLGA (PLGA 50:50 and a molecular weight of 38-54kDa was used) dissolved in NMP. The implants prepared with PLGA-PEG-PLGA showed a significantly lower burst effect (≈6%) than the systems prepared just with PLGA (≈14%) which can be attributed to the interaction between PEG and NMP. Nevertheless, these authors found that the formulation containing PEG exhibited a faster overall release. While PLGA formulation exhibited a controlled drug release for around one month, PLGA-PEG-PLGA system released 100% of buprenorphine within 3 weeks [88]. The presence of PEG could facilitate the penetration of water into the polymeric deposit and accelerate the overall drug release.

6. ISFIs developed for the treatment of mental disorders

Low therapeutic compliance represents the main problem of treating mental disorders as it leads to worsening of symptomatology and relapse of the disease [24]. It is difficult for these patients to have good adherence to treatment, as they often forget the doses. The use of non-patient-dependent dosage forms, such as ISFIs may resolve this problem. Compared with oral medications, patients receiving ISFI-based medications are, in general, better controlled, need fewer hospital check-ups, and show an improved quality of life [89, 90]. Therefore, ISFIs are an excellent tool for the treatment of mental disorders. To date, three different ISFIs of risperidone (Perseris®, Uzedy® and Okedi®) are approved for the treatment of schizophrenia (Table 3).

Commercial name	Drug	Polymer/Solvent	Duration	Inidication	Approval year
Atridox®	Doxycycline	PLA; NMP	7 days	Periodontitis	FDA 1998 EMA 2001.
Eligard®	Leuprolide acetate	PLGA (50:50); NMP	1 month	Hormone-dependent metastatic prostate cancer	FDA 2002; EMA 2004.
		PLGA (75:25); NMP	3-4 months		
		PLGA (85:15); NMP	6 months		
Sublocade®	Buprenorphine	PLGA (50:50); NMP	1 month	Sever opioid addiction	FDA 2017 EMA 2019
Perseris®	Risperidone	PLGA (80:20); NMP	1 month	Adult schizophrenia	FDA 2018 EMA 2021
Uzedy®	Risperidone	m-PEG-b-PLA and PLA-PEG-PLA; DMSO	1-2months	Adult schizophrenia	FDA 2023
Okedi® **Risvan®**	Risperidone	PLGA (50:50); DMSO	1 month	Adult schizophrenia	EMA 2023 FDA 2024

Table 3: ISFIs currently approved by the FDA and EMA.

6.1. Perseris®

Perseris ® is an in situ forming implant formulation of risperidone approved for the treatment of schizophrenia in adults [91]. It is based on Atrigel® technology, which was developed by Dunn and coworkers in 1987 and licensed by Vipont Research Laboratories (Atrix Laboratories). Atrigel® consists of a viscous polymeric solution of PLA or PLGA in NMP at concentrations ranging from 10 to 80% (w/w) [92–95].

Perseris ® formulation consists of two syringes: i) one syringe containing the polymeric solution (PLGA 80:20 dissolved in NMP at a polymer concentration of approximately 45%) and ii) a second syringe containing risperidone. Two strengths of risperidone (90 and 120 mg) are currently available. Both syringes are mixed before the administration and injected subcutaneously using an 18G needle in the abdomen or back of the upper arm where form a deposit [96] that releases risperidone in a controlled manner for 1 month.

6.2 Uzedy®

Uzedy® is an in situ forming implant of risperidone recently approved by the FDA for the treatment of adult schizophrenia [97]. This formulation is based on BEPO® technology that consists of a prefilled syringe containing a polymeric solution of methoxy-poly(ethylene glycol)-co-poly(D,L-lactide) (15% w/w), and poly(D,L-lactide)-co-poly(ethylene glycol)-co-poly(D,L-lactide) (PLA-m-PEG-PLA) (10% w/w) dissolved in DMSO (45% w/w). This formulation is available at different strengths of risperidone, ranging from 50 to 250 mg, that is dissolved in the above polymeric solution. This formulation is administered subcutaneously in the abdomen or the back and outer area of the upper arms using a 21G needle. Uzedy® can be administered once monthly (at doses of 60mg, 75mg, 100mg, 125mg) or every two months (at doses of 100mg, 150mg, 200mg and 250mg) [98]. This formulation has been shown to be effective in reducing the symptoms of schizophrenia and delaying relapses [99]. Furthermore, the wide range of available doses enables treatment to be tailored to the specific needs of each patient [100].

6.3. Okedi®

Okedi® is another formulation available for the treatment of schizophrenia in adults approved by EMA [101]. It was recently approved by FDA (2024) as Risvan® [102]. This formulation is based of In-Situ-Microimplant® (ISM®) technology and consists of two prefilled syringes that are mixed just before administration. One syringe contains risperidone and PLGA (50:50), and the other the solvent (DMSO). There are two available dosage strengths: 75 mg and 100 mg of risperidone that are administered via intramuscular injection in the gluteus or deltoid area using a 20 and 21G needle, respectively, where they form micro-deposits that release risperidone in a controlled manner during approximately 28 days.

6.4. Formulations developed at the preclinical level

In addition to risperidone, formulations of other antipsychotic drugs have also been developed. Wang and collaborators developed ISFIs of paliperidone, a drug approved for the treatment of schizophrenia. These formulations were prepared using PLGA 50:50 or PLGA 75:25 with different molecular weights (58-105 KDa) as polymer and NMP or DMSO as organic solvent (Table 4). The NMP-based-formulations showed a faster in vitro drug release. They were only able to control the release of paliperidone for around 7 days. However, the formulations prepared with DMSO exhibited a more controlled drug release,

especially the implants prepared with PLGA 50:50 with the highest molecular weight (105kDa). This formulation exhibited a controlled and sustained paliperidone release for approximately 2 months. Nearly 100% of the drug was released by this time. In vivo studies in a mouse schizophrenic behavior model demonstrated that this formulation administered subcutaneously at single dose was able to control symptomatology for around 38 days, indicating its utility as a long-lasting effect system [70].

Avachat et. al. prepared implants of asenapine, another antipsychotic approved for the treatment of schizophrenia and bipolar disorder. These implants were made of PLGA 50:50 with a molecular weight of 58kDa and NMP at different polymer concentrations (350-700 mg/ml). All the formulations exhibited a controlled asenapine release for around 3 weeks. The higher the polymer concentration, the slower the drug release. Interestingly, the implants were successfully formed ex-vivo in the extensor digitorum chick muscle after their injection using a 20G needle. Finally, in vivo studies in a psychosis model induced in rats by injecting ketamine reported that asenapine ISFIs improved the symptomatology (locomotor activity and behavioral response) of psychotic rats and that their response was significantly higher compared to the administration of oral asenapine solution. While asenapine-loaded ISFIs were single administered via intramuscular route, the asenapine oral solution was administered every 5 day 30 minutes before the evaluation of the locomotor activity [103].

Disease	Drug	Polymer	Solvent	Additives	Reference
Schizophrenia	Paliperidone	PLGA 50:50 PLGA 75:25	NMP or DMSO	-	[70]
	Asenapine	PLGA 50:50	NMP	-	[103]
Periodontal disease	Tinidazole	PLA (≈7.5kDa)	NMP	PEG-400	[87]
	Doxycycline and secnidazole	PLA (75-120kDa) PLGA50:50 (40-75kDa)	NMP	-	[79]
	Dexamethasone	PLGA 50:50 (12-28 KDa) PLA (12-28 KDa)	DMSO	-	[104]
	Ibuprofen and Chlorhexidine	PLGA 50:50	NMP	Acetyltributyl citrate and hydroxypropyl methylcellulose	[105]
Cancer	Paclitaxel	PLGA 50:50 (12-46 kDa)	NMP	-	[78]
	Doxorubicin	PLGA 75:25 (28.8 kDa)	NMP	-	[106]
	Cannabidiol	PLGA 50:50 (7-38 kDa) PLA (10-28 kDa)	NMP and DMSO	-	[107]
Addictions	Buprenorphine	PLGA-PEG-PLGA	NMP	PEG	[88]

		(PLGA 50:50 of 38-54kDa)			
	Naltrexone	PLGA 50:50 (38-54kDa) PLGA 75:25 (76-115kDa)	NMP	-	[108]
Arthrosis and Arthritis	Triamcinolone acetonide Sodium diclofenac	PLGA 50:50 (15 KDa) PLGA 75:25 (15 KDa)	NMP	benzyl benzoate and α- tocopherol	[86]
Bone injury	Raloxifene hydrochloride	PLGA 50:50 PLGA 75:25	DMSO	-	[109]
Macular degeneration, diabetic macular oedema, uveitis, and glaucoma	Fluocinolone acetonide	PLGA 50:50 (38-54kDa) PLGA 75:25 (76-115kDa)	NMP	-	[110]
	Dexamethasone	PLGA 50:50	NMP	-	[72]

Table 4: Solvent-exchange-based ISFIs developed at the preclinical level

7. ISFIs developed for the treatment of periodontal diseases

In the treatment of periodontal diseases, ISFIs offer a promising strategy for delivering drugs directly to the periodontal pocket. These formulations are minimally invasive, they are administered using conventional needles and provide local therapeutical levels for extended periods with a single administration. At the same time, the systemic drug concentrations, and consequently adverse effects, are minimized [111, 112]. Commonly employed drugs for the treatment of periodontal diseases include antimicrobials such as doxycycline or metronidazole, anti-inflammatory agents, both non-steroid anti-inflammatories and corticosteroids, and tissue-regenerative compounds like growth factors. The administration

of all these drugs in ISFIs implants is very interesting. In fact, an ISFI of doxycycline (Atridox®) is currently approved for the treatment of chronic periodontitis (Table 5).

7.1 Atridox ®

Atridox® is an ISFI of doxycycline approved for the treatment of chronic periodontitis, an inflammatory disease that affects the tissues that support the teeth [113]. It is also based on Atrigel® technology and consists of two prefilled syringes, one containing 450 mg of a solution composed of PLA (36.7%) and NMP (63.3%) and the other containing 50mg of doxycycline hyclate [114], a broad spectrum antibiotic active against bacteria associated with periodontal disease [115]. These two syringes are connected and mixed immediately before their use. This formulation is administered subgingivally into the periodontal pocket using a 23G blunt cannula without anaesthesia, where it solidifies, forming a deposit that releases the antibiotic in a controlled manner for 7 days. During this period, the local concentrations of doxycycline remained above 6.0 µg/mL, the minimum inhibitory concentration for pathogens related to periodontal disease. These local concentrations of doxycycline could not be achieved using oral medications. Nevertheless, the plasma levels of this antibiotic were minimized with concentrations lower than 0.1 µg/mL[116]. These data support the use of ISFI formulation for the treatment of local periodontal diseases.

7.2. Formulations developed at the preclinical level

Apart from the currently marketed ISFIs of doxycycline (Atridox®), other formulations for the delivery of antibiotics in periodontitis have been developed (Table 4). Qin and collaborators developed ISFIs of tinidazole, a second-generation 2-methyl-5-nitroimidazole class antibiotic with high efficacy against anaerobic bacteria [117] that are putative pathogens in periodontitis. These implants were prepared using PLA of low molecular weight (≈7.5KDa) and NMP. PEG-400 was added at different concentrations as an additive to control the burst effect. The formulation containing PEG-400 showed a slower drug release, with a controlled tinidazole release for around 7 days. Formulations containing PEG-400 showed a slower drug release. This formulation was injected into the periodontal pocket of beagle dogs (0.5 g of formulation was injected) once a week for 4 weeks and showed a reduction in periodontitis symptoms [87].

ISFIs of anti-inflammatory drugs have also been developed. Batool and collaborators developed ISFIs loaded with both ibuprofen and chlorhexidine. These formulations were

prepared with PLGA 50:50 dissolved in NMP at 28% (w/w). Acetyltributyl citrate and hydroxypropyl methylcellulose (at concentrations of 10% w/w) were also added as plasticizers and adhesion excipients, respectively. Both ibuprofen and chlorhexidine were released in a controlled manner for at least 30 days [105]. This formulation exhibited antimicrobial activity against periodontal pathogens such as Streptococcus sanguinis, Actinomyces odontolyticus, Fusobacterium nucleatum and Porphyromonas gingivalis, among others after 48 hours of treatment [118]. Moreover, in vivo studies indicated an anti-inflammatory activity and an improvement in periodontal wound healing 7 and 15 days after their intra-periodontal pocket administration into mice [119].

8. ISFIs developed for cancer treatment

ISFIs also represent a highly valuable technology for delivering anticancer agents. On the one hand, these systems deliver the drugs in a controlled manner with a single administration, which is useful in cancer therapy as this disease requires long-term treatments. On the other hand, these systems could be injected directly into the tumour area, which would allow the localization of anticancer drugs in the action site. This may increase their anticancer activity and decrease their systemic side effects, especially when compared with intravenous administration [120]. Nowadays, an ISFI formulation of leuprolide acetate (Eligard®), a potent inhibitor of gonadotropin (GnRH) secretion, is currently approved for prostate cancer (Table 5)[121]. This formulation was developed to obtain a long-lasting effect with a single administration.

8.1. Eligard ®

Eligard® is a long-acting depot formulation of leuprolide acetate, a potent inhibitor of gonadotropin (GnRH) secretion that is approved for the treatment of prostate cancer [121]. This formulation is composed of two prefilled syringes based on the Atrigel® technology. The first syringe contains a PLGA solution in NMP and the second leuprolide acetate. Both syringes are mixed immediately before administration. There are four different Eligard® formulations on the market, differing in the dose of leuprolide and the type (ratio of lactic-to-glycolic acid) and concentration of PLGA: i) formulation containing 7.5 mg of leuprolide acetate, 82.5 mg of PLGA 50:50 at a polymer concentration of 34%, ii) formulation with 22.5 mg of leuprolide acetate, 158.6 mg of PLGA 75:25 at a concentration of 45% iii) a formulation of 30 mg of leuprolide acetate, 211.5mg of PLGA 75:25 at a concentration of 45% and iv) a formulation with 45mg of leuprolide acetate, 165mg of PLGA 85:15 at 50% [122]. These

formulations are administered subcutaneously using 20G needles and provide a controlled release of leuprolide acetate from 1 to 6 months, depending on each composition [116]. As explained above, the concentration of the polymer and the lactic-to-glycolic acid ratio can be adjusted to obtain a prolonged drug release and, therefore, a prolonged therapeutic effect. The higher the polymer concentration and the lactic-to-glycolic acid ratio, the slower and more prolonged drug release. While the formulation prepared with PLGA 50:50 and the lowest polymer concentration (34%) has a duration of 1 month, the systems containing PLGA 75:25 last for 3 or 4 months depending on the drug content. The formulation prepared with PLGA 85:15 showed the longest duration, around 6 months.

8.2. Formulations developed at the preclinical level

Jeganathan et. al. prepared ISFIs of doxorubicin and P-glycoprotein inhibitors (valspodar or pluronic P85). The implants were made of PLGA 75:25 (28.8KDa) and NMP and showed a controlled release of all actives for at least 21 days (more than 60% of the drugs were released by this time). These implants were successfully formed when injected intratumorally (single administration) in a colon cancer subcutaneous model developed in mice (this model was created by injecting HCT-15 cells), and a tumour growth reduction was observed in more than 50% compared with non-treated mice. ISFIs prepared with doxorubicin and valspodar were more effective compared to all other treatment groups (not treated, free doxorubicin and doxorubicin+P85 implant group) [106].

9. ISFIs developed for the treatment of addictions

ISFIs are also useful for managing addictions. Their main advantage relies on the long-acting effect. These systems offer a prolonged release of the drugs, achieving therapeutical levels for extended periods (weeks to months) after a single administration, avoiding the need for daily dosing. The treatment adherence is significantly improved, and consequently, the risk of relapse is reduced [123]. These systems have been widely investigated for the treatment of opioid abuse disorders, with an ISFI formulation of buprenorphine (Sublocade®) in the market (Table 5).

9.1. Sublocade ®

Sublocade® is an ISFI formulation of buprenorphine approved by the FDA and EMA for the treatment of moderate to severe opioid use disorder. This formulation is composed of PLGA 50:50 dissolved in NMP at a polymer concentration of 39%. It is available in two

buprenorphine dosage strengths: 100mg and 300mg [124]. Both formulations are administered subcutaneously using 19G needles in the abdomen, forming a deposit that provides a therapeutic effect for 1 month. Sublocade® resulted in a significant advance in the treatment of opioid dependence compared to sublingual daily therapies, as it provides reliable and consistent buprenorphine levels after a single administration. The continuous and stable concentrations achieved with this system are critical to reducing the risk of relapse. Moreover, this formulation improves medication adherence [125].

10. ISFIs developed for the treatment of other diseases

To the best of our knowledge, no other formulation of in situ forming implants has been approved. However, these systems are also interesting in the treatment of other pathologies, and several formulations have been developed at the preclinical level. For instance, in the management of chronic pain, ISFIs incorporating analgesics or anti-inflammatory agents offer prolonged pain relief with a single administration, reducing the frequency of dosing and improving treatment adherence. They can be especially useful in the treatment of arthrosis and arthritis. Lin and collaborators developed ISFIs of triamcinolone acetonide prepared with PLGA 50:50 at a molecular weight of 15kDa, NMP as organic solvent and benzyl benzoate as additive. This formulation exhibited a controlled drug release for at least one month. When injected into the knee of arthritic mice (intraarticular injection) showed a reduction of the swelling, pain, and motor discoordination related to this pathology for at least 10 days. Moreover, it also suppressed the progression of the disease [86].

ISFIs have also been developed to improve bone degeneration. For example, Elkasabgy and collaborators prepared raloxifene hydrochloride ISFIs to improve bone injury in older people or osteoporotic patients. These formulations were prepared using PLGA 50:50 or PLGA 75:25 (end capper with ester group) dissolved in DMSO at a polymer concentration of 10% (w/v). PLGA 50:50 and PLGA 75:25 implants showed a controlled raloxifene release for around 20 and 30 days, respectively. The slowest release rate of this latter formulation can be attributed to the higher hydrophobicity of this PLGA due to the higher lactic to glycolic acid ratio. To extend drug release, liquid lipids (Labrasol® or Maisine®) were added to the formulation at a DMSO: lipid ratio of 90:10. Interestingly, these additives slowed down the drug release of raloxifene, especially in PLGA 50:50 formulations, extending drug release up to 50 days. PLGA75:25 implants containing Maisine® as lipid showed the best release

profile and were tested in vivo in a rat model of bone injury in the tibia. These implants were singly administered subcutaneously and shown to promote bone regeneration [109].

Ocular pathologies, especially those affecting the posterior segment of the eye, such as age-related macular degeneration, diabetic macular oedema, uveitis, and glaucoma, are another area of interest for the development of ISFIs. The treatment of these disorders through ocular or systemic (oral or intravenous) routes is very challenging due to the anatomical and physiological barriers that limit drug penetration and retention in the affected area. ISFIs offer a minimally invasive strategy to deliver the drug directly to the affected ocular tissue in a controlled manner for weeks to months. Karimi and coworkers developed formulations of fluocinolone acetonide using PLGA-504H (PLGA 50:50 with a molecular weight of 38-54kDa and carboxylic acid terminated groups), PLGA-756S (PLGA 75:25 with a molecular weight of 76-115kDa and ester terminated groups) and their mixtures (ratios of 1:1, 1:3 and 3:1 were tested) as polymer and NMP as solvent. All the formulations exhibited a controlled drug release for at least 4 weeks. The implants prepared with both polymers at a ratio 1:1 showed a better release profile. Around 60% of fluocinolone acetonide was released within the first 3 days. Then, a sustained release rate of 0.36 μg/day was appreciated from the third day to two months. This formulation was injected intraocularly in rabbits and exhibited a mean residence time of fluocinolone acetonide of ≈ 20 days and a clearance of ≈0.3 ml/h for the vitreous humor, suggesting a sustained and slow absorption of this corticoid [110].

10. Conclusions

Solvent-exchange based ISFIs represent an interesting solution for sustained drug delivery with a single administration. By modifying the formulation components, such as polymer characteristics, polymer concentration, solvent choice or incorporation of some additives, drug release kinetics can be fine-tuned to meet the specific therapeutical requirements, extending or shortening the duration of the therapeutic effect.

NMP is the most used solvent in the development of these implants. In fact, except for Uzedy® and Risvan®/ Okedi® (which have been recently approved in 2023 or 2024 by the FDA or EMA) the rest of the commercialized formulations contain NMP. It should be noted that in general, DMSO allows better control of the release of the drug during the first time points and its use is very interesting. Regarding polymers, PLGA is the most used. The ratio

of lactic acid to glycolic acid and the polymer concentration are the factors that commonly change to adapt the formulations to specific uses.

The main advantage of ISFIs relies on their ability to maintain the therapeutic effect over extended periods, from days to months, with a single administration using a conventional needle. This, together with the reduction of side effects by reducing fluctuations in drug levels in biophase, generally improves therapeutic adherence allowing better disease management in chronic conditions. Currently, ISFIs are highly valuable for the treatment of mental disorders like schizophrenia, addictions, and cancer and several ISFI formulations have been approved by the FDA and/or EMA to treat these conditions. Additionally, they are useful for achieving local therapeutic levels at the administration site while minimizing systemic plasma levels. In this regard, they have great potential for the treatment of periodontal diseases. In fact, a formulation for this application is currently approved by FDA and EMA. They are also useful for the treatment of bone diseases, such as osteoporosis and arthritis, being useful for the administration of analgesics or bone regenerators, as well as for ocular pathologies. However, the formulations developed for these disorders are still at the preclinical level.

Despite all these applications, ISFIs still face challenges to wider adoption. The variability in implant formation due to injection conditions (tissue characteristics and injection speed) affects the release profile consistency and, consequently, the therapeutic outcome. An exhaustive study of these aspects is required.

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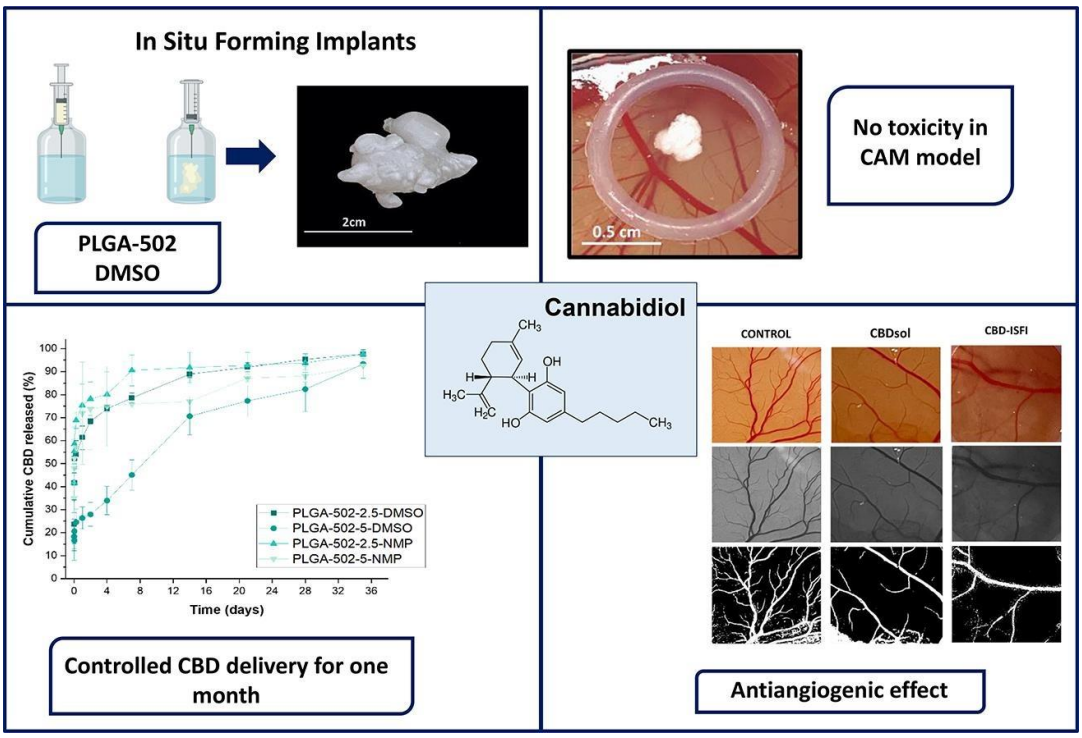
CHAPTER 2

In situ forming PLA and PLGA implants for the parenteral administration of cannabidiol



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ABSTRACT

Cannabidiol (CBD) is the main non-psychotropic cannabinoid. It has attracted a great deal of interest in the treatment of several diseases such as inflammatory disorders and cancer. Despite its promising clinical interest, its administration is very challenging. In situ forming implants could be a simple and cheap strategy to administer CBD while obtaining a prolonged effect with a single administration. This work aims to design, develop, and characterize for the first time ISFIs for the parenteral administration of CBD with potential application in cancer disease. Formulations made of PLGA-502, PLGA-502H, and PLA-202 in NMP or DMSO and PLA-203 in DMSO at a polymer concentration of 0.25 mg/µL and loaded with CBD at a drug: polymer ratio of 2.5:100 and 5:100 (w/w) were developed. The formulations prepared with NMP exhibited a faster drug release. CBD implants elaborated with PLGA-502 and DMSO with the highest CBD: polymer ratio showed the most suitable drug release for one month. This formulation was successfully formed in ovo onto the chorioallantoic chick membrane without exhibiting signs of toxicity and exhibited a superior antiangiogenic activity than CBD in solution administered at the same doses. Consequently, implants made of PLGA-502 and DMSO represent a promising strategy to effectively administer CBD subcutaneously as combination therapy in cancer disease.

Keywords: Angiogenesis, CAM model, Cannabinoids, Drug delivery, In situ-forming implants, Polymeric formulations.

1. INTRODUCTION

In-situ forming implants (ISFIs) have gained a great deal of attention for parenteral controlled drug delivery resulting in an excellent alternative to classical implants [1, 2]. Unlike conventional preformed implants that require surgery or the use of large gauge needles for their administration, ISFIs can be easily injected into patients using conventional needles. According to the mechanism responsible for in-vivo solidification of the implants, these systems are grouped into four categories: i) thermo-sensitive, ii) in-situ cross-linked, iii) in-situ solidifying organogels, and iv) in-situ phase separation formulations [3]. Among all of them, these latter systems represent one of the most studied and exploited for drug delivery. They consist of a polymeric solution composed of a biocompatible and biodegradable polymer such as poly-lactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA) and a water-miscible solvent, being N-methyl-2-pyrrolidone (NMP) the most commonly used solvent [4, 5]. The drug can be suspended or dissolved into this polymeric solution [6]. When ISFI formulations are injected into the patients, the implants are formed by desolvation. Upon contact with the aqueous fluids of the administration site, the water-miscible solvent diffuses in the surrounding area which triggers the precipitation of the polymer as it is not soluble in water. The drug is entrapped into this precipitated polymeric matrix from which is released in a controlled manner mainly due to drug diffusion through the polymeric matrix, drug dissolution, and polymer degradation [4]. It should be noted that the manufacturing of these ISFIs is simple and relatively cheap especially when compared with other drug delivery systems such as microparticles or nanoparticles [5]. In addition, if the patients suffer allergic reactions or other adverse effects that require the removal of the implant, the formed implants can be easily removed by surgery with the use of local anaesthesia [7]. This is not possible when micro or nanoparticles are administered.

ISFIs have been exploited for the administration of a broad range of drugs, both water-soluble and liposoluble compounds such as antiseptic agents (e.g., chlorhexidine), anti-inflammatory drugs (e.g. ibuprofen and dexamethasone) [6, 8, 9], antibiotics (e.g. doxycycline) [10], antiretrovirals (e.g. ritonavir, lamivudine, and efavirenz) [11] and chemotherapeutics (e.g. doxorubicin) [12] among others. Several ISFI-based formulations are currently approved by the FDA and/or EMA for the treatment of various diseases: Eligard®, Sublocade®, Perseris®, Atridox®, Uzedy®, Okedy® (in FDA is approved as Risvan®) and Brixadi® [13-17]. Their composition and specific therapeutic indications are displayed in Table 1.

	Polymer	Solvent	Drug	Administration route	Indication
Eligard®	PLGA	NMP	Leuprorelin	Subcutaneous	Prostate cancer
Sublocade®	PLGA	NMP	Buprenorphine	Subcutaneous	Opioid use disorder
Perseris®	PLGA	NMP	Risperidone	Subcutaneous	Schizophrenia
Atridox®	PLA	NMP	Doxycycline Hyclate	Subgingival	Periodontitis
Uzedy®	m-PEG-b-PLA and PLGA	DMSO	Risperidone	Subcutaneous	Schizophrenia in adults
Brixadi®	Soybean phosphatidylcholine/ glycerol dioleate	NMP	Buprenorphine	Subcutaneous	Opioid dependence
Risvan® (FDA) Okedi® (EMA)	PLGA	DMSO	Risperidone	Intramuscular	Schizophrenia in adults

Table 1: Characteristics of ISFI formulations that are currently approved by FDA and/or EMA.

Cannabinoids have become promising drugs for the treatment of a broad range of medical conditions such as neurological and neurodegenerative diseases, addictions, pain, cancer, and inflammatory disorders, among others [18-20]. Cannabidiol is one of the most studied cannabinoids. It lacks psychotropic effects, and it has been demonstrated to be, in general, well tolerated by patients [21, 22]. There are currently two formulations containing CBD that are approved by the FDA and/or EMA: i) Epidiolex®, an oral solution of pure CBD used for the treatment of childhood epilepsies, and ii) Sativex®, an oromucosal spray of nabiximols (a cannabis extract containing CBD and Delta-9-tetrahydrocannabinol at a ratio 1:1) approved for the management of spasticity in multiple sclerosis patients [23].

Cancer is one of the pathologies where CBD has an important therapeutic potential [24]. It has been demonstrated that this cannabinoid inhibits tumour growth as monotherapy and potentiates the effect and reduces the adverse effects of conventional antineoplastics such as paclitaxel or doxorubicin [25-27]. Inflammatory diseases are another important area of interest for medicinal cannabidiol [28]. Several studies have shown the usefulness of this drug in the treatment of chronic diseases such as psoriasis, osteoarthritis, and rheumatoid arthritis among others [29-31].

Nevertheless, the administration of CBD is very challenging. It is a highly lipophilic drug (Log P 6.3) [32] that shows a low and erratic bioavailability (around 6%) when administered by oral administration route [23, 33]. In this context, the development of subcutaneous ISFIs could be a simple and cheap strategy to administer CBD while obtaining a prolonged effect with a single administration which is desirable in chronic diseases like cancer [34].

The objective of the present research is to design, develop, and characterize a long-lasting ISFI for the parenteral administration of CBD with potential application as combination therapy in cancer disease. Different PLA and PLGA polymers and two solvents, NMP and dimethyl sulfoxide (DMSO), are used, with the aim of achieving a system that is easily injectable and provides a prolonged, preferably sustained, release of CBD of about one month. To the best of our knowledge, this is the first work proposing the use of ISFIs for the administration of cannabinoids.

2. MATERIALS AND METHODS

2.1. Materials

Poly (D, L-lactic-co-glycolic acid) RG 502, RG 502H and RG 503 and Poly (lactic acid) RG 202 and RG 203 were supplied from Evonik® Industries (Essen, Germany); N- methyl-pyrrolidone (NMP) and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Madrid, Spain). Cannabidiol was obtained from THC Pharm (Frankfurt, Germany). Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), Potassium dihydrogen phosphate (KH2PO4), and Polyethylene glycol sorbitan monooleate (Tween80) were supplied from Panreac (Barcelona, Spain). Methanol and acetonitrile were obtained from Scharlab S.L. (Madrid, Spain); Demineralised Milli-Q® water (Millipore, Madrid, Spain) was used.

2.2. Preliminary studies

Before implant formation, for selecting the most appropriate PLA and PLGA polymers and the most suitable polymer concentration, injectability, and viscosity studies were carried out using PLA 202 and 203 and PLGA 502, 502-H and 503. The characteristics of these polymers are displayed in Table 2.

Polymer	Composition	End Group	Molecular Weight Range (KDa)	Inherent viscosity (dl/g)	Degradation timeframe	Tm (°C)	Tg (°C)
PLA 202	Poly(D,L-lactide)	Ester	10-18	0.16 - 0.24	< 9 months	Amorphous	38-42
PLA 203	Poly(D,L-lactide)	Ester	18-28	0.25 - 0.35	< 12 months	Amorphous	46-50
PLGA-502	Poly(D,L-lactide-co-glycolide) 50:50	Ester	7-17	0.16-0.24	< 3 months	Amorphous	42-46
PLGA 502-H	Poly(D,L-lactide-co-glycolide) 50:50	Free carboxylic acid	38-54	0.16-0.24	< 3 months	Amorphous	42-46
PLGA 503	Poly(D,L-lactide-co-glycolide) 50:50	Ester	24-38	0.32-0.44	< 3 months	Amorphous	44-48

Table 2: Characteristics of PLGA and PLA polymers used. Data obtained from the supplier (Evonik® Industries)

2.2.1. Injectability studies: panel test

The injectability of the solutions prepared with the different polymers (Table 2) and water-miscible solvents (NMP and DMSO) at polymer concentrations of 0.66, 0.33 and 0.25 mg/µL was evaluated using the protocol described in [35]. Briefly, in a blind study, 7 volunteers received 1 mL syringes (Injekt-F, B-Braun, Germany) coupled with 23G (0,6x 25mm, Icoplus3, Peroxfarma, Spain) and 25G (0,5x 16 mm, Icoplus3, Peroxfarma, Spain) needles filled with 1 mL of the different polymeric solutions. Then, the volunteers were asked to evaluate the injectability of each solution and to provide a score (1-4) based on ease of injectability and flow through 23G and 25G needles according to the description in Table 3.

	Description	
Score	**Injection**	**Flow**
1	Very difficult	No flow, dropwise
2	Difficult	Initially dropwise, then continuous
3	Moderate	Continuous
4	Easy	Continuous

Table 3: Scores of the injectability study.

2.2.2. Viscosity studies.

The viscosity of the polymeric solutions elaborated with each polymer (PLA 202, PLA 203, PLGA 502, PLGA 502H, and PLGA 503), and NMP or DMSO was determined at 25ºC using a viscometer Brookfield Ametek (Ametek Brookfield, Massachusetts, USA). Different polymer concentrations (0.66; 0.33 and 0.25 mg/µL) were tested.

2.3. Preparation of ISFIs

Unloaded and CBD-loaded ISFIs (CBD-ISFIs) were prepared by direct medium injection technique using PLA-202, PLA-203, PLGA-502, PLGA-502H, or PLGA-503 as polymer and NMP or DMSO as solvent [6]. Briefly, each polymer was dissolved in NMP or DMSO at a polymer concentration of 0.25 mg/µL by vortexing. In the case of CBD-ISFIs, the drug was then added at a drug: polymer ratio of 2.5:100 (w/w) (CBD-ISFI-2.5) and 5:100 (w/w) (CBD-ISFI-5) to the polymeric solution, and the mixtures were vortexed. To form implants, 100 µL

of this polymeric solution (without or with CBD) was injected in 2.5 mL of phosphate buffer solution (PBS) pH 7.4 using a standard syringe coupled to a 25G needle gauge.

2.4. Characterization of ISFIs.

2.4.1. Implant morphology

The morphology of unloaded and CBD-loaded recently prepared formulations was evaluated by optical microscopy. 15 minutes after implant formation following the protocol described in section 2.3, the excess water was removed, and the implants were observed using a Stereo Blue SB.1302-P microscope (Euromex, Holland).

Scanning electron microscopy (SEM) (JEOL 6400 JSM, Tokyo Japan) analyses were also carried out to evaluate the surface and inner structure of the implants. Briefly, 15 minutes after implant formation as described in section 2.3, the excess water was removed, and the implants were placed onto aluminium stubs and dried for 48 hours. Then, they were coated with gold for 180 seconds and examined by SEM microscope.

2.4.2. Drug release studies

Initially, the solubility of CBD in PBS pH 7.4 containing Tween 80 as a solubilizing agent was analysed. 50 mg of CBD was added to glass closed tubes containing 5 mL of PBS, PBS-Tween 80 0.05%, PBS-Tween 80 0.1%, PBS-Tween 80 0.5% or PBS-Tween 80 1%. These CBD solutions were placed into an orbital shaking water bath (ST 30 Nuve Sanayi, Turkey) and maintained under stirring (100 rpm) at 37 ±0.5ºC for 24 hours. Then, the samples were filtered using a cellulose 0,45µm pore size filter and dissolved CBD was quantified by HPLC (1200 Infinity Series Agilent Technologies) using the protocol validated and described in [36]. Briefly, the mobile phase was composed of a mixture of methanol, acetonitrile, and water (52%,30%, and 18%) and the column was a Teknokroma Mediterranea SEA C18 5µm 15 x 0,46. A flow of 1.8 mL/min and a wavelength of 228 nm were used.

The release of CBD from ISFIs was evaluated in PBS pH 7.4 containing Tween 80 at the concentration necessary to maintain sink conditions. Implants were formed by injecting 200 µL of the CBD polymeric solutions in closed glass tubes containing 5 mL of release medium. Then, the samples were immediately placed in an orbital shaking water bath (ST 30 Nuve Sanayi, Ankara, Turkey) at 37 ± 0.5°C and 100 rpm. At predetermined time points (15 min, 1 h, 2 h, 6h, 1, 2, 4, 7, 14, 21,28, and 35 days) the supernatant was removed, and fresh

medium was added. The samples were filtered using a cellulose 0,45µm pore size filter and analysed by HPLC as aforementioned.

2.5. In ovo studies

2.5.1. HET-CAM assay

The toxicity of the selected CBD-ISFI formulation was evaluated using a modified HET-CAM assay (Hen's Egg Test- Chorioallantoic Membrane) [37]. Briefly, fertilized chicken eggs (Granja Santa Isabel, Cordoba, Spain) were placed in an automated incubator at 37°C and 47% humidity in a rotating mode. On embryo development day (EDD) 4, a little hole in the eggshell was performed and sealed with tape to prevent desiccation. Then, the eggs were subsequently placed in the incubator at the stationary mode with the same levels of temperature and humidity. On EDD 10, the window was enlarged to expose the chorioallantoic membrane. Then, it was hydrated with 1 mL of PBS (pH 7.4). A silicone O-ring (1 cm) was placed on the membrane and the eggs were topically treated with 20 µL of NMP, DMSO, saline solution (negative control of toxicity), NaOH 0.5M (positive control of toxicity), and the CBD-ISFI selected formulation. Then, the membrane status of each egg was monitored by evaluating the appearance of haemorrhage, coagulation, and lysis for 5 minutes. Irritation scores of each treatment were calculated as follows as toxicity indicators:

$$Irritation\ score\ (IS): \left(\left(301 - \frac{Ht}{300}\right)x5\right) + \left(\left(301 - \frac{Lt}{300}\right)x7\right) + \left(\left(301 - \frac{Ct}{300}\right)x9\right) \quad \text{(Eq.5)}$$

where Ht, Lt, and Ct are the times of the appearance of haemorrhage, lysis, and coagulation respectively. At least 5 eggs per treatment were evaluated.

2.5.2. Angiogenesis study

Angiogenesis studies were also carried out using a CAM model assay. Fertilized chicken eggs were placed in an egg incubator with rotation at 37°C and 47% humidity. On EDD4, a little hole in the eggshell was made and sealed with tape. Then, the eggs were placed again in the incubator without rotation. On EDD 10, the chorioallantoic membrane was exposed. A silicone 1 cm O-ring was placed on this membrane to delimit the treatment area. The eggs were treated for 48 hours with CBD in solution (6.3 µg of CBD were daily administered) or the equivalent amount of selected CBD-ISFIs (single administration of 20µL of CBD-PLGA-502-5-DMSO polymeric solution to get a CBD release of around 12.6 µg in 48 hours). Eggs treated with unloaded implants (20µL of PLGA-502-5-DMSO polymeric solution), and PBS pH 7.4 (negative control of angiogenesis) were also evaluated. After 48 hours, egg

membranes were photographed and the treated area of each egg was analysed by counting the total number of blood vessels [38] and by calculating the vascular density (%). For vascular density analysis, the images of the treated area were transferred to the ImageJ-Fiji image processing software and converted into an 8-bit image type. Then, the threshold was adjusted, and the percentage of vascular density was automatically obtained by the program according to the ratio of black and white colour differences [39]. At least 5 eggs per treatment were analysed.

2.6. Statistical analysis

All the data are expressed as the mean ± standard deviation (SD) of at least three different experiments. For descriptive results (scores of the injectability study) a Mann-Whitney U test was used and performed on IBM SPSS Statistics 28 software (IBM, Chicago, Illinois). Student's t-tests were used to compare the two groups while ANOVA test followed by Duncan's multiple range tests were used to evaluate the significance of the differences detected among multiple groups. These tests were performed using Statgraphics 19 Centurion (Statgraphics Technologies, Inc., The Plains, Virginia). The graphs were prepared using Origin 2019 software (Origin lab, Massachusetts, USA).

3. RESULTS AND DISCUSSION

3.1. Preliminary studies: injectability and viscosity of the polymeric solutions

The viscosity of the polymeric solutions can condition their injectability, and consequently the formation of the implants. Therefore, to select the most appropriate polymer and the most suitable polymer concentration to prepare CBD-loaded ISFIs, the injectability and viscosity of the different polymeric solutions in NMP and DMSO were studied. Table 4 describes the median scores obtained for each solution in the injectability panel test, in which the volunteers were asked to evaluate the injectability of 1 mL of all the polymeric solutions in terms of ease of injection and flow through 23G and 25G needles which are commonly used for subcutaneous administration. A score of 3 or 4 through both needles must be provided by all participants to consider that the solution showed adequate injectability. Table 5 displays the results of the viscosity study.

		INJECTABILITY					
		NMP			DMSO		
	Polymer conc. (mg/µL)	23G needle[a] (n=7)	25G needle[a] (n=7)	Adequate injectability[b]	23G needle[a] (n=7)	25G needle[a] (n=7)	Adequate injectability[b]
PLA-202	0.66	3-4 (3)	2-3 (2)	N	2 (2)	1-2 (1)	N
	0.33	3-4 (3)	2-3 (3)	N	3-4 (3)	2-3 (2)	N
	0.25	3-4 (4)	3-4 (4)	Y	3-4 (4)	3-4 (4)	Y
PLA-203	0.66	1-3 (1)	1-2 (1)	N	1-2 (1)	1-2 (1)	N
	0.33	2-3 (2)	2-3 (2)	N	3-4 (3)	2-3 (2)	N
	0.25	2-4 (3)	2-4 (2)	N	3-4 (4)	3-4 (3)	Y
PLGA-502	0.66	1-2 (2)	1-2 (2)	N	1-2 (2)	1-2 (1)	N
	0.33	3-4 (3)	2-3 (2)	N	3-4 (3)	1-2 (1)	N
	0.25	3-4 (3)	3-4 (3)	Y	3-4 (4)	3-4 (3)	Y
PLGA-502 H	0.66	1-3 (2)	1-2 (1)	N	2-3 (2)	1-2 (2)	N
	0.33	3-4 (4)	2-3 (2)	N	2-4 (3)	2-4 (3)	N
	0.25	4 (4)	3-4 (4)	Y	3-4 (4)	3-4 (4)	Y
PLGA-503	0.66	1-2 (1)	1-2 (1)	N	1-2 (1)	1 (1)	N
	0.33	2-3 (2)	1-2 (2)	N	2-3 (2)	1-2 (1)	N
	0.25	2-3 (3)	2-3 (2)	N	2-3 (2)	1-2 (1)	N

Table 4: Results obtained in the injectability panel test.

[a] Score range (median) of every solution

[b] Adequate injectability: Yes (y) or No (N)

	Concentration (mg/µL)	Viscosity (cP)	
		NMP	DMSO
PLA-202	0.66	297.78 ± 2.98	304.58 ± 5.00
	0.33	70.62 ± 0.99	126.86 ± 0.59
	0.25	35.97 ± 0.35	53.15 ± 5.12
PLA-203	0.66	948.00 ± 26.85	838.95 ± 21.09
	0.33	249.60 ± 3.70	156.13 ± 1.13
	0.25	205.57 ± 1.16	98.06 ± 1.75
PLGA-502	0.66	589.88± 6.62	485.58± 14.23
	0.33	92.57 ± 0.91	158.93 ± 10.71
	0.25	40.38 ± 3.38	94.69 ± 6.48
PLGA-502 H	0.66	594.33 ± 5.88	583.23 ± 6.93
	0.33	119.40 ± 2.25	113. 65 ± 2.59
	0.25	37.98 ± 1.25	98.04 ± 1.49
PLGA-503	0.66	8249.60 ± 3.70	7000.50 ± 8.66
	0.33	5473.63 ± 5.14	1119.75 ± 26.32
	0.25	205.57 ± 1.16	98.06 ± 1.75

Table 5: Viscosity obtained for the different polymers dissolved in DMSO or NMP.

When NMP was used as a solvent, the polymeric solutions made of PLA-202, PLGA-502, and PLGA-502H showed adequate injectability properties in the panel test, with moderate-easy injection, and continuous flow (scores 3-4) through both 23G and 25G needles at a polymer concentration of 0.25 mg/µL. Statistically significant differences between the scores obtained for both needles were not detected (p value>0.05). It should be pointed out that these polymeric solutions also showed acceptable injectability at a polymer concentration of 0.33 mg/µL but only when 23G needles were used. These results are in agreement with those found by Patki and coworkers who indicated that polymeric solutions of PLGA in NMP at a concentration higher than 0.25 mg/µL do not show good injectability through needles of a calibre lower than 23G [35].

These results of the injectability panel test follow the viscosity data obtained per each polymeric solution in NMP. While both PLA-203 and PLGA-503 solutions at a polymer concentration of 0.25 mg/µL that had a poor-moderated injectability showed viscosity values of 190.56± 1.22 cP and 205.57±1.16 cP respectively, PLA-202, PLGA-502, and PLGA-502H showed a significantly lower viscosity in the range of 35-41 cP.

When the solutions were prepared at a polymer concentration of 0.25 mg/µL using DMSO as a solvent, except PLGA-503, all the polymers showed acceptable injectability as scores 3-4 were provided for all the participants using both 23G and 25G needles. In the case of PLA-202 PLGA-502 and PLGA-502H solutions, statistically significant differences in the injectability scores between both needles were not detected (p value>0.05). However, in the PLA-203 solution, a significantly higher score was obtained in the 23G needle (p value=0.01). These results are also correlated with the viscosity values obtained for each solution. At a polymer concentration of 0.25 mg/µL except PLGA-503, all the polymeric solutions showed a viscosity lower than 100 Cp. It should be mentioned that PLA-203 showed a significantly lower viscosity in DMSO than in NMP (p value<0.01) which explains why it had an acceptable injectability dissolved in DMSO but not when dissolved in NMP (Tables 4 and 5).

Due to their good injectability properties PLA-202, PLGA-502, and PLGA-502H dissolved in NMP or DMSO and PLA-203 dissolved in DMSO at a polymer concentration of 0.25 mg/µL were selected for the elaboration of CBD-loaded ISFIs.

3.2. Development and characterization of CBD-loaded ISFIs

CBD-loaded PLA and PLGA-based implants with a drug: polymer ratio of 2.5 and 5 (w/w) were successfully formed using both NMP and DMSO as solvents. CBD was soluble in all these selected polymeric solutions (Cs >25 mg/mL). Upon contact with the simulated physiological medium (PBS pH 7.4), a quick precipitation of the polymer occurred triggering the formation of soft implants. CBD was entrapped in the polymeric matrix. No differences in the formation of both PLGA and PLA implants when using NMP or DMSO as solvent were appreciated.

3.2.1. Morphology of the implants

Figures 1 and 2 display the macroscopic images of both unloaded and CBD-loaded ISFIs prepared using NMP and DMSO respectively. All formed implants showed an irregular shape, similar to those obtained by other authors using direct medium injection technique for implant preparation [40]. Notably, no differences were observed between implants prepared with DMSO and with NMP, nor between implants with and without CBD.

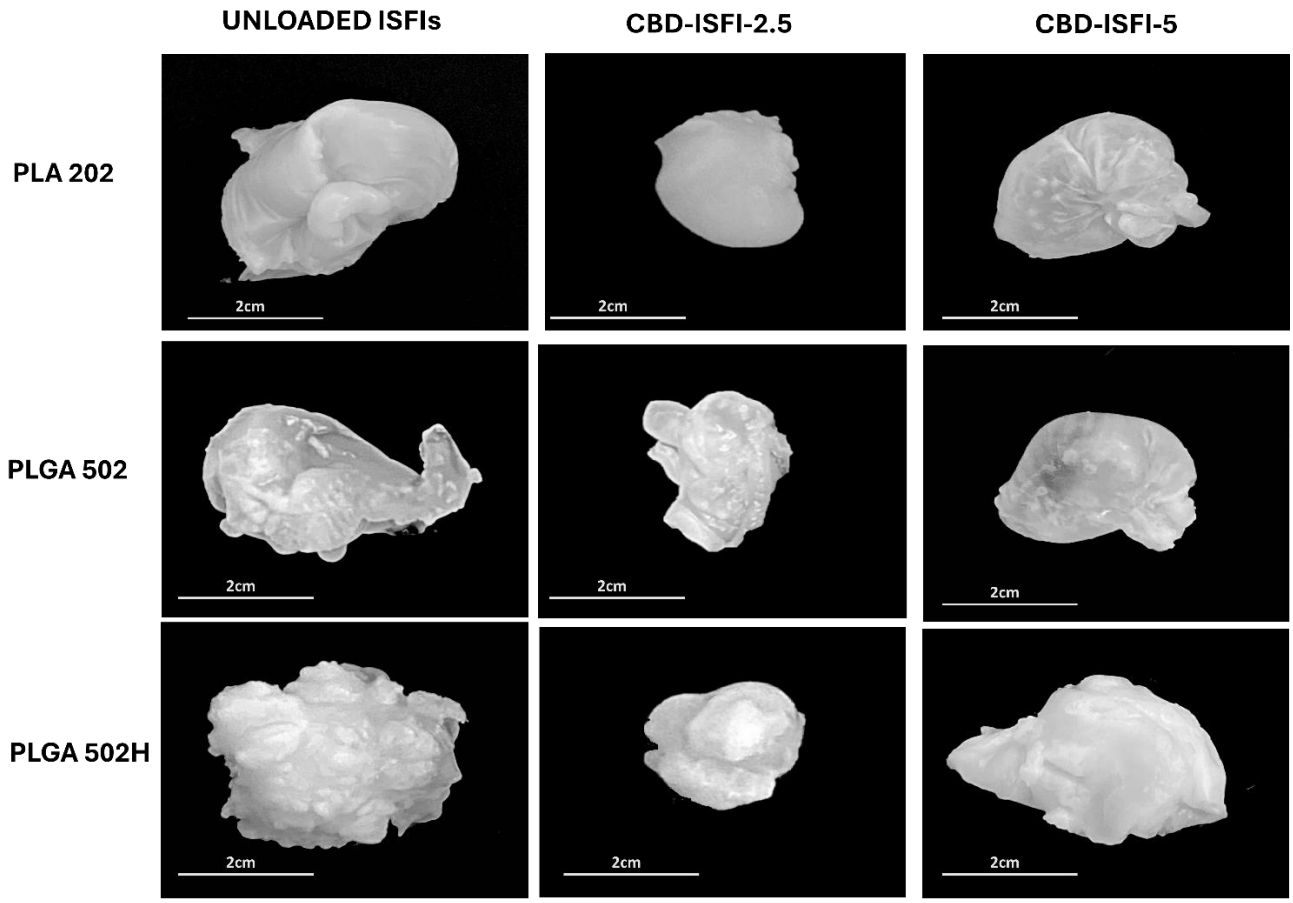

Figure 1: Optical images of recently prepared ISFIs without and with CBD using NMP. Images were captured 15 minutes after implant formation.

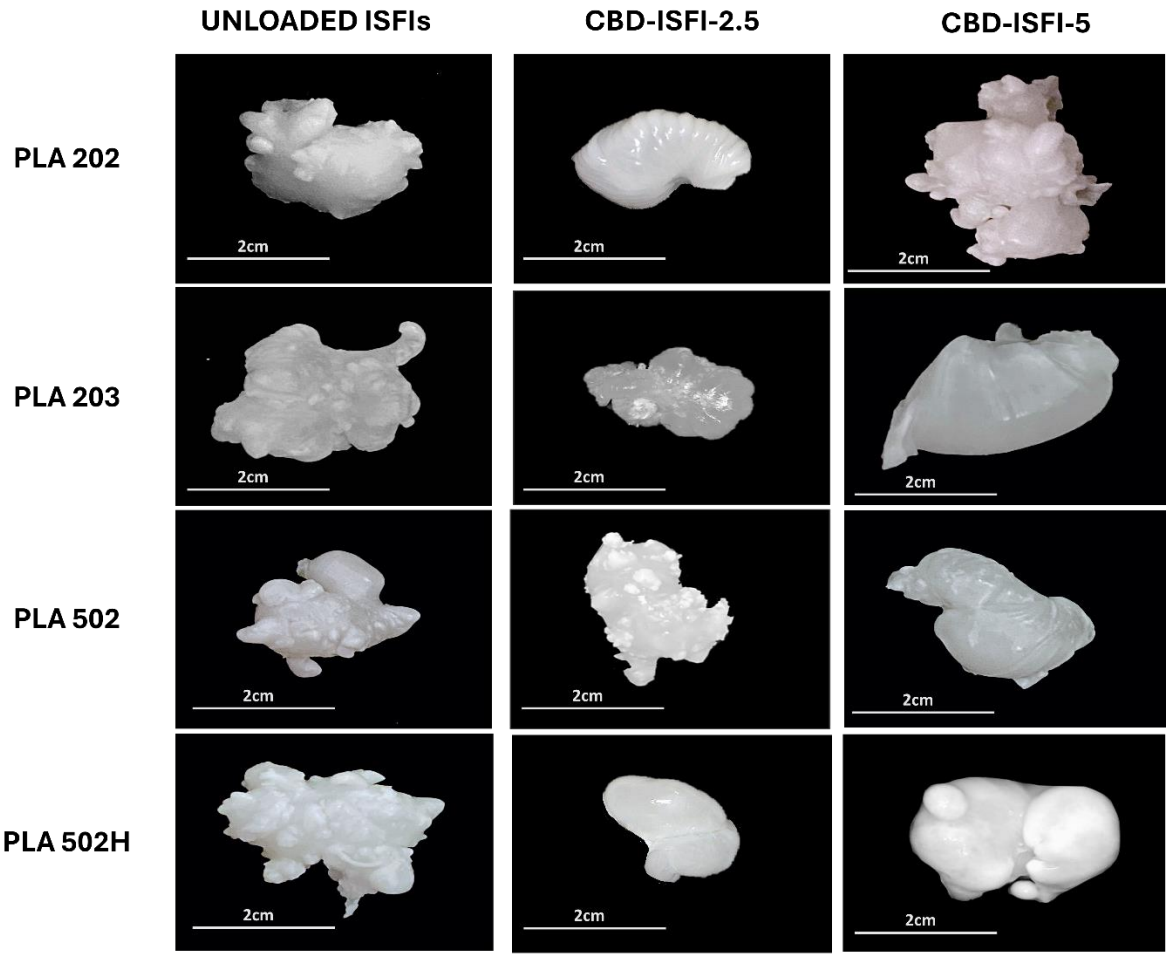

Figure 2: Optical images of recently prepared IFIs without and with CBD using DMSO. Images were captured 15 minutes after implant formation.

Nevertheless, differences in the surface of ISFIs prepared with NMP and DMSO were found when examined by scanning electron microscopy. While unloaded, and CBD-loaded in situ forming implants prepared with NMP (Figure 3) showed a slightly rough surface, the surface of the implants elaborated with DMSO was smooth (Figure 4). Moreover, the presence of pores in the surface of both NMP and DMSO ISFIs is observed, being more evident in the latter ones. This porous structure is attributed to NMP and DMSO as it has been demonstrated that hydrophilic solvents such as NMP and DMSO tend to form porous implants [41]. Zhan and co-workers demonstrated that the solvent diffusion rate from PLA and PLGA depots of NMP is slower than DMSO, with an average solvent exchange of around 47% and 70% respectively during the first day after implant formation [42]. This faster removal of DMSO can explain the presence of some pores in the surface of the implants

prepared with this solvent. When the inner structure of the CBD-loaded implants was examined (Figure 5) the presence of this characteristic microstructure was also appreciated. No differences in the morphology and inner structure of the unloaded and CBD-loaded implants were appreciated.

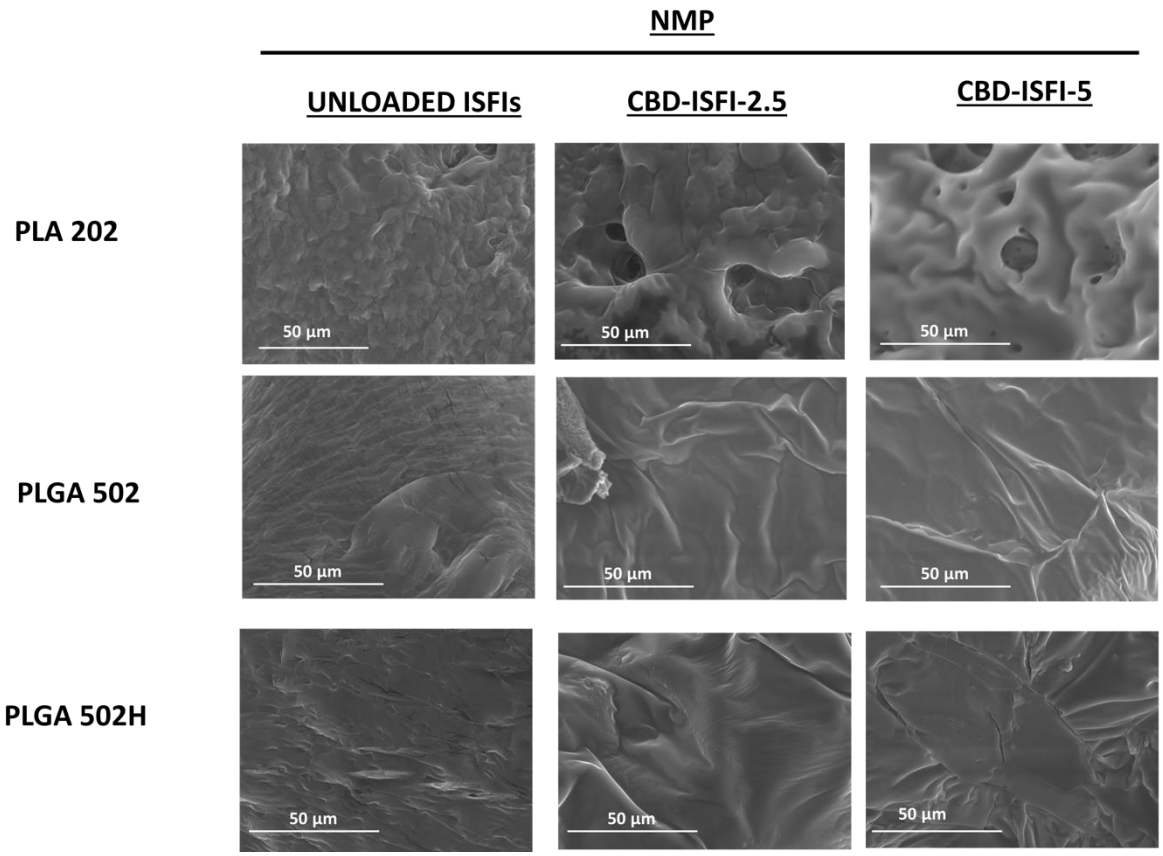

Figure 3: Images of the surface of the ISFI formulations unloaded and loaded with CBD prepared with NMP obtained by scanning electron microscopy. The implants were picked up 15 minutes after their formation and dried for 48 hours before their examination.

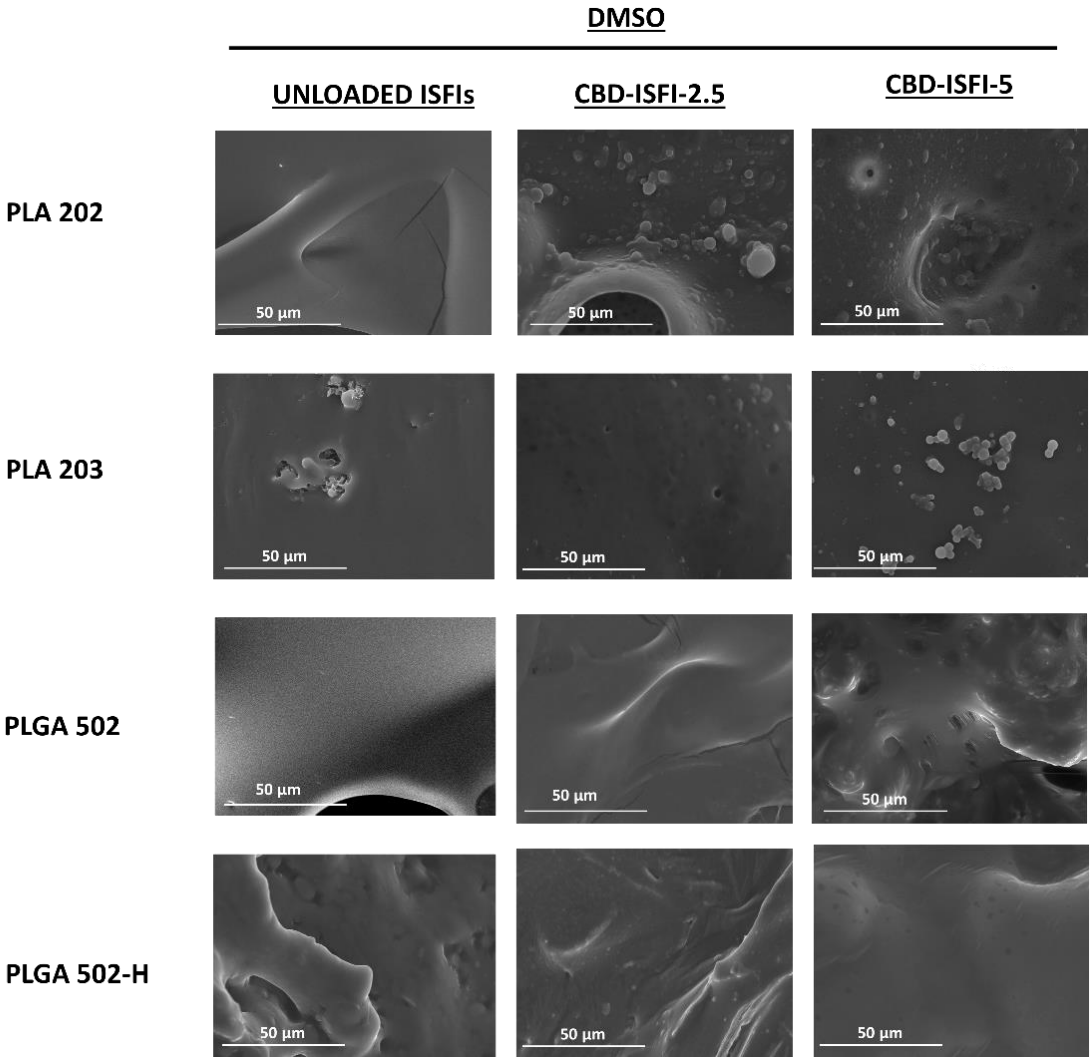

Figure 4: Images of the surface of the ISFI formulations unloaded and loaded with CBD prepared with DMSO obtained by scanning electron microscopy. The implants were picked up 15 minutes after their formation and dried for 48 hours before their examination by SEM.

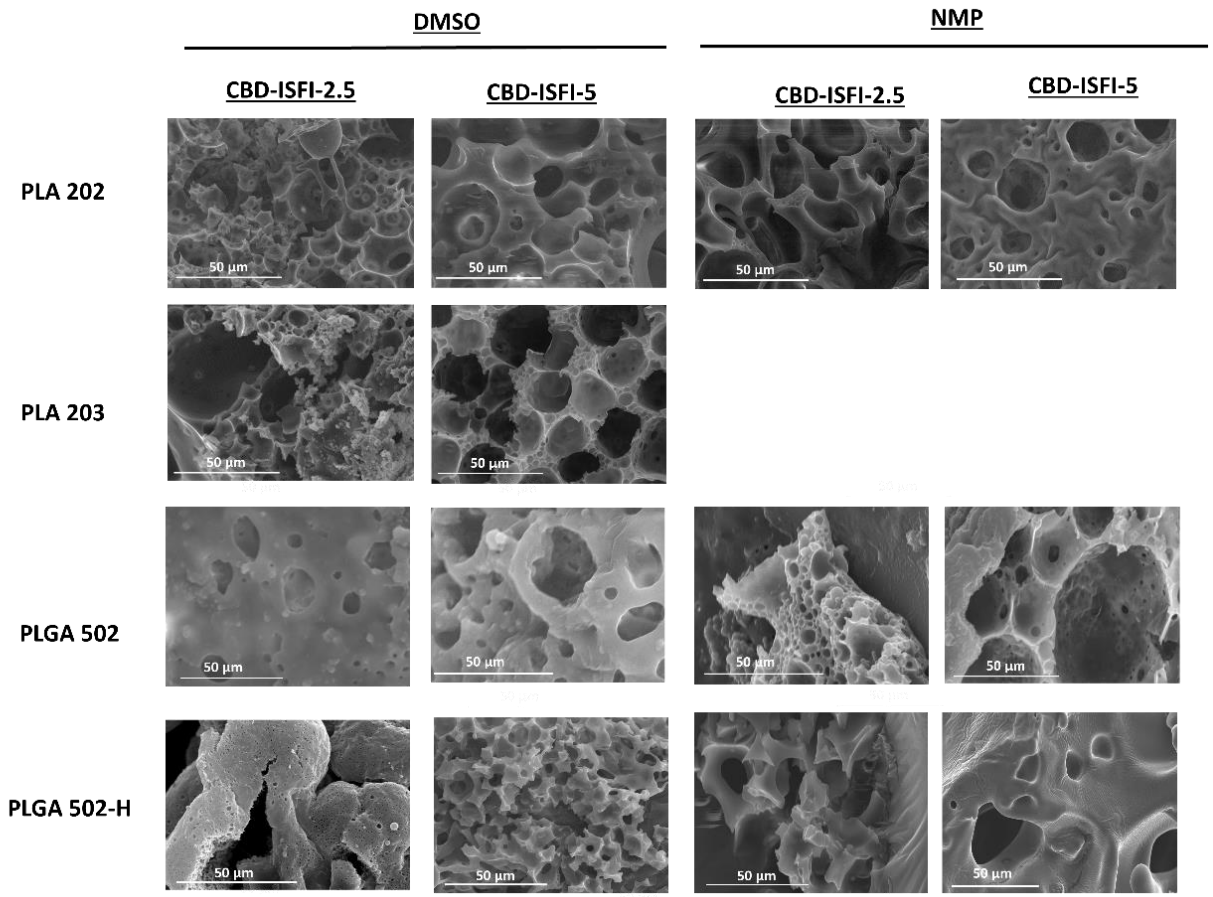

Figure 5: Images of the cross-section of the CBD-loaded ISFIs prepared with DMSO or NMP obtained by scanning electron microscopy. The implants were picked up 15 minutes after their formation and dried for 48 hours before their examination by SEM.

3.2.2. Drug release

To the adequate design of the release studies of CBD from the ISFIs the solubility of this cannabinoid in PBS pH 7.4 with and without the addition of Tween 80 at 37ºC was evaluated. CBD was practically insoluble in PBS ($<6.6 \cdot 10^{-4}$ mg/mL) but the addition of Tween 80 significantly improved its solubility. A linear correlation was established between the concentration of Tween 80 and the solubility of CBD (Supplementary material Figure S1). In PBS-Tween 80 1% the solubility of CBD was found to be 7.9 mg/mL. This was the selected medium to maintain sink conditions during the release study.

The CBD release profile from the different PLGA and PLA ISFIs prepared with NMP or DMSO are displayed in Figure 6.

Amongst all the developed formulations, the fastest release was appreciated in the implants prepared with PLGA-502 and NMP, especially in CBD-PLGA-NMP-2.5-ISFIs that exhibited

more than 41% of the CBD released in 15 minutes, around 75% in 1 day and around 91% in 14 days. CBD-PLGA-502-5-NMP showed a slightly slower release with around 35% of CBD released in 15 minutes, 71% in 1 day, 77% within 14 days, and approximately 85% in 21 days. A significantly slower (p value<0.05) drug release was detected with both CBD-PLGA-502-2.5-DMSO and CBD-PLGA-502-5-DMSO. These formulations exhibited around 24% and 18% of CBD released in 15 minutes, 61% and 26% in 1 day, and 90% and 70% in 14 days respectively. In CBD-PLGA-502-5-DMSO approximately 85% of the CBD was released in 28 days (Figure 6A).

Compared to PLGA-502-ISFIs, a slightly faster release but with a slightly lower burst effect was appreciated in PLGA-502-H formulations (Figure 6B). CBD-PLGA-502-H-2.5-NMP and CBD-PLGA-502-H-5-NMP showed a similar drug release during the first 2 days, with around 15% of the CBD released in 15 minutes, and around 68% in 2 days. However, from this moment a faster release was appreciated in the implants elaborated with a higher CBD content. While CBD-PLGA-502-H-2.5-NMP showed around 85% of the CBD release in 21 days, in CBD-PLGA-502-H-5-NMP this percentage was released in approximately 7 days. The implants prepared with DMSO exhibited a slightly slower drug release, especially in CBD-PLGA-502-H-5-DMSO formulation. CBD-PLGA-502-H-2.5-DMSO showed a CBD release of around 6% in 15 minutes, 40% in 1 day, and around 90% in 14 days. In CBD-PLGA-502-H-5-DMSO ISFIs CBD release percentages of around 3% in 15 minutes, 20% in 1 day, and around 85% in 14 days were detected (Figure 6B).

CBD-ISFIs prepared with PLA showed a slower release than PLGA formulations. In fact, 85% of the drug released was not achieved with any of the PLA implants developed in this work. This slower release rate from PLA formulations could be attributed, at least in part, to the hydrophobicity of the polymer as the presence of glycolic acid reduce the hydrophobicity of PLGA. In fact, D,L-PLA polymers show a degradation time lower than 9-12 months while the degradation time of 50:50 PLGA polymers is lower than 3 months (Table 2) [43]. The CBD-loaded implants made of PLA-202 and DMSO showed the slowest and most controllable drug release, especially the formulation prepared with the highest CBD concentration. CBD-PLA-202-2.5-DMSO exhibited around 9% of the drug released in 15 min, around 35% in 1 day, and around 45% in 14 days. At these times, CBD-PLA-202-5-DMSO showed CBD release percentages of 9%, 24%, and 36% respectively. At the end of the assay (35 days), both ISFIs showed a CBD release of just around 55% (Figure 6C).

PLA-202 ISFIs also exhibited a faster drug release when elaborated using NMP. In PLA-202-2.5-NMP formulation around 25% of CBD was released in 15 minutes, around 45% in 1 day, and around 65% in 14 days. PLA-202-5-DMSO showed a slightly faster release with around 25% released in 15 minutes, around 65% in 1 day, and around 70% in 14 days (Figure 6C).

It has been reported that the higher the molecular weight of the polymer, the slower drug release [39]. Nevertheless, CBD-PLA-203 ISFIs showed a faster drug release than CBD-PLA-202-DMSO formulations. In CBD-PLA-203-2.5-DMSO formulation 8% of the CBD was released in 15 min, around 35% in 1 day, and around 60% in 14 days. CBD-PLA-202-2.5-DMSO exhibited a faster burst effect with 21% of the CBD released in 15 min and around 50% in 1 day. Around 66% of the drug was released on day 14 (Figure 6D).

CBD is a highly lipid-soluble molecule with a low molecular weight (314.47g/mol). During implant formation, before being completely entrapped into the polymeric matrix, this cannabinoid could be transferred into the aqueous medium with solvent and trigger a fast drug release. As aforementioned, NMP shows a slower solvent escape rate than DMSO [42]. In this context, a faster drug release due to a higher burst effect could be expected from the implants developed with DMSO. However, CBD-loaded PLGA and PLA-ISFIs exhibited a faster drug release when developed with NMP (more than 20% of this cannabinoid was released in 1 hour in all formulations) than when prepared using DMSO. Like in our study, PLGA-ISFIs loaded with leuprolide acetate also showed a faster drug release when prepared using NMP instead of DMSO [44]. The higher drug release from NMP formulations could be attribute to a higher solubility of the drug in NMP (logP=-0.38) than in DMSO (logP=-1.35) in such a way that the partition of CBD (logP =6.1) between PLGA and each solvent is more favourable for NMP. It seems that CBD tends to be more trapped in the polymeric matrix of the implants made with DMSO, while in implants made with NMP it tends to diffuse together with the solvent, locating on the surface of the implant.

When comparing the implants developed with different amounts of CBD, except in CBD-PLA-203-DMSO-ISFIs, the higher the CBD content the slower drug release. This has been also appreciated in PLGA microparticles loaded with this cannabinoid [25, 26]. CBD is a very lipophilic compound that could hinder the diffusion of the release medium into the implant, slowing down the release of the drug.

CBD-PLGA-502-5-DMSO ISFIs had the most suitable CBD release profile and were selected for ovo experiments. This work aims to develop a formulation with potential cancer

application that allows a controlled release of CBD for one month. PLGA-502-2.5-DMSO, PLGA-502-NMP, and PLGA-502H-NMP ISFIs exhibited a very fast CBD release as more than 70% of this drug was released within 4 days and these formulations were discarded. PLGA-502-H-DMSO formulation exhibited a slower drug release compared to all these, but also relatively fast as around 85% of CBD was released in 14 days and for this reason, it was not selected either. On the contrary, ISFIs prepared with PLA-202 and NMP or DMSO exhibited a very slow drug release and were also discarded. Finally, it should be noted that CBD-PLA-203-5-DMSO also showed a controlled drug release for one month and could be also an interesting formulation. However, this formulation exhibited a significantly higher initial drug release than CBD-PLGA-502-5-DMSO (around 25% and 50% in 1 day) being also discarded.

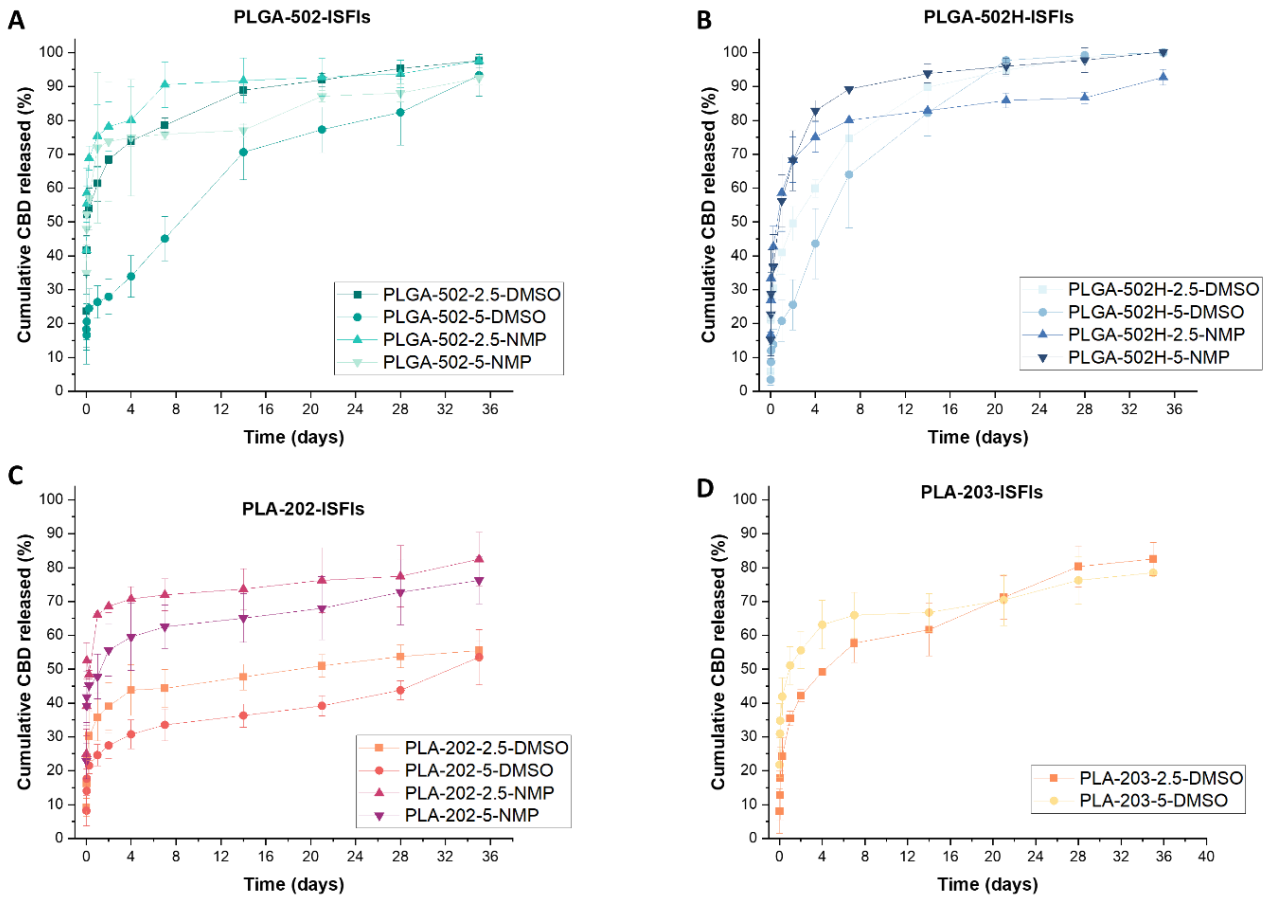

Figure 6: Drug release profile of CBD-loaded PLGA-502-ISFIs (A), PLGA-502H-ISFIs (B), PLA-202-ISFIs (C), and PLA-203-ISFIs (D).

3.3. In Ovo studies

3.3.1 Toxicity studies

The toxicity of the CBD-PLGA-502-5-DMSO-ISFI formulation has been evaluated in ovo using HET-CAM assay which is one of the most useful models to perform toxicity studies [45, 46].

Interestingly, while no damage to the blood vessels on the chorioallantoic membrane surface after 5 minutes of contact with DMSO was appreciated (IS=0), the membrane of NMP-treated eggs exhibited signs of toxicity, as the appearance of haemorrhage, lysis, and coagulation of the blood vessels was detected (Figure 7). Nevertheless, its toxicity was lower than the appreciated with NaOH 0.5N, which was used as a positive control. The IS of NMP (6.7±0,76) was significantly lower (p value=0,0012) than the detected with NaOH solution (Table 6).

Treatment	Irritation score (IS)	Meaning
NaCl [a]	0	No irritant
NaOH 0.5M [b]	19.11±1.83	Strongly irritant
DMSO	0	No irritant
NMP	6.74 ± 0.77	Irritant
CBD-PLGA-ISFI-5	0	No irritant

[a] Negative control of toxicity

[b] Positive control of toxicity

Table 6: Irritation scores obtained in HET-CAM assay and used as toxicity indicators.

After verifying the absence of toxicity of the DMSO in the CAM model, the toxicity of the PLGA-502-5-DMSO was also evaluated. As displayed in Figure 7, these implants were successfully formed onto the CAM without showing signs of damage to the blood vessels of the membrane in terms of haemorrhage, lysis, and coagulation after the contact. All these results indicate that this formulation could be adequate for subcutaneous administration of CBD.

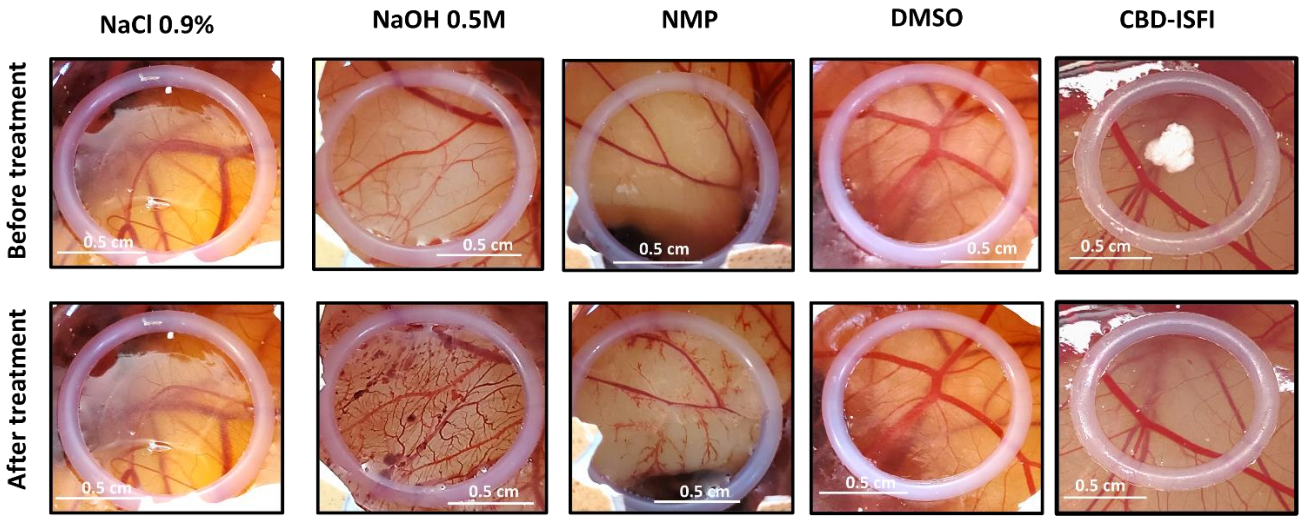

Figure 7: Images obtained of the HET-CAM toxicity assay before and 5 minutes after the addition of each treatment (A) and of a PLGA-502-5-DMSO implant successfully formed on the CAM membrane (topical administration).

3.3.2. Angiogenesis studies

It has been demonstrated that abnormal angiogenesis, the growth of new blood vessels from preexisting ones, plays an important role in the development and pathogenesis of many diseases including cancer and inflammatory disorders such as psoriasis and rheumatoid arthritis [47-49]. In this context, the use of agents with an antiangiogenic effect could be interesting. It has been proven that CBD has an antiangiogenic effect [50]. In this manuscript, the potential antiangiogenic effect of CBD in solution and CBD-PLGA-502-ISFI-5 has been evaluated in the CAM model, one of the established models to carry out angiogenesis studies [51].

Figure 8A displays original and processed images of the chorioallantoic membrane of eggs after their treatment. Eggs treated with both CBD in solution and CBD-PLGA-502-5-DMSO at a daily CBD concentration of 100µM exhibited a lower quantity of blood vessels and consequently a lower vascular density compared with the control. Statistically significant differences (p value< 0.05) between the control and each CBD treatment were detected (Figure 8B and 8C). It should be noted that CBD-loaded in situ forming implants exhibited a slightly higher antiangiogenic effect than CBD in solution administered at the same doses with reduction percentages in the number of blood vessels of 74.28 ± 7.23 % and 60.49 ± 15.05 % respectively. However, statistically significant differences in the percentages of inhibition between CBD treatments (p value>0,05) were not detected. Unloaded ISFIs did not exert any antiangiogenic effect. Statistically significant differences in the number of blood

vessels or the vascular density between unloaded-PLGA-502-DMSO-ISFI and the control were not detected (Figure 8B and 8C).

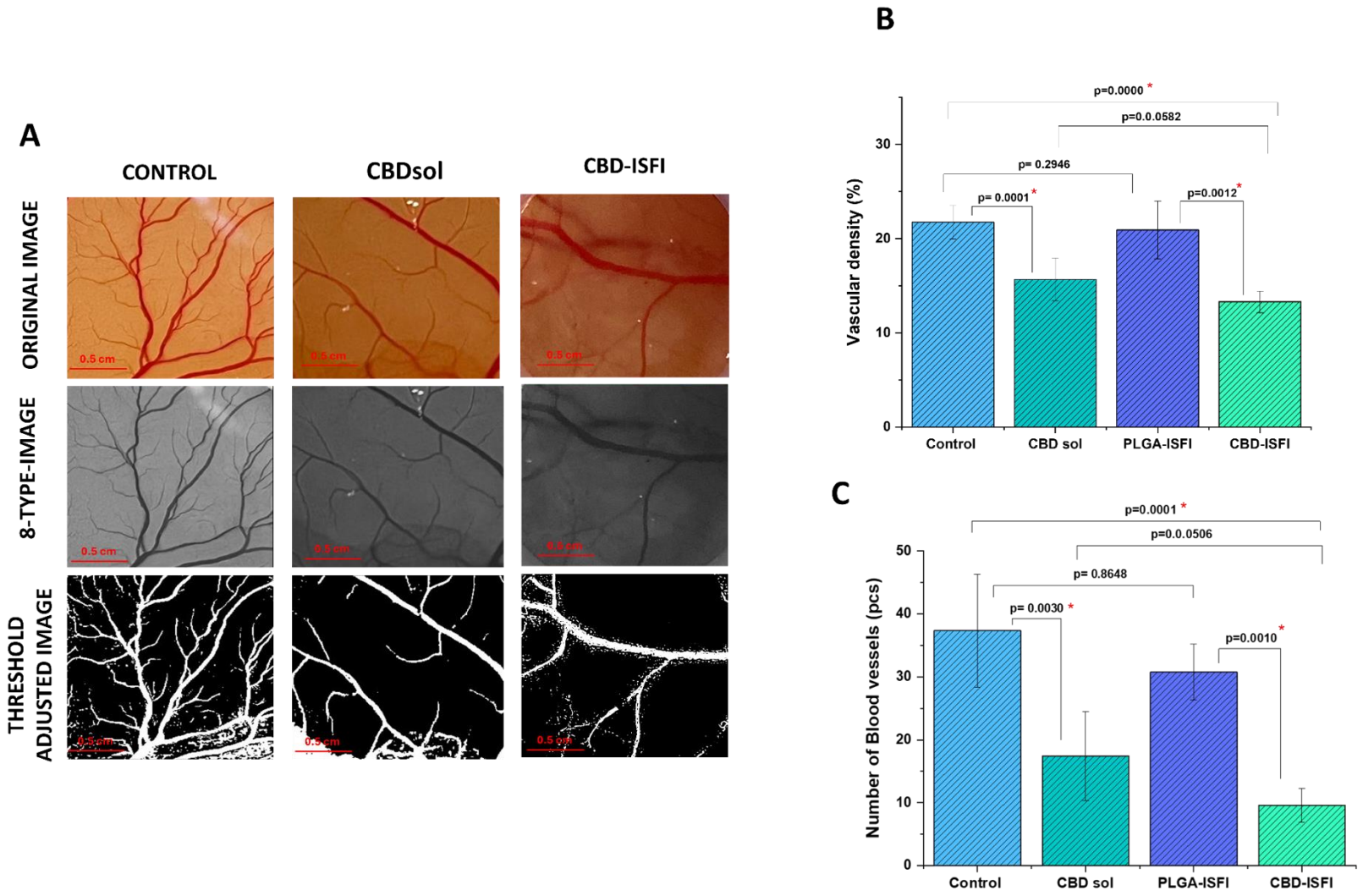

Figure 8: Results of angiogenesis study developed in the CAM model: A) Image processing carried out with ImageJ-Fiji software, B) vascular density (%) analysis, and C) number of blood vessels detected after each treatment. The images and all the calculations correspond to the treated area of the CAM. * Indicates statistically significant differences between the treatments.

The superior activity of CBD-loaded in situ forming implants compared with CBD in solution could be attributed to the controlled drug release achieved with these implants. It is seen that the constant release of the drug is more effective than the daily administration of free CBD. This has been also reported in other drugs. Celecoxib-loaded PLGA nanoparticles that exhibited a controlled drug release for around 28 days also showed a higher antiangiogenic effect than the celecoxib in solution daily administered at the same doses [38]. Similar results were also found in the polyphenols caffeic acid and pinocembrin when microencapsulated in composites made of nanoporous silicon, chitosan, and a β-cyclodextrin polymer. In both cases, microencapsulated polyphenols were more active. While caffeic acid in solution exhibited a significant antiangiogenic effect at concentrations of 150 µM, caffeic acid-loaded microparticles were effective at lower concentrations (50 µM). In the case of pinocembrin, it exerted an antiangiogenic effect at concentrations above 20 µM. However, pinocembrin-loaded microparticles were active when administered at a polyphenol concentration of 10µM [52].

4. CONCLUSIONS

ISFIs represent an interesting strategy for parenteral administration, offering many advantages over conventional formulations such as their easy formulation and relatively comfortable administration, their high tolerability, and their long-lasting effect after a single administration due to the controlled delivery of the drugs. These systems have proven to be suitable for the administration of CBD by subcutaneous route. The polymeric solutions of PLGA 502, PLGA 502H, PLA 202, or PLA 203 in DMSO, and PLGA 502, PLGA 502H, or PLA 202 in NMP using a polymer concentration of 0.25 mg/µL showed good injectability properties through both 23G and 25G needles and could be adequate for the elaboration of subcutaneously administered CBD-loaded-ISFIs. All the PLGA and PLA-developed implants allowed a controlled CBD delivery for at least ≈7 days. Implants developed using PLGA 502 and DMSO at a CBD: polymer ratio of 5:100 (w/w) showed the most controlled and CBD release with an initial burst release of less than 25%, followed by a nearly constant drug release rate for approximately one month. This formulation was successfully formed in ovo without causing signs of toxicity in the CAM assay and showed a marked and slightly higher antiangiogenic effect than CBD in solution. Due

to these results, CBD-PLGA-502-5-DMSO ISFI could be adequate for the subcutaneous administration in a cheap and easy way and to get a long-lasting effect for at least one month, being useful in the treatment of diseases involving angiogenesis. This is of particular relevance in cancer disease where angiogenesis plays a pivotal role. In addition, the ability of CBD to potentially enhance the efficacy of conventional antitumor drugs reinforces its potential usefulness in this pathology. Angiogenesis also plays a key role in the development of many chronic inflammatory diseases such as rheumatoid arthritis, and therefore the formulation developed in this work would also be useful in the treatment of these other pathologies.

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SUPPLEMENTARY MATERIAL

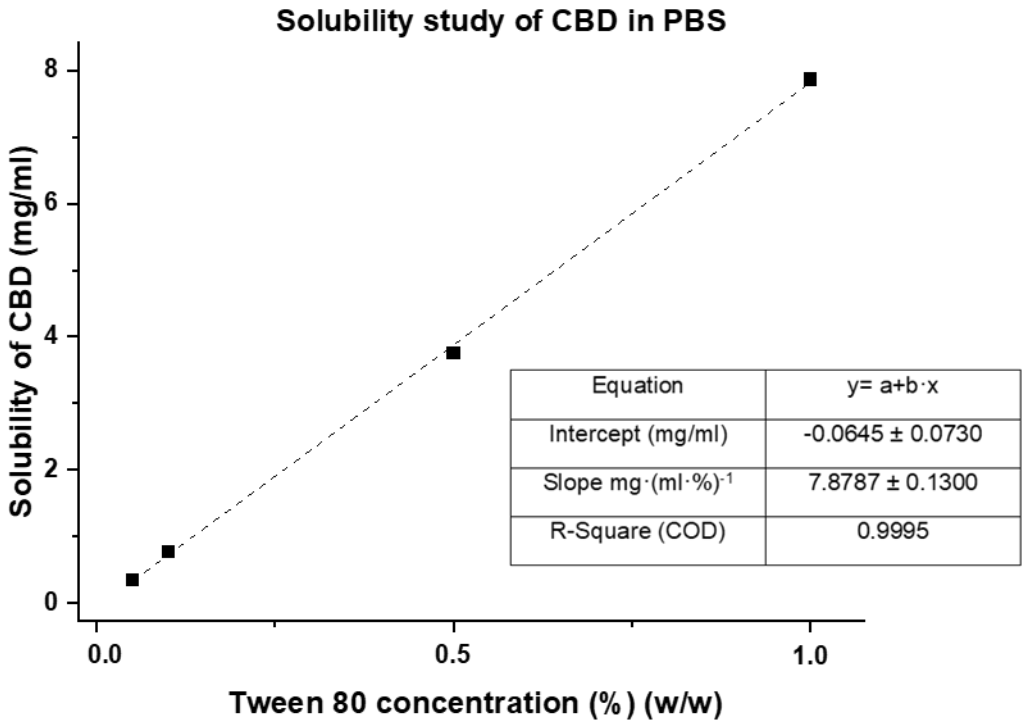
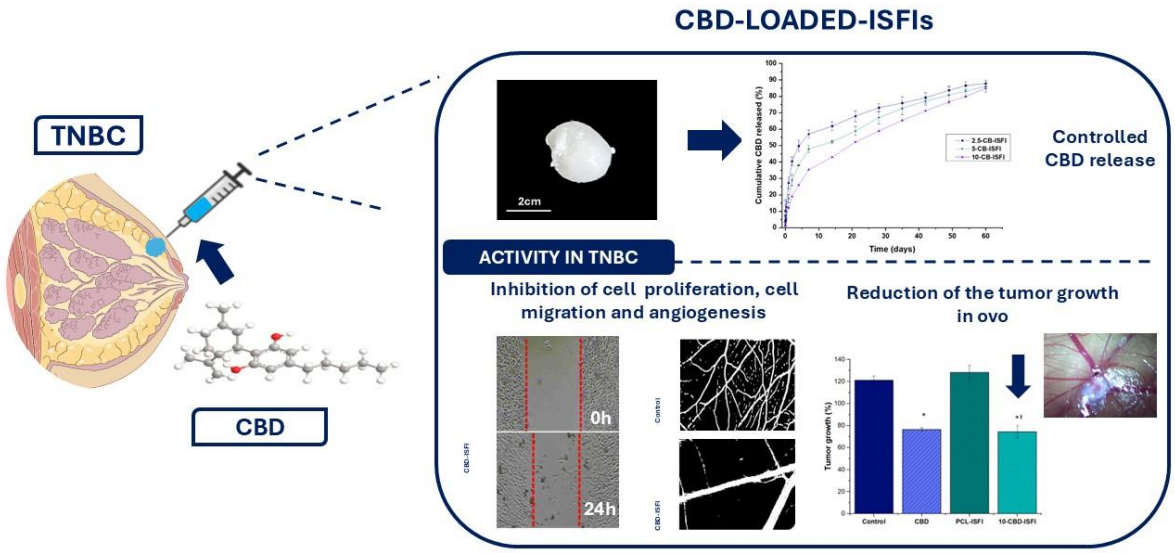


Figure S1: Data of the solubility study of CBD in PBS plus Tween 80 at different concentrations

CHAPTER 3

Cannabidiol-loaded-injectable depot formulation for the treatment of triple-negative breast cancer: design, development, in-vitro and in-ovo evaluation of its anticancer activity.



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ABSTRACT

Triple-negative breast cancer (TNBC) is an invasive and difficult-to-treat carcinoma that represents 15-20% of breast malignancies and is frequently diagnosed in younger women. Chemotherapy is the mainstay treatment approach. Cannabidiol (CBD), the main non-psychoactive cannabinoid, has shown a potential anticancer activity in TNBC enhancing the effect of conventional antineoplastics. This research aims to develop in situ forming implants (ISFIs) as a long-acting depot formulation of CBD with potential application in TNBC. This formulation is intended to be administered in the tumour site during chemotherapeutic regimens, allowing a controlled CBD release. ISFIs were elaborated with 100 mg of PCL and 2.5 mg (2.5-CB-ISFI), 5 mg (5-CB-ISFI) or 10 mg (10-CB-ISFI) of CBD dissolved in 400 µL of NMP. All the formulations exhibited a controlled drug release for around two months. 10-CB-ISFI formulation with the highest CBD content and the most suitable CBD release profile was selected for biological studies. This formulation inhibited the proliferation and migration of MDA-MB-231 and 4T1 cells and exerted an antiangiogenic effect in ovo. Interestingly, the antiangiogenic activity of 10-CB-ISFI was higher compared with CBD in solution administered at the same concentration, showing vascular inhibition percentages of around 80% and 60%, respectively. Finally, this formulation reduced the growth of MDA-MB-231-derived tumours developed in the CAM model. The single administration of 10-CB-ISFI exhibited a similar antitumor efficacy to the daily administration of CBD in solution (≈60% of tumour growth inhibition). Therefore, the injectable depot formulation of CBD developed in this work showed a promising utility in TNBC treatment.

Keywords: Breast Cancer, Cannabinoids, Drug delivery, Injectable formulations, In situ forming implants.

1. INTRODUCTION

Breast cancer (BC) is the most common cancer in women worldwide, accounting for approximately 11.6% of new cancer diagnoses in this population, and represents a leading cause of cancer-related deaths [1, 2]. It can be categorized into three groups based on their molecular patterns: i) Hormone-Receptor-expressing BC that express oestrogen receptors and/or progesterone receptors, ii) Human Epidermal Receptor type 2 (HER-2) expressing BC, and iii) Triple-negative breast Cancer (TNBC) that lacks oestrogen, progesterone, and HER-2 receptors [3].

TNBC represents approximately 15-20% of all breast malignancies, being frequently diagnosed in younger women, especially those under 50 years old. While breast tumours expressing progesterone or oestrogen receptors, in general, respond well to hormone therapy and those overexpressing HER-2 are effectively treated with HER-2 targeted therapies, TNBC does not respond to these treatments, so fewer treatment options are available [4, 5]. Nowadays, chemotherapy remains the mainstay pharmacotherapy for TNBC. It is often administered before surgery (neoadjuvant chemotherapy) to reduce the tumour size and/or after surgery (adjuvant chemotherapy) to kill any remaining tumour cells and reduce the chance of recurrence [6, 7]. Moreover, TNBC often grows and spreads more rapidly than other types of BC, leading to a poorer prognosis and a higher likelihood of recurrence [8]. All this makes TNBC a significant health problem.

In recent decades, cannabinoids have garnered a great deal of attention as potential therapeutic agents in cancer disease [9, 10]. Several Cannabis sativa based preparations (such as Tilray®, Bediol®, Bedrocan® and Bedica®) are currently available in Australia, Canada and some countries of Europe as palliative agents to treat nausea and vomiting related to chemotherapy. Moreover, FDA has approved three medications containing dronabinol, synthetic Δ^9-THC, (Marinol® and Syndros®) or nabilone, Δ^9-THC analogue (Cesamet®), as active for this purpose [11, 12]. Cannabinoids have also demonstrated anticancer activity per se by inhibiting tumour growth and metastases and exerting an antiangiogenic effect [13].

Cannabidiol (CBD) is the most abundant non-psychoactive cannabinoid and has shown promising antitumor activity in breast cancer, including TNBC [14, 15]. Numerous research works have demonstrated that CBD inhibits the proliferation of TNBC cells by inducing apoptosis and generating Reactive Oxygen Species (ROS). Moreover, it reduces the invasiveness of tumour cells, down-regulating the activity of Inhibitor Differentiation Protein 1 (ID-1) [16], which is overexpressed in TNBC and associated with larger tumour sizes, higher invasiveness and poorer prognosis [17]. Furthermore, it has been reported that CBD enhances the efficacy of conventional antineoplastic drugs such as paclitaxel and doxorubicin [18, 19]. For example, Patel and collaborators demonstrated in a xenograft tumour model of TNBC (developed by injecting MDA-MB-231 cells) that CBD encapsulated in extracellular vesicles at doses of 5 mg/Kg significantly improved the antitumor efficacy of doxorubicin administered at doses of 2 mg/Kg [20]. Moreover, CBD alleviates some of the major side effects associated with these antineoplastics, like the peripheral neuropathy of paclitaxel and cardiotoxicity of doxorubicin [21-23], reinforcing the utility of combining CBD and conventional antineoplastics.

Nevertheless, CBD exhibits high lipophilicity (Log P 6.3), high instability and low and erratic oral bioavailability. All this complicates its handling and administration, making drug delivery systems an interesting strategy to administer this drug [24].
In-situ forming implants (ISFIs), defined as liquid formulations that form solid or semi-solid depots upon the administration site, have garnered significant interest as long-acting formulations becoming an alternative to traditional surgical implants [25] due to, among other advantages, their simple manufacturing process, their easy and simple administration way to patients and their ability to control drug release over extended periods (from days to months depending on the formulation) [26] which allows a long-lasting effect with a single administration. This prolonged activity is worth considering in the treatment of cancer, where there is a need for prolonged therapies [27, 28].

ISFIs can be classified according to their formation mechanism at the injection site into thermally induced systems, pH-responsive formulations, cross-linked implants and systems based on phase separation due to solvent exchange [29]. The solvent

exchange process is one of the most used mechanisms of formation, consisting of the precipitation of the polymer in solution and the formation of a deposit at the injection site because of the diffusion of the solvent into the surrounding tissues and the penetration of water into the formulation. The drug is entrapped into this deposit [30]. The FDA and/or EMA have approved several ISFI formulations based on this mechanism: i) Eligard® a formulation of leuprorelin approved for the treatment of prostate cancer, ii) Sublocade® and Brixadi® (approved by EMA as Buvidal®) ISFIs that contain buprenorphine and are used for opioid dependence, iii) Perseris®, Uzedy® and Risvan® (EMA approved this latter as Okedi®) that consist of formulations of risperidone approved for schizophrenia and iv) Atridox® that contains doxycycline hyclate and is used for the treatment of periodontitis. Except for Brixadi®, prepared with soybean phosphatidylcholine/glycerol dioleate, all these formulations contain polylactide (PLA) or poly-lactic-co-glycolic acid (PLGA) as polymeric matrix dissolved in N-methyl-2-pyrrolidone (NMP) or dimethyl sulfoxide [31-33].

This research aims to design, develop, and characterize CBD-loaded ISFIs as a long-acting injectable formulation with potential application in treating TNBC. This formulation is composed of polycaprolactone (PCL), an aliphatic polyester polymer widely used in the development of drug delivery systems, as it is a biodegradable and highly biocompatible polymer [34] and NMP as a water-soluble solvent. To the best of our knowledge, this is the first work to develop a PCL-based injectable depot implant to administer cannabinoids and to evaluate their utility to inhibit the proliferation and migration of breast turmors.

2. MATERIALS AND METHODS

2.1. Materials

Polycaprolactone (PCL) (Resomer® C202) was obtained from Evonik® Industries (Essen, Germany). Cannabidiol was supplied by THC Pharm (Frankfurt, Germany). Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), Potassium dihydrogen phosphate (KH_2PO_4), and Polyethylene glycol sorbitan monooleate (Tween 80) were purchased from Panreac (Barcelona, Spain). NMP, Gibco™ RPMI 1640 medium, Gibco™ Trypsin-EDTA (0.25%), Mitomycin C, Gibco™ PBS pH 7.4, Gibco™ Fetal

Bovine Serum (FBS), Gibco™ Geltrex Matrix and Molecular Probes™ MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Fisher Scientific (Madrid, Spain). Methanol and acetonitrile were purchased from Scharlab S.L. (Barcelona, Spain). Milli-Q® water obtained using a Millipore system (Massachusetts, USA) was used.

2.2. Injectability and viscosity studies

ISFIs were prepared using PCL as polymer and NMP as solvent. Firstly, the injectability and viscosity of the polymeric solutions at different polymer: solvent ratios (w/v) (100 mg:150 µL, 100 mg:300 µL, or 100 mg:400 µL) were analyzed.

The injectability was tested in a double-blinded study. Briefly, seven volunteers were asked to evaluate the injectability of 1ml of each solution in 1 mL syringes (Injekt-F, B-Braun, Germany) coupled with 23G and 25G needles and assign a score (ranging from 1 to 4) to each sample based on the ease of injectability and the flow through the needle: score 1: injectability extremely hard and no-flow; score 2: injectability hard and flow starts with droplets, then steady; score 3: injectability relatively easy and flow steady and score 4: injectability very easy and flow steady.

The viscosity of each polymeric solution was evaluated at 25ºC in a viscometer DVNext Brookfield Ametek (Massachusetts, USA).

2.3. Elaboration of ISFIs

CBD-loaded-ISFIs (CB-ISFI) were elaborated by the direct medium injection technique. PCL (100 mg) and CBD (2.5, 5 or 10 mg) were dissolved in 400µL of NMP. Then, 200 µL of each solution was injected into a vial containing 5 ml of phosphate buffer (PBS) pH 7.4 using a standard 25G needle gauge syringe. Formulations without CBD (PCL-ISFI) were also prepared.

2.4. Characterization of ISFIs

2.4.1. Morphology

Implant morphology was examined by both optical microscopy (Ninyoon 4K microscope, Evatost, Ireland) and scanning electron microscopy (SEM) (JEOL 6400 JSM, Tokyo, Japan) 15 minutes after ISFIs preparation as described above (section

2.3). The excess water was removed before examination. For optical microscopy, implants were directly examined (Ninyoon 4K microscope, Evatost, Ireland). However, for SEM analyses, the implants were placed onto aluminium stubs, dried for 48 hours in a desiccator, and coated with gold for 180 seconds.

2.4.2. Drug Release

The release of CBD from 2.5-CB-ISFI, 5-CB-ISFI, and 10-CB-ISFI was evaluated in PBS pH 7.4 containing Tween 80 at 1% to maintain sink conditions. Implants were prepared by injecting 200 µL of each CBD-PCL solution in NMP into 5 mL of release medium and placed in a thermostatic shaking bath at 37 ± 0.5 °C and at 100 rpm. At specific times (15 min, 1h, 2h, 6h, 1, 2, 4, 7, 14, 21, 28, 35, 49, and 60 days), the supernatant was gently removed, filtered with 0.45µm cellulose syringe filters and analyzed by HPLC [35]. Fresh-release medium was added at each time.

2.5. Cell culture studies

2.5.1. Cell lines

MDA-MB-231 (ATCC HTB-26, human origin) and 4T1 (CRL-2539, mouse origin) cell lines were selected as models of TNBC. Both cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin and incubated at 37ºC and 5% CO_2.

2.5.2. Antiproliferative activity

MDA-MB 231 cells were seeded in 6-well plates at a cell density of 350000 cells/well. 24 hours after seeding, the medium was removed, and cells were treated with CBD in solution and 10-CB-ISFI at a CBD concentration of 15 µM. 4T1 cells were seeded at a cell density of 150000 cells/well and treated 24 hours after seeding with CBD in solution and 10-CB-ISFI at a CBD concentration of 7.5 µM. These concentrations were selected according to the IC_{50} value of CBD in solution (Supplementary Material Figures S1 and S2) in each cell line. For this study, 10-CB-ISFIs were formed in parallel and maintained in a complete RPMI-1640 medium. At predetermined time points after formation, 0 days (T0-2), 2 days (T2-4), 4 days (T4-6), and 6 days (T6-8), implants were collected. Then, the cells were treated with these collected implants. The effect of recently prepared (T0) unloaded PCL-ISFIs

was also evaluated. The implants were removed 48 hours after the treatment, and the cells were washed with PBS. Then, cell viability was evaluated using an MTT assay. Briefly, 600 µL of MTT solution in complete RPMI medium (1.0 mg/mL) were added to each well, and after 3 hours of incubation, the medium was carefully removed. Finally, 600 µL of DMSO were added to each well to dissolve the formazan crystals, plates were stirred for 10 minutes at 100 rpm, and absorbance was measured using a plate reader spectrophotometer (Varioskan 3020-2023 LUX, Thermo Fisher Scientific, Madrid) at 570 nm.

2.5.2. Migration study: scratch assay.

The effect of CBD and 10-CB-ISFIs on cell migration was assessed using the scratch assay [36]. Briefly, MDA-MB-231 and 4T1 cells were seeded in 24-well plates at a cell density of 50000 cells/well and 30000 cells/well, respectively. When 90-100% of confluence was reached, the cells were treated with mitomycin (10 µg/mL) for 40 minutes to stop cell proliferation. Then, each well was scraped with a sterile 10 µL pipette tip, and the cells were washed with PBS to remove cellular debris. Then, CBD in solution and 10-CB-ISFI (recently prepared in complete cell culture medium) were administered at a CBD concentration of 5 and 7.5 µM in 4T1 cells and 10µM and 20 µM in MDA-MB-231 cells. These CBD concentrations showed a cell death lower than 30% after 24 hours of treatment (Supplementary material Figures S1 and S2). Cells treated with a complete RPMI medium were used as control. Cells were photographed before (T0) and 24 hours after the administration of each treatment (T24) using a microscope Eclipse TE300 Nikon microscope (Melville, USA). The percentage of wound closure was calculated using ImageJ-Fiji software using the following equation:

$$Wound\ closure\ (\%) = \frac{\text{Area T0} - \text{Area T24}}{Area\ T0}\ x\ 100 \quad \text{(Eq. 1)}$$

The migration ratio of each treatment was also calculated as follows:

$$Migration\ ratio\ = \frac{\text{Wound closure treatment}}{Wound\ closure\ control} \quad \text{(Eq. 2)}$$

2.6. In Ovo Studies

2.6.1. Angiogenesis study

The antiangiogenic effect of 10-CB-ISFIs was studied in the chorioallantoic membrane (CAM) model [37]. Briefly, fertilized leghorn chicken eggs (Granja Santa Isabel, Cordoba, Spain) were incubated at 37°C and 47% humidity. On embryo development day 4 (EDD4), the eggshell was gently drilled, and the hole was sealed with tape. On EDD 10, the CAM was exposed, and a silicone O-ring with a diameter of 1 cm was positioned on this membrane to define the treatment area. The eggs were then treated for 48h with CBD in solution at 100 µM (daily administration of 7.78 µg of CBD dissolved in RMPI-medium) or the equivalent amount of 10-CB-ISFIs (single administration of 4.5 µL of CBD-ISFI polymeric solution to get a CBD release of around 16 µg in 48h). Eggs treated with PCL-ISFIs and PBS pH 7.4 (negative control for angiogenesis) were also evaluated. The treated area of each egg was photographed 48 hours after the treatments and analyzed by calculating the total number of blood vessels and the vascular density [38].

Vascular density was calculated using ImageJ-Fiji software. Firstly, images were converted to an 8-bit image type, and the threshold was adjusted. Then, vascular density was automatically calculated according to the black-and-white colour differences ratio.

2.6.2. Antitumor Efficacy

The antitumor efficacy of CBD-ISFIs was also tested in ovo in MDA-MB-231-derived tumours formed onto the CAM. Fertilized leghorn chicken eggs were incubated as described in section 2.6.1. On EDD 4, the eggshell of each egg was carefully drilled and closed with tape [33]. On EDD 7, the hole was opened, and CAM was exposed. Then, it was gently scratched, and MDA-MB-231 cells (2 x 10^6 cells suspended in Geltrex® matrix, 30µL per egg) were inoculated. On EDD 9, MDA-MB-231-derived tumours were already formed. They were surrounded with a silicone o-ring (1cm in diameter), photographed (Ninyoon 4K microscope, Evatost, Ireland) and topically treated with PBS pH 7.4 (used as negative control), CBD in solution (daily administration of 7.78 µg of CBD dissolved in RMPI-medium), 10-CB-ISFI (single

administration of 4.5 µL of CBD-ISFI polymeric solution to get a CBD release of around 16 µg in 48 h) or PCL-ISFI (single administration of 4.5 µL of PCL solution without CBD). On EDD 11, the tumours were photographed again.

The tumour growth (%) was obtained by calculating the tumour area before (Tumour area initial) and after (Tumour area final) the treatments using ImageJ-Fiji software as follows [39]:

$$Tumour\ growth\ (\%) = \frac{\text{Tumour area final}}{Tumour\ area\ initial}\ x\ 100 \quad \text{(Eq. 3)}$$

2.7. Statistical analysis

Data are displayed as mean ± standard deviation (SD) of at least three independent experiments. Cell culture studies were performed in quadruplicate, and in the studies in ovo, at least 5 eggs per treatment were analyzed. The injectability study scores were statistically compared using the Mann-Whitney U test using IBM SPSS Statistics 28 software (IBM, Chicago, Illinois). In the rest of the experiments, the significance of the differences detected between the two groups was evaluated using a student's t-test. ANOVA test was used to compare the differences detected between multiple groups. Both tests were performed using Statgraphics 19 Centurion (Statgraphics Technologies, Inc., The Plains, Virginia). Finally, the graphs were elaborated using Origin 2019 software (OriginLab, Massachusetts, USA).

3. RESULTS AND DISCUSSION

3.1. Injectability and viscosity studies

The injectability and viscosity of the polymeric solutions were first examined to determine the most suitable polymer: solvent ratio for preparing CB-ISFIs. Table 1 displays the scores obtained from the injectability study in which volunteers scored the ease of injectability and the flow of the different polymeric solutions (100 mg of PCL dissolved in 150, 300 or 400µL of NMP) through 23G and 25G needles and the results of viscosity measurements. Only in the solutions prepared with 100 mg of PCL and 400 µL of NMP, all participants indicated good injectability properties (score 3 or 4) through 23G and 25G needle gauges without detecting statistically significant differences between both needles (p-value > 0.05). The better injectability properties of this solution can be attributed to its lower viscosity. While the PCL solution

prepared with a PCL: NMP ratio of 100 mg:400µL showed a mean viscosity value of around 65 cP, the solutions elaborated with a lower volume of NMP showed a significantly higher viscosity above 100 cP. Therefore, a PCL: NMP ratio of 100 mg:400µL was selected to prepare CB-ISFIs.

	Injectability study			Viscosity (cP)
Polymer: solvent ratio	**Score range (median) 23G needle (n=7)**	**Score range (median) 25G needle (n=7)**	**Suitable Injectability**	**Mean ± SD (n=3)**
100 mg:150 µL	1-2 (1)	1-2 (1)	No	582.23±2.54
100 mg:300 µL	2-3 (3)	2-3 (3)	No	134.05±3.85
100 mg:400 µL	3-4 (4)	3-4 (4)	Yes	65.6±1.10

Table 1: Results of injectability and viscosity studies.

3.2. Elaboration and characterization of ISFIs

CB-ISFIs were elaborated with 100 mg of PCL and 2.5 mg (2.5-CB-ISFI), 5 mg (5-CB-ISFI) or 10 mg (10-CB-ISFI) of CBD, dissolved in 400 µL of NMP. All these solutions were clear and transparent to yellowish. All the implants were rapidly formed when these solutions were injected into the simulated physiological medium (PBS, pH 7.4).

3.2.1. Optical and SEM microscopy

Figure 1 displays the appearance of recently prepared ISFIs. All the formulations exhibited an irregular shape, typical of the implants elaborated by the direct injection technique [40]. No differences were observed in the aspect of PCL-ISFIs and CB-ISFIs, nor in the appearance of the implants prepared with varying amounts of CBD.

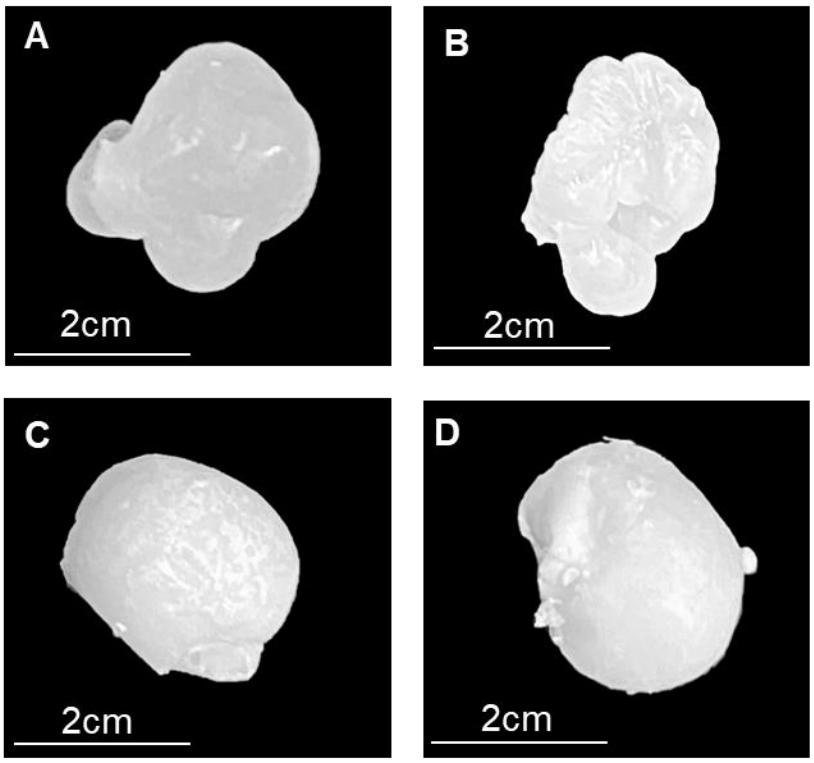


Figure 1: Optical images of recently prepared PCL-ISFI (A), 2.5-CB-ISFI (B), 5-CB-ISFI (C) and 10-CB-ISFI (D) formulations.

When the surface of the implants was examined by SEM (Figure 2), a smooth surface was observed in both unloaded and CBD-loaded ISFIs. It should be noted that some pores can be appreciated on the surface of all the formulations, which are attributed to the removal of the solvent during the solidification process. NMP is a hydrophilic solvent that rapidly diffuses into the aqueous medium when the polymeric solution is injected into an aqueous environment, triggering the formation of porous implants [41]. It has been demonstrated that the faster solvent removal, the higher porosity of the polymeric matrix. Unloaded PCL-ISFIs showed a more porous surface than CBD-loaded formulations, probably due to the plasticizer effect of CBD on PCL [45].

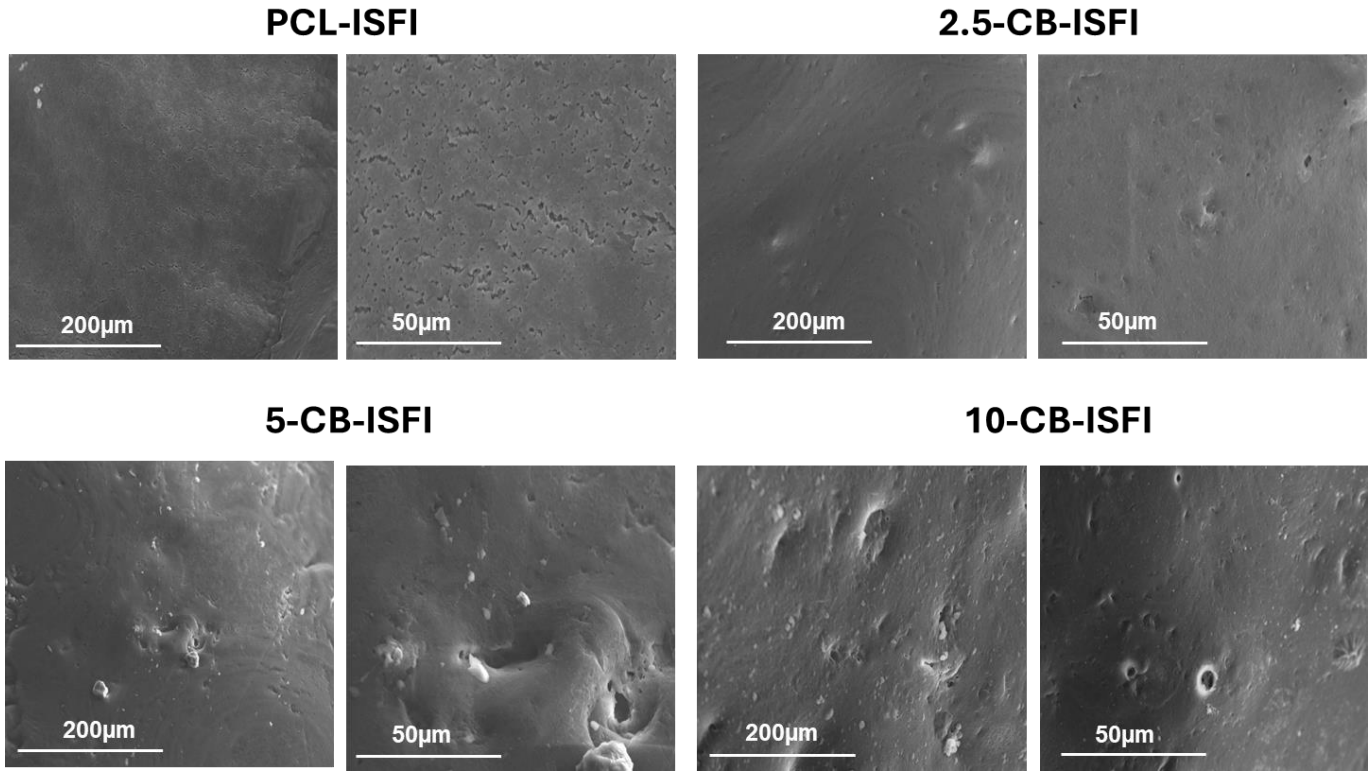


Figure 2: Surface morphology of PCL-ISFIs and CB-ISFI implants examined by SEM.

3.2.2. In Vitro Drug Release Study

As depicted in Figure 3, CBD-loaded ISFIs exhibited a controlled drug release over approximately 60 days, with more than 80% of CBD being released by this time. Typically, the higher the drug content, the higher the release rate. For instance, in PLGA-ISFIs loaded with cabotegravir, the release rate of this drug increased from 3.35 to 11.20 µg/day in implants prepared with drug concentrations of 112 and 290 mg/ml, respectively [42]. Similar results were found in PLGA implants prepared with 10% and 20% of insulin (w/w) [43]. However, CB-ISFIs showed an inverse relationship: the higher the CBD content, the slower the drug release. This aspect could be related to the plasticizer effect of this cannabinoid on PCL [45]. It should be noted that this phenomenon has also been appreciated in other CBD-loaded polymeric formulations. For example, in microparticles containing 10% (w/w) of CBD, around 60% of this cannabinoid was released within 21 days, whereas in

formulations with 20% (w/w) of CBD, this release percentage was achieved in 31 days [44].

2.5-CB-ISFIs exhibited the fastest drug release with a relatively high burst effect; approximately 40% of the CBD was released within the first 48 hours. The burst effect in 5-CB-ISFIs and 10-CB-ISFIs was significantly lower, with around 28% and 18% of the CBD released by this time. Both 2.5-CB-ISFIs and 5-CB-ISFIs showed a biphasic release profile with an initial first faster release phase from day 2 to day 7 (around 57% and 47% of the CBD was released after 7 days from 2.5-CB-ISFIs and 5-CB-ISFIs respectively), followed by a second phase up to day 60 in which CBD is release slowly. This second phase can be fitted to a zero-order release kinetics (2.5-CB-ISFIs: K_0=7.325 µg/day, R^2=0.9857; 5-CB-ISFIs K_0=18.62 µg/day, R^2=0.9806). Nevertheless, the CBD release from 10-CB-ISFIs was more controlled, detecting a constant release phase from day 2 to day 60 fitted to a zero-order release kinetics (K_0=53 µg/day, R^2=0.967).

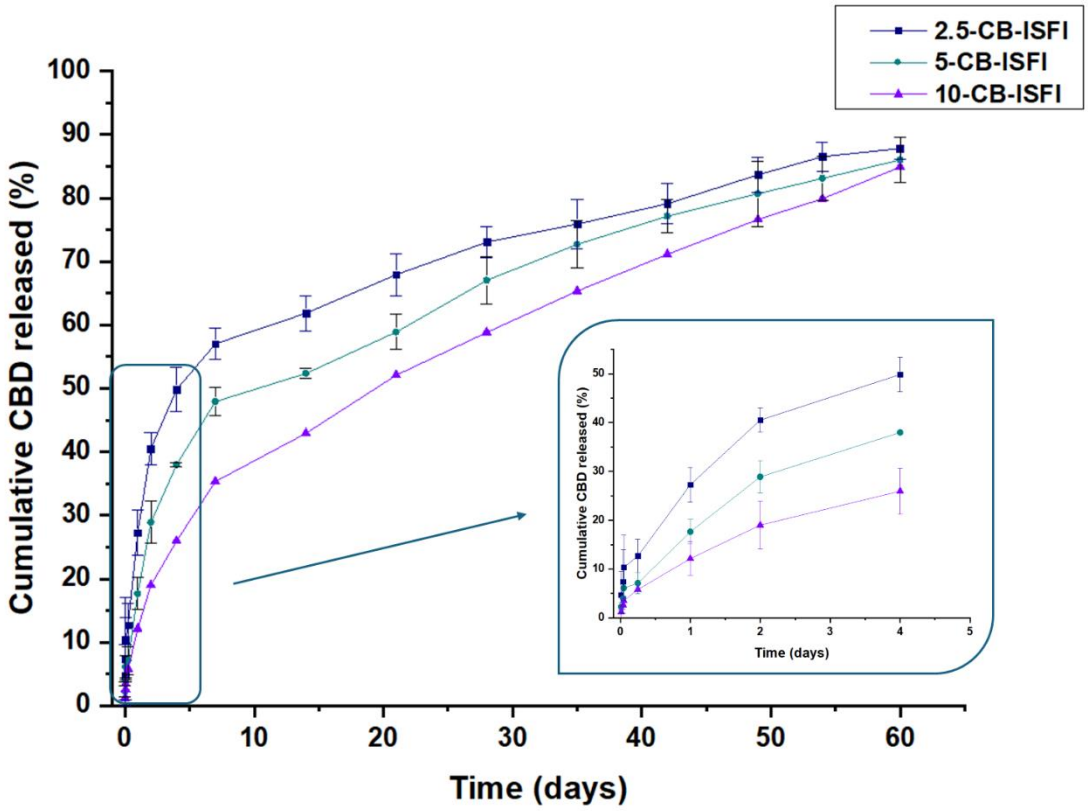


Figure 3: Drug release profile of CBD-loaded-ISFIs.

A previous study showed that CBD-loaded ISFIs prepared with other polymers (PLGA-502, PLGA-502H or PLA-202) exhibited a notably faster CBD release than the PCL formulations developed in this work. While PCL-ISFIs exhibited less than 30% of CBD released within the first day, PLGA and PLA formulations released more than 45% of this cannabinoid by this time [45]. This difference could be attributed to the crystallinity of the polymers. While PLGA and PLA are amorphous, PCL is semicrystalline [46, 47], and drug release is usually faster in polymers with higher amorphous content due to the difficulty of water penetration, which is responsible for the bulk erosion of the polymer [48].

3.3 Cell culture experiments

CBD has attracted considerable interest because of its potential anti-cancer properties, as it inhibits the proliferation, migration, and invasion of cancer cells and reduces tumour growth and metastases [49]. One of the tumours where it has excellent potential is breast cancer, including TNBC. This study evaluated the antiproliferative and anti-migration activity of 10-CB-ISFIs in MDA-MB-231 and 4T1 cells. This formulation was selected as it showed the highest CBD content (10 mg per 100 mg of polymer) and exhibited the most suitable CBD release profile (lowest burst effect and more controlled CBD release for around 60 days). This controlled drug release would provide a long-acting effect with a single administration.

3.3.1 Antiproliferative activity

The antiproliferative activity of 10-CB-ISFIs was evaluated for 8 days. For this study, MDA-MB-231 and 4T1 cells were treated with CBD in solution and in 10-CB-ISFI at a drug concentration of 15µM and 7.5µM, respectively. These concentrations correspond approximately to the IC_{50} values (16.5 µM ± 5.2 in MDA-MB-231 cells and 7.4 µM ± 3.4 in 4T1 cells) of CBD in solution after 48 hours of incubation in both cell lines (Figure S1 and S2). The cytotoxicity of PCL-ISFIs was also tested.

While PCL-ISFIs did not exhibit cytotoxic effects in MDA-MB-231 and 4T1 cells, 10-CB-ISFIs demonstrated a prolonged and constant antiproliferative activity for at least 8 days (Figure 4). 10-CB-ISFIs showed a lower antiproliferative activity (cell death percentage of around 25% and 30% in MDA-MB-231 and 4T1 cells, respectively) than CBD in solution. The controlled drug release of CBD can explain this lower

activity. The lower effect of the entrapped drug has also been appreciated in viability studies of PLGA microparticles loaded with CBD or paclitaxel in SKOV-3 and A549 cells, respectively [44, 50]. In both cases, higher cell death was observed in cells treated with free drug (≈ 40 and 45% for paclitaxel and CBD, respectively) than in PLGA-loaded microparticles (≈ 25% in both cases).

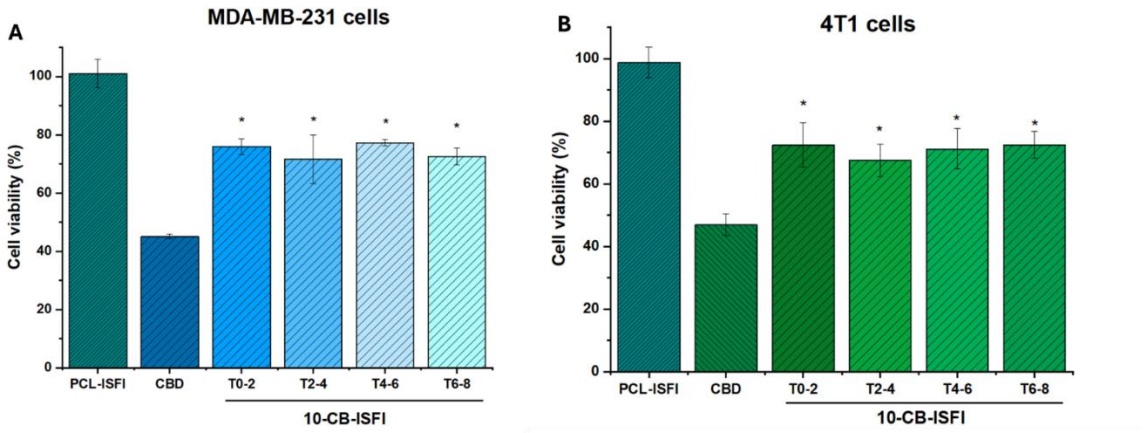


Figure 4: Cell viability studies of 10-CB-ISFIs in MDA-MB-231 cells (A) and 4T1 cells (B). * indicates statistically significant differences (p-value <0.05) between PCL-ISFIs and 10-CB-ISFI at each incubation time.

3.3.2 Migration study: scratch assay

The migration of cancerous cells from the primary site of the tumour leads to metastasis, which is the main cause of death in cancer patients [51]. Its inhibition is an essential step in cancer treatment, and CBD has exhibited an anti-migration activity in some cancer types [52]. In this study, the effect of CBD-loaded-ISFIs on the migration of TNBC cells has also been evaluated.

Untreated MDA-MB-231 cells (control group) exhibited a wound closure percentage of approximately 50%. In contrast, cells treated with 10-CB-ISFI showed statistically significant (p-value < 0.01 compared to the control) lower wound closure. It should be noted that CBD-loaded-ISFIs exhibited a similar activity compared with CBD in solution, with wound closure percentages of around 20% and 17% at concentrations of 10 µM and 20 µM, respectively (Figure 5). Significant differences between CBD in solution and 10-CB-ISFI at each concentration were not achieved (p-value > 0.05).

Migration ratios of CBD treatments were also calculated (Table S1). Ratios less than 1 indicate inhibited cell migration, while ratios greater than 1 indicate enhanced migration. Migration ratios of around 0.40 and 0.35 were obtained with both CBD in solution and 10-CB-ISFI at concentrations of 10 μM and 20 μM respectively, corroborating the ability of CBD and CBD-loaded implants to inhibit the migration of MDA-MB-231 cells.

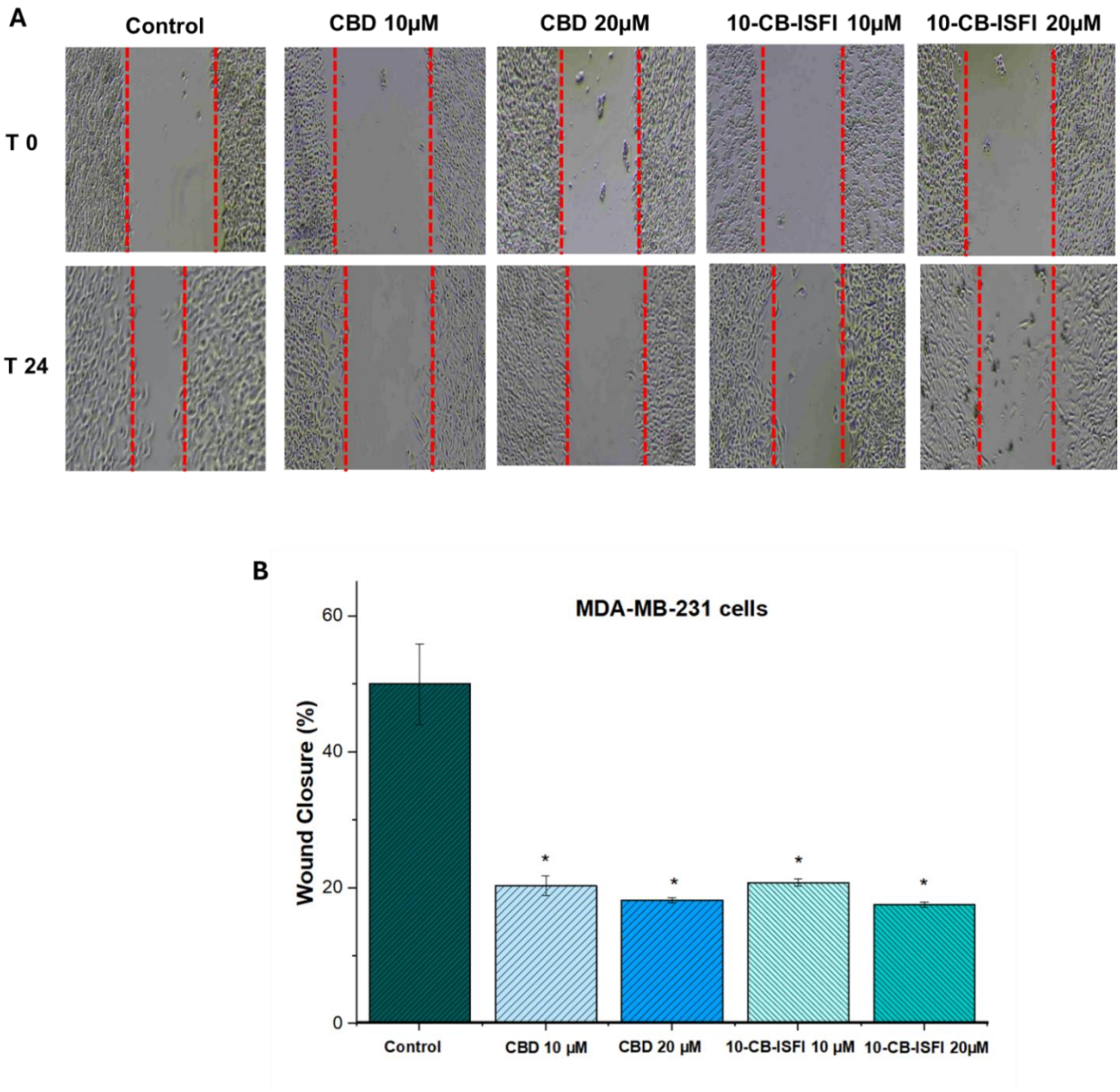


Figure 5: In vitro scratch wound healing assay in MDA-MB-231 cells. Images at time 0 (T0h) and after 24 hours (T24) of treatment (A). Wound closure percentage of each treatment (B). * Indicates statistically significant (p-value<0.05) differences with the control (cells treated with complete growth medium).

A lower anti-migration activity was detected in 4T1 cells compared to MDA-MB-231 cells. As depicted in Figure 6A, in the control group of 4T1 cells, the generated wound was almost completely closed, with a closure percentage of around 93%

(Figure 6B). However, cells treated with CB-ISFIs exhibited a statistically significant (p-value<0.05) lower wound closure. Like in MDA-MB-231 cells, CBD in solution and 10-CB-ISFI showed a similar and non-statistically significant (p-value> 0.05) effect with wound closure percentages of around 65% and 57% at concentrations of 5 µM and 7.5 µM respectively. Migration ratios lower than 1 (around 0.74 and 0.6 at CBD concentrations of 5 µM and 7.5 µM) were appreciated (Table S2).

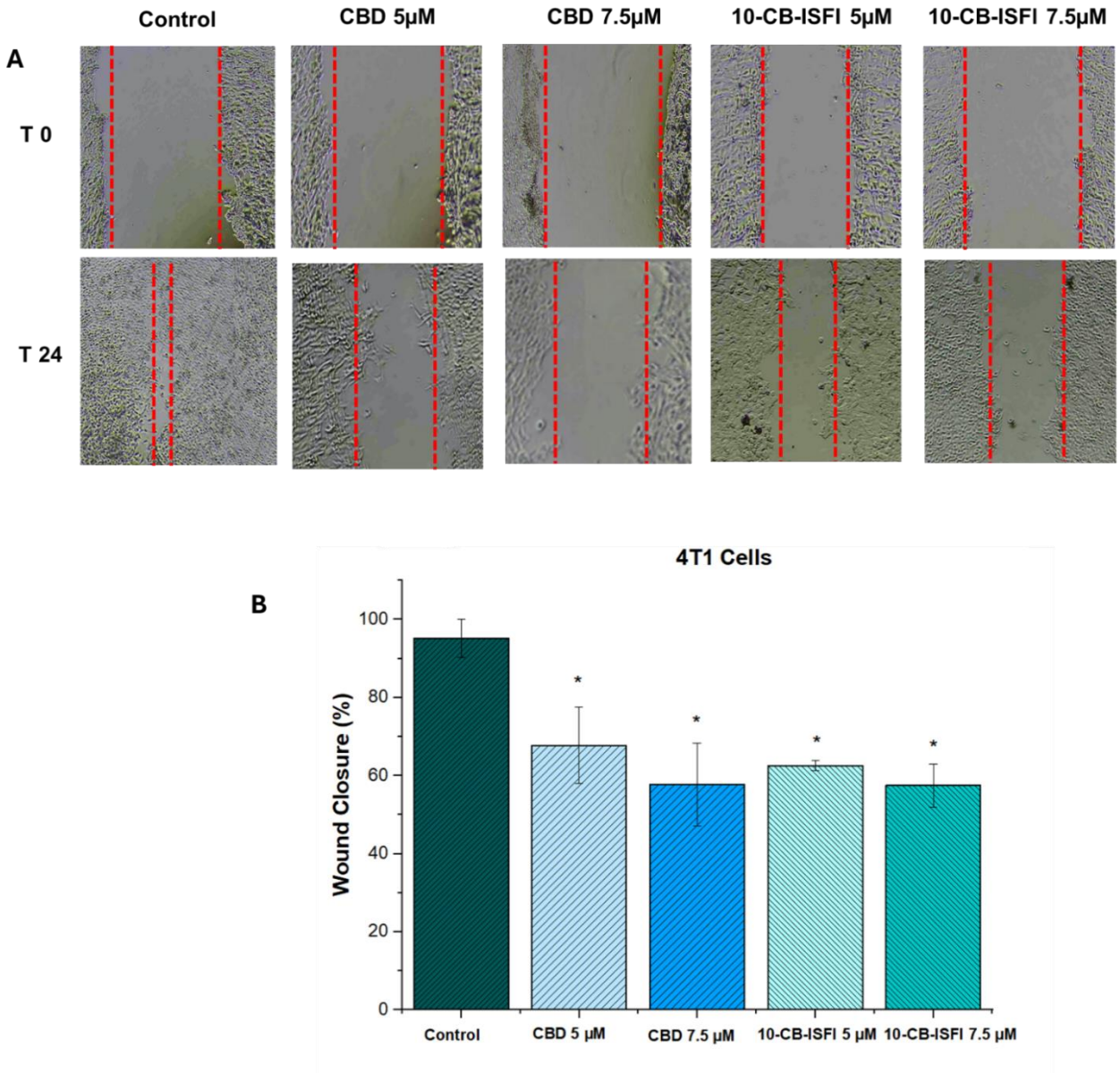


Figure 6: In vitro scratch wound healing assay in 4T1 cells. Images at time 0 (T0h) and after 24 hours (T24) of treatment (A). Wound closure percentage of each

treatment. * indicates statistically significant (p-value<0.05) differences with the control (cells treated with complete growth medium).

Other researchers have previously evaluated the effect of CBD on the migration of TNBC cells. In contrast to our data, D'Aoia and collaborators reported that CBD did not have any activity on the migration of MDA-MB-231 cells after 10 hours of incubation when administered at concentrations of 5µM [53]. Nevertheless, other authors demonstrated its efficacy even at lower concentrations (CBD 2 µM). In their study, while non-treated cells exhibited a wound closure of around 60% after 24 hours, cells treated with CBD at 2µM showed a wound closure of around 40% [54]. These results are in accordance with the results of our study, as at 10µM CBD in solution showed a wound closure percentage of around 20%. Elbaz and collaborators also evaluated the effect of CBD in the migration of 4T1 cells. In this case, the administration of CBD at 6µM did not exert any anti-migration activity. However, in this study, the authors used a "transwell model" to evaluate the migration and calculated the migration after 48 hours [55]. Using another model could explain the differences found in our study.

3.4 In Ovo studies

3.4.1 Angiogenesis studies

Angiogenesis is a complex and dynamic process crucial for tumour growth and dissemination. Antiangiogenic compounds have become essential in cancer therapy [56, 57]. Several studies have demonstrated the ability of CBD to inhibit angiogenesis [58]. This study analyzed the antiangiogenic activity of ISFIs using the CAM model.

Implants were successfully formed on the CAM of fertilized chicken eggs. It should be mentioned that CAM assay is a validated model for evaluating the toxicity of drugs and formulations. Notably, 10-CB-ISFIs showed no signs of toxicity, as no damage (e.g., haemorrhage or lysis) was observed, as displayed in Figure 7A (original image).

Concerning the antiangiogenic activity of 10-CB-ISFI, a statistically significant (p-value<0.001) lower vascularisation was detected in the eggs treated with the

implants compared to the control group (eggs treated with PBS pH 7.4), with vascular density values of around 16% ± 1.49 and around 13% ± 1.2 in CBD in solution and 10-CB-ISFI treated eggs. Eggs treated with non-loaded implants did not exhibit any effect. Similar and non-statistically significant (p-value>0.05) vascular density compared with the control (21.5% ± 1.3) was obtained.

Interestingly, the single administration of 10-CB-ISFIs was more effective than the daily administration of CBD in solution at the same concentration of 100µM (Figure 7D). While CBD in solution showed a vascular inhibition percentage of around 65%, CBD-loaded implants showed an inhibition percentage of around 80%. However, statistically significant differences between these treatments were not observed (p-value > 0.05). It should be noted that similar data in terms of antiangiogenic effect was also observed in the CBD-loaded ISFIs elaborated with PLGA-502 and DMSO [59].

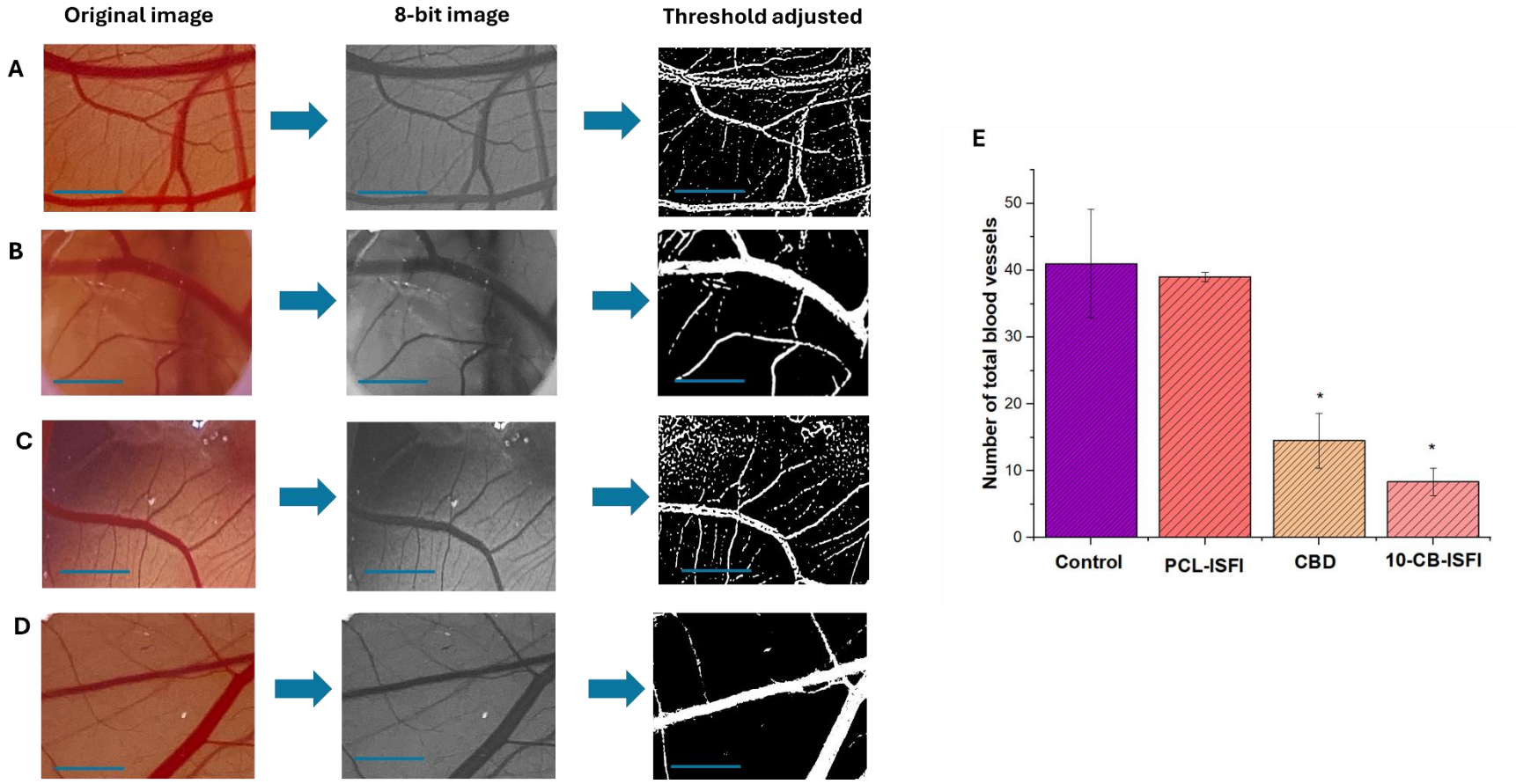
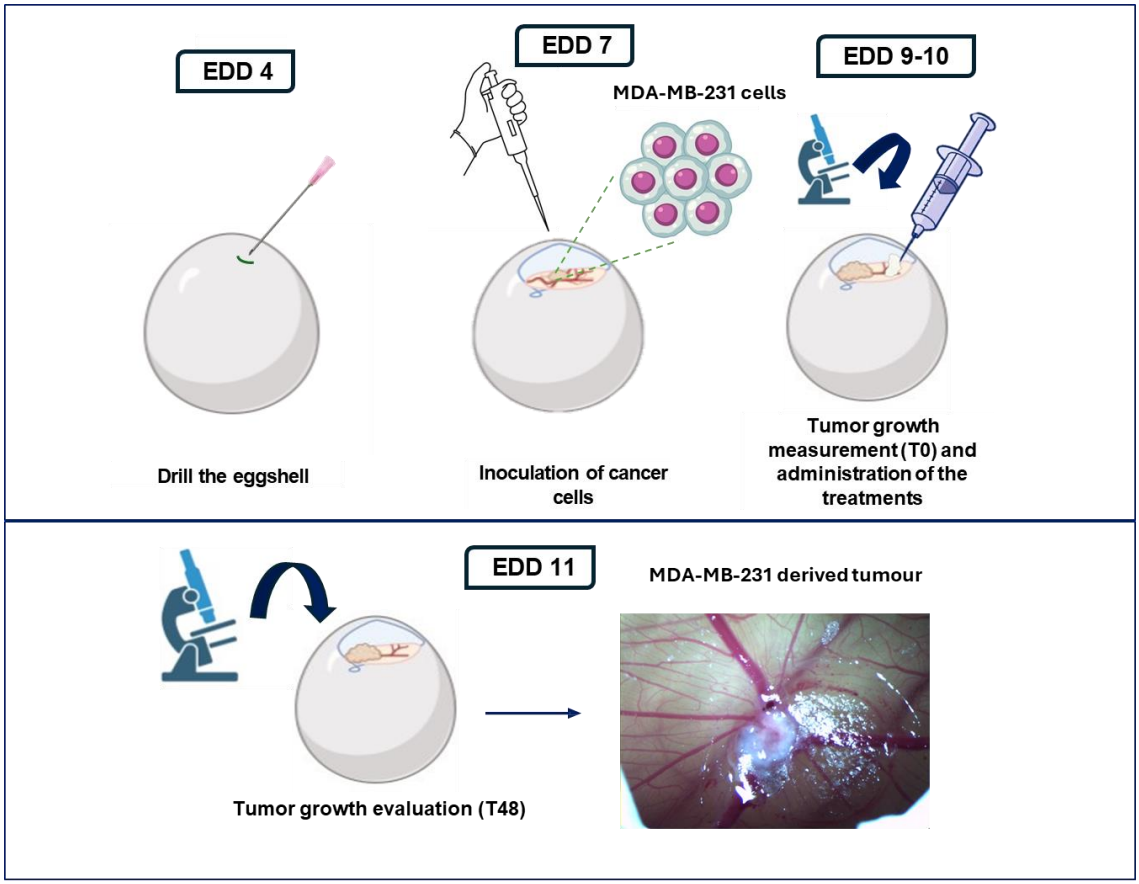


Figure 7: Angiogenesis study. Original and Image-J-Fiji processed images of the CAM membrane of PBS (control) (A), CBD in solution (B), PCL-ISFI (C) and 10-CB-ISFI (D) treated eggs. Images were captured 24 hours after the administration of each treatment. Graph showing the number of total blood vessels (E). * indicates statistically significant (p-value<0.05) differences between 10-CB-ISFI and the control.

3.4.2 Antitumour Efficacy

The CAM model offers a straightforward, fast, and cost-effective method for evaluating cancer characteristics, including treatment responses. Tumour cells can be easily grafted onto the CAM, with tumours forming rapidly within a few days. Figure 8A illustrates this model schematically. The anticancer effectiveness of 10-CB-ISFIs was tested on MDA-MB-231-derived tumours developed using this model. MDA-MB-231 cells were selected due to their human origin compared to 4T1 cells, which are murine cells.

48 hours after the treatment, the control group (eggs treated with PBS pH 7.4) showed tumour growth of around 21%. In contrast, the tumours treated with CBD in solution (7.7 µg of CBD were daily administered) or 10-CB-ISFIs (single administration of a CBD dose of 16 µg) not only stopped their growth but also were reduced by around 60% (Figure 8B). Unloaded implants did not exert anticancer activity, with tumour growth percentages slightly higher than the control (≈28%). Nonetheless, significant differences between the control and PCL-ISFIs were not detected (p-value >0.05).



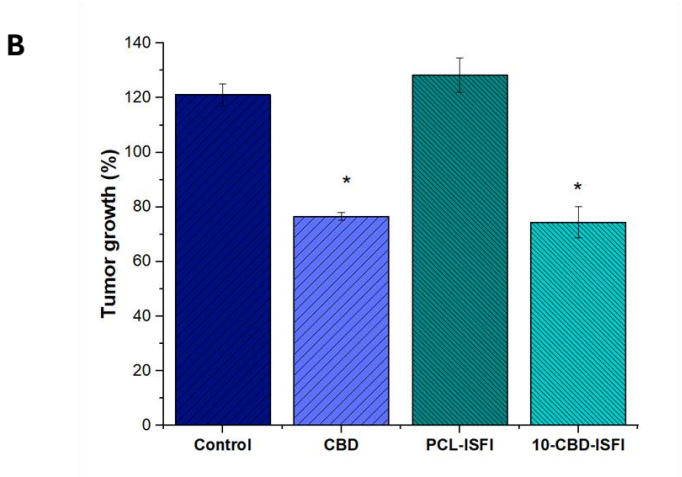


Figure 8: Schematic representation of tumour formation in the CAM model, including an image of a developed MDA-MB231-derived tumour (A). Tumour growth percentage (%) 48 hours after the administration of each treatment: PBS pH 7.4 (control), CBD in solution, PCL-ISFI and 10-CB-ISFI (B). * symbol shows statistical significance (p-value<0.05) between 10-CB-ISFI and the control.

CBD is an emerging anti-cancer agent that could potentially be administered in combination with other therapies, such as conventional antineoplastics [60] in many cancer types, including TNBC. The CBD-loaded implant formulations developed in this work proved to be

at least as effective as CBD in solution, exerting an antiangiogenic effect and inhibiting the growth of MDA-MB-231-derived tumours.

4. CONCLUSIONS

The polymeric solution composed of 100 mg of PCL and 400 µL of NMP showed a good injectability through conventional needles (23G and 25G), being suitable for parenteral administration. CBD-loaded implants prepared with this polymeric solution and containing 2.5 mg (2.5-CB-ISFI), 5 mg (5-CB-ISFI), or 10 mg (10-CB-ISFI) of CBD were rapidly formed when injected into a simulated physiological medium. They would form a deposit at the injection site and provide a controlled drug release after a single administration. 10-CB-ISFIs was selected as the optimal formulation because it would allow the administration of a higher amount of CBD with the same amount of polymer and provide a more controlled and prolonged release of CBD. This formulation showed an extended antiproliferative activity for at least 8 days and an anti-migration effect in both MDA-MB-231 and 4T1 cells (used as a model of TNBC). Additionally, in ovo studies demonstrated the ability of this formulation to inhibit vascularisation and reduce the size of MDA-MB-231-derived tumours. Interestingly, the single administration of CBD-loaded ISFIs was at least as effective as the daily administration of CBD in solution at the same concentrations. These results highlight the promising utility of the developed CBD-loaded implants for treating TNBC. This simple and easy-to-administer formulation can be injected at the tumour site during chemotherapy regimens, providing a long-lasting anticancer activity that could enhance the effect of currently available drugs.

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SUPPLEMENTARY MATERIAL

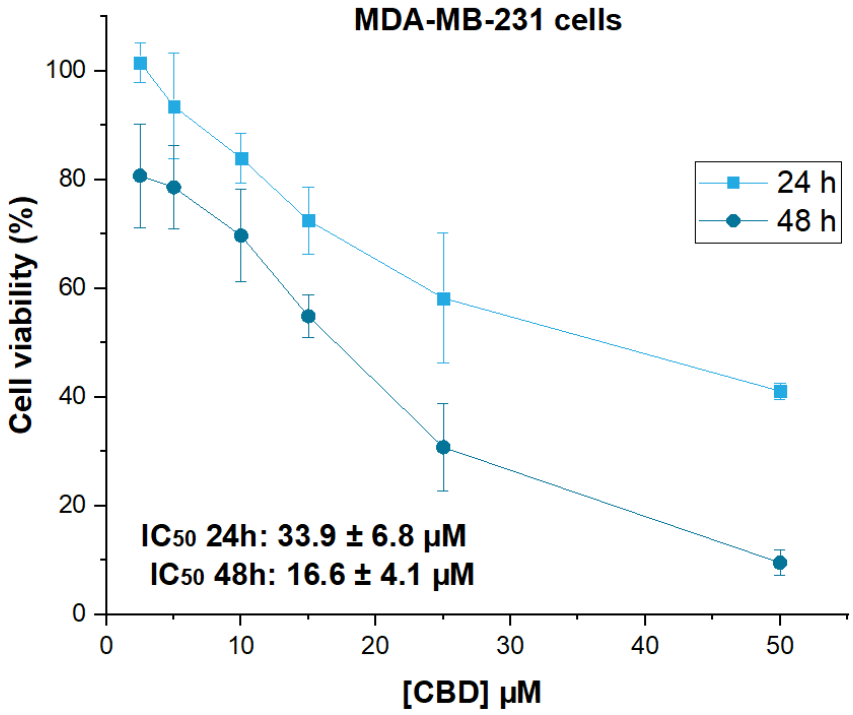

Figure S1: Effect of free CBD on MDA-MB-231 cells after 24 and 48 hours of treatment.

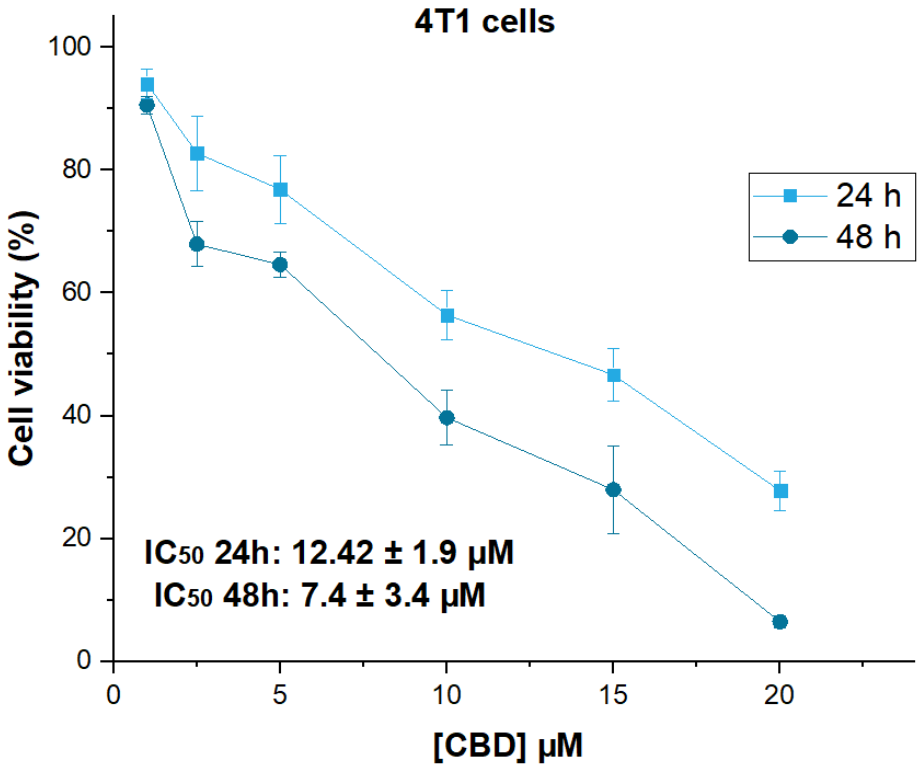

Figure S1: Effect of free CBD on 4T1 cells after 24 and 48 hours of treatment.

Table S1: Migration ratios in MDA-MB-231 cells.

	Migration ratios
CBD in solution 10 µM	0.40 ± 0.03
CBD in solution 20 µM	0.35 ± 0.02
10-CB-ISFI 10 µM	0.41 ± 0.02
10-CB-ISFI 20 µM	0.34 ± 0.02

Table S2: Migration ratios in 4T1 cells.

	Migration ratios
CBD in solution 5 µM	0.74 ± 0.11
CBD in solution 7.5 µM	0.63 ± 0.10
10-CB-ISFI 5 µM	0.73 ± 0.20
10-CB-ISFI 7.5 µM	0.58 ± 0.12

PART 2: Niosomes

INTRODUCTION

Niosomes are lipid-based vesicles (similar to liposomes) consisting of a bilayer membrane of synthetic non-ionic surfactants (primary ingredients in niosomes), and lipidic components that surround a hydrophilic (aqueous) core (Figure 1). Non-ionic surfactants like sorbitan esters (Spans and polysorbates (Tweens) are typically used and preferred over cationic, anionic or amphoteric surfactants due to their lower toxicity, higher biocompatibility and enhanced stability [1]. Cholesterol is the main lipidic ingredient used in niosomes. It enhances the stability of the vesicles by increasing the rigidity of the membrane and reducing its permeability [2, 3]. Niosomes are able to encapsulate both hydrophilic and lipophilic drugs in the inner-core and lipid bilayer respectively and show great potential as drug delivery systems.

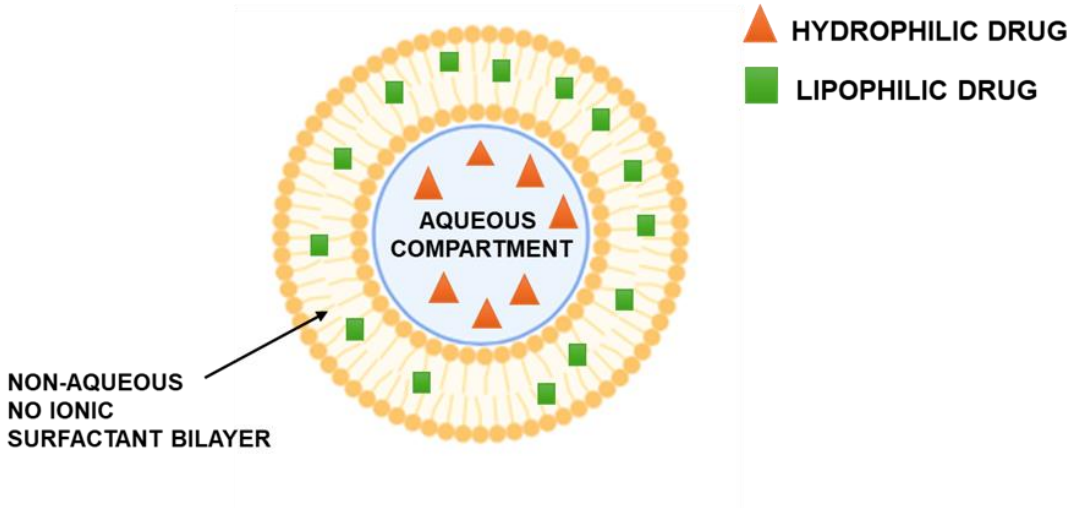

Figure 1: Schematic representation of a niosome

The manufacturing process of niosomes is similar to liposomes and also scalable at the industrial level. However, the production of niosomes is cheaper, as non-ionic surfactants used in the manufacturing of niosomes are less expensive than the phospholipids required for liposome production [4]. Moreover, niosomes are more stable, even at room temperature, due to their surfactant-based composition, which is less prone to hydrolysis and oxidative degradation compared to phospholipids [5]. This improved stability translates into longer shelf life and reduced risk of drug degradation during storage [6]. Additionally, niosomes can be more easily tailored for controlled and sustained drug release [7]. All these advantages make niosomes a compelling alternative to liposomes in drug delivery applications.

There are several methods available for the manufacturing of niosomes. Common techniques include the thin-film hydration method, the reverse phase evaporation method and the solvent injection technique (Table 1). In the thin-film hydration method (the most

used technique), non-ionic surfactants and cholesterol are dissolved in an organic solvent, typically chloroform or methanol. Then, the solvent is evaporated under reduced pressure to form a thin film on the walls of a round-bottomed flask. This film is subsequently hydrated with an aqueous medium, such as water, phosphate-buffered saline or HEPES buffer. The hydration process leads to the spontaneous formation of the bilayer vesicles. This method is advantageous due to its simplicity, scalability, and the ability to control the size of the niosomes by adjusting the ratio of surfactants, cholesterol, and hydration buffer. Sonication can be also used to reduce the size of niosomes [9, 10].

The reverse phase evaporation method is a technique based on creating water-in-oil emulsion. Non-ionic surfactants and cholesterol are dissolved in an organic solvent like dichloromethane. Then, an aqueous phase is added to form an emulsion. As the solvent is removed, the emulsion undergoes phase inversion, leading to the formation of stable niosomes. This method is particularly useful for encapsulating hydrophobic drugs and can produce niosomes with high encapsulation efficiencies and controlled size distributions [11, 12].

Finally, in the solvent injection method non-ionic surfactants and cholesterol are dissolved in an organic solvent, typically ethanol or chloroform. Then, they are injected into the aqueous phase. The solvent is diluted in the aqueous phase and the surfactant molecules self-assembled into bilayer structures, encapsulating the drug within the vesicles. This method is very simple and easily scalable at the industrial level. It is particularly effective for encapsulating hydrophilic drugs [13, 14].

Method of preparation	Characteristics	Particle size	Entrapment efficacy (%)
Thin film hydration	Widely used and simple Requires additional steps (i.e. sonication) for obtaining smaller particles with a low polydispersion	≈100-500nm	30-100
Reverse phase separation	High entrapment efficiencies Particularly useful for encapsulating hydrophobic drugs. More complex	≈50-200nm	90-100
Solvent injection	Simple. Particularly useful for encapsulating hydrophilic drugs. Typically, bigger particles are obtained with this technique.	≈250nm-3.9μm	40-90

Table 1: Main characteristics of the techniques that are commonly used for the elaboration of niosomes.

As mentioned earlier, niosomes have significant potential as drug delivery systems, being exploited for the administration of a broad range of drugs including antineoplastics. For instance, Pourmoghadasiya and collaborators developed paclitaxel-loaded niosomes using the thin-film method. The optimized niosomes were prepared using Span60 (6mM) and cholesterol (1mM) and showed a particle size of around 194nm and entrapment efficacy (EE) of 94%. This formulation exhibited a controlled paclitaxel release for 72h. At pH 7.4, approximately 50% of paclitaxel was released in 72 hours. However, a higher release was observed at acidic pHs values (approximately 65% and 80% of paclitaxel was released by this time at pH of 6.4 and 5.5 respectively). Interestingly, paclitaxel-loaded-niosomes exhibited a higher antiproliferative effect than free paclitaxel in estrogen positive breast tumor (MCF-7 and T-47D cells), HER-2 positive breast cancer (SkBr3 cells) and triple negative breast cancer (MDA-MB-231 cells). For example, in MCF-7 cells the IC_{50} of free paclitaxel after 72 hours of treatment was 69.28 μg/ml and of paclitaxel loaded niosomes of 25.35μg/ml. The higher antiproliferative activity of nanoencapsulated paclitaxel could be related to the internalization of the niosomes by tumor cells [15].

Niosomes loaded with doxorubicin have also been developed These niosomes were composed of Span60, Tween60 and cholesterol and prepared using the thin film hydration method. They showed a particle size of 170 nm and an entrapment efficacy of around 78% and a controlled doxorubicin release. At pH 7.4 only 35% of this drug was released after 72 hours. Its anticancer activity was evaluated in MCF-7 cells. Both, free doxorubicin and doxorubicin-loaded niosomes decreased the viability of these cells. However, the effect of free doxorubicin was slightly higher compared with doxorubicin loaded niosomes. While after 72 hours of incubation, the IC_{50} of doxorubicin was around 129 μg/ml, the value of the niosomal formulation was slightly higher of around 138 μg/ml. Several studies have reported that curcumin enhances the anticancer activity of doxorubicin. For this reason, niosomes loaded with both curcumin and doxorubicin were also prepared. This formulation exhibited a higher particle size than doxorubicin-loaded niosomes (≈ 298 nm) and high encapsulation efficiencies of both drugs (around 80%). These niosomes also exhibited a controlled release of both drugs with around 18% of curcumin and 25% of doxorubicin released in 72 hours. Like in the previous study, the niosomal formulation exhibited a slightly lower antiproliferative effect on MCF-7 cells than the co-administration of these drugs in solution, showing IC_{50} values of around 56,8 μg/ml and 50 μg/ml, respectively after 72 hours of incubation [16]. The lower activity of these niosomal formulations could be attributed to the controlled release of the drugs. Finally, Salem et al. developed niosomes loaded with tamoxifen citrate for breast cancer treatment. These nanoparticles were also prepared using the thin-film hydration method and exhibited an average size of 317 nm and an encapsulation efficiency (EE) of 89%. In vivo studies in a murine model of breast cancer developed by inoculating the subcutaneous tissue Ehrlich ascites tumoral cells (mammary adenocarcinoma) revealed a significant tumor growth inhibition in the mice treated with niosomes (this formulation was administered by intra-tumor injection at doses of 10 mg/kg after 1 and 6 days of tumor development). While, the control group displayed a tumor volume of 2100.93 ± 1.34 mm^3, and the mice group treated with free tamoxifen had a tumor volume of 1116.55 ± 1.24 mm^3 (tumor growth inhibition of 47.5% compared with the control group), the mice treated with tamoxifen loaded niosomes exhibited a significantly lower tumor volume of 510.57 ± 0.9 mm^3 (tumor growth inhibition 75.33% compared to the control group) [17].

Regarding cannabinoids, the use of niosomes has not been extensively exploited. Recently, Gugleva et al. developed cannabidiol (CBD) loaded niosomes. These niosomes were composed of non-ionic surfactants (Span20, Span60, Spa80, or a mixture of Span60 and

Tween60) and cholesterol and were prepared by the thin-film hydration method. The formulation prepared with span60 and tween60 as non-ionic surfactants exhibited the lowest particle size (around 150 nm) and the highest entrapment efficiency (around 85%). These niosomes showed a controlled CBD release for more than 72 hours. The effect of this formulation on cell proliferation was evaluated in T-24 cells (urinary bladder carcinoma), HIT-78 and MJ (cutaneous T-cell lymphoma). While empty niosomes did not showed any effect on the proliferation of these cells, CBD-loaded formulations exhibited an antiproliferative effect that was lower compared to the administration of free CBD. For example, in T24 cells the IC_{50} after 72 hours of incubation of free CBD was 12.2 µg/ml while the IC_{50} of CBD-loaded niosomes was 58.9 µg/ml [18].

In this doctoral thesis, a niosomal formulation of CBD **(Chapter 4)** has been developed as a strategy to improve the activity of this cannabinoid in triple-negative breast cancer. This formulation was prepared using Tween85 as a surfactant plus cholesterol. To the best of our knowledge, no niosome-based formulations of CBD have been previously tested in breast cancer.

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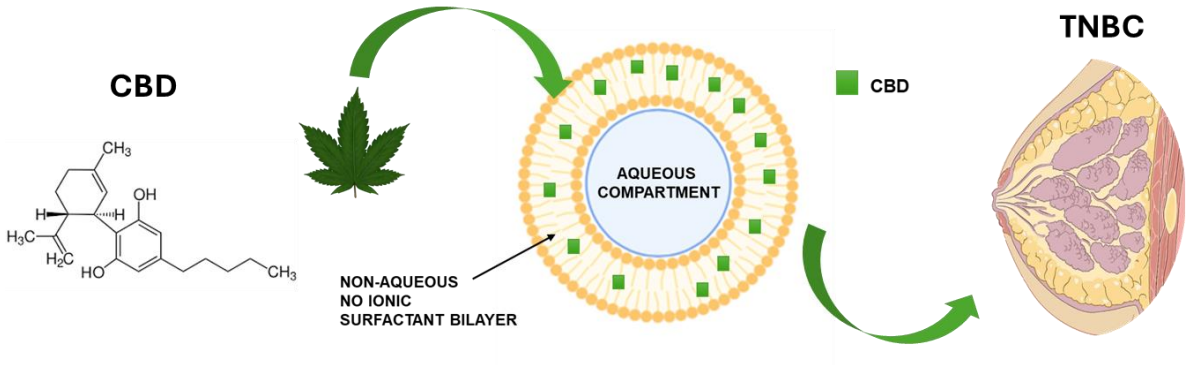
Chapter 4

Design and development of cannabidiol-loaded-niosomes as a strategy to improve the anticancer activity in triple negative breast cancer.



ABSTRACT

Triple-negative breast cancer (TNBC) is an aggressive and invasive subtype of breast cancer characterized by the absence of estrogen, progesterone and HER2 receptors, making it more challenging to treat than other forms of mammary carcinoma. Cannabidiol (CBD), the main non-psychoactive cannabinoid, has demonstrated promising anti-cancer properties against TNBC. However, its therapeutic use is hindered by its lipophilic nature. The use of nanocarriers may address this challenge and improve its anticancer effect. This study aims to design, develop and characterize niosomes for the delivery of CBD (CBD-Nio) to enhance its activity against TNBC. CBD-Nio, composed of Tween85 and cholesterol, were elaborated using the thin film hydration method. These niosomes exhibited a particle size of 113nm, a low polydispersity (PDI value of 0.2), a drug loading of 0.92±0.025mg per ml of formulation, a high entrapment efficiency (~92%) and a controlled CBD release for 24 hours. Compared to free CBD, CBD-Nio demonstrated an enhanced antiangiogenic effect in ovo and an improved antiproliferative activity in MDA-MB-231 and 4T1 cells (models of TNBC) with lower IC_{50} values. Furthermore, they reduced the migration of these cells. Finally, CBD-Nio were capable of reducing the growth of MDA-MB-231 derived tumors developed on the chorioallantoic membrane of chick embryos, exhibiting slightly greater activity than free CBD. Although further studies are required, all these findings suggest that niosomes are a promising nanocarrier for CBD delivery with the potential to improve its therapeutic activity in TNBC.

Keywords: Triple negative breast cancer, cannabidiol, nanoparticles, niosomes.

1. INTRODUCTION

Cannabis sativa L. plant has been used for medical purposes for thousands of years. Cannabinoids are the main compounds responsible for their therapeutic effects. Among all cannabinoids, cannabidiol (CBD) is one of the most interesting in therapeutics, as it is the main non-psychoactive and the second most abundant cannabinoid [1]. CBD has shown antioxidant, anticonvulsant, anti-inflammatory, neuroprotective and anticancer properties among others [2]. In the context of cancer disease, CBD has demonstrated to inhibit the growth and metastases of a broad range of tumors such as lung cancer, gastrointestinal malignancies, brain tumors, melanoma and breast carcinomas. Moreover, it shows an antiangiogenic activity [3]. Precisely one of the tumors in which CBD exhibits promising anticancer activity is breast cancer, including triple-negative breast cancer (TNBC).

TNBC is one of the most studied cancers. It represents around 15-20% of all breast cancer diagnoses. More than 350000 women were diagnosed with this disease worldwide and 150000 died in 2022 worldwide [4, 5]. TNBC is characterized by a lack of expression of both hormone (estrogen and progesterone) and Human Epidermal Receptor type 2 (HER-2) receptors [6]. This carcinoma is very aggressive and difficult to treat [7] compared with other breast tumors with lower survival rates (five-year survival rate is around 65% in regional breast cancer and 11% in distant or metastatic disease) [8]. This carcinoma is usually treated with chemotherapy in neoadjuvant (administered before the surgery) or adjuvant (administered after the surgery) settings [9].

Several studies have demonstrated that CBD inhibits the proliferation of TNBC cells (e.g. MDA-MB-231, MDA-MB-468 and 4T1 cell lines) due to the promotion of reactive oxygen species and the subsequent induction of apoptosis [10–12]. Moreover, it decreases the migration and invasion capability of these cells [13]. This latter effect has been attributed to the downregulation of Inhibitor Differentiation Protein 1 (ID-1), which is overexpressed in TNBC [14]. CBD also potentiates the effect of conventional antineoplastics, including the activity of paclitaxel and doxorubicin, drugs that are commonly used in TNBC [15, 16]. Studies conducted on MDA-MB-231 cells demonstrated that the inhibitory concentration 50 (IC_{50}) after 48 hours of incubation with paclitaxel or doxorubicin co-administered with CBD was reduced by approximately 2 to 7 times depending on the CBD concentration compared to the administration of these antineoplastics alone. The efficacy of paclitaxel (85.39 µg/ml) in combination with CBD (31.4 µg/ml) was also evaluated in ovo using MDA-MB-231 derived tumours. Notably, the combination treatment led to a significantly greater reduction in tumor

growth than paclitaxel alone, with inhibition percentages of approximately 60% and 40%, respectively [17]. Furthermore, the combination of doxorubicin (2 mg/kg) plus CBD (5 mg/kg) was tested in vivo in a subcutaneous MDA-MB-231 tumor model. After two weeks of treatment, the tumour volume was significantly smaller in the doxorubicin plus CBD treated group compared to the doxorubicin treated group. Mice treated with the combination exhibited an average tumor volume of approximately 4000 mm^3, whereas the mice receiving doxorubicin alone had a volume of around 4500 mm^3 [18]. These results demonstrate that the combination of CBD with paclitaxel and doxorubicin is very interesting, as it allows to reduce the dose of these anticancer drugs administered, and therefore their adverse effects, without impacting their anticancer efficacy. Moreover, it has been reported that CBD alleviates some chemotherapy-induced side effects, such as doxorubicin cardiotoxicity and paclitaxel peripheral neuropathy [19] making the combination even more interesting [20].

Despite the potential therapeutical interest of CBD, it has a low water solubility (Log P 6.3), low stability and erratic and poor oral bioavailability hindering their administration. Its nanoencapsulation could be a good technological resource to address these issues and administer CBD [21]. Furthermore, due to the Enhanced Permeability and Retention (EPR) effect, nanomedicines tend to accumulate in the tumors. Tumor vasculature is deficient, and these blood vessels are more permeable than normal vessels, allowing nanoparticles to pass through more easily and accumulate at the tumor site. Additionally, the interstitial pressure within the tumor is higher, which enhanced the retention of extravasated nanoparticles. This phenomenon can potentially improve the effectiveness of nanoencapsulated drugs [22].

Niosomes (Nio) are lipid-based vesicular structures, similar to liposomes, consisting of a bilayer membrane composed of non-ionic surfactants (their main constituents) and lipidic compounds (mostly cholesterol), surrounding an aqueous core. They were first introduced in the seventies for cosmetic use by L'Oréal, Paris. However, in the last decade, they have gained considerable popularity as drug delivery systems, especially as an alternative to liposomes [23, 24]. Niosomes are biocompatible, biodegradable and capable of encapsulating both hydrophilic and lipophilic drugs (they are located in the lipid bilayer and the inner aqueous core respectively) [25]. Compared with liposomes, niosomes are more stable and more reproducible and less expensive to manufacture especially at the industrial level [26, 27].

Several researchers have developed niosomes for the targeted delivery of antineoplastics in TNBC. For instance, Akbarzadeh et al. developed tamoxifen loaded niosomes composed

of sorbitan esters (spans) and cholesterol. While unloaded niosomes did not affect the viability of MDA-MB-231 cells, tamoxifen loaded niosomes exhibited a significant antiproliferative effect. Notably, their activity was superior to that of non-encapsulated tamoxifen, with IC_{50} values of approximately 60 μg/ml for tamoxifen-loaded niosomes, compared to 140 μg/ml for the free drug after 72 hours of incubation [28]. Similar results were obtained by Ajdari and collaborators who developed niosomes loaded with docetaxel. In this case, while free docetaxel exhibited an IC_{50} value of 12 μg/ml after 48 hours of incubation, the niosomal formulation showed a lower value of around 5 μg/ml [29]. These studies underscore the potential of niosomes for the effective delivery of anticancer agents, including cannabinoids. Notably, in a previous study, Gugleva et al. prepared CBD-loaded niosomes composed of cholesterol and various sorbitan esters (Span 20, 60, or 80) or a combination of Span 60 and polysorbate 80 (Tween 80). The antiproliferative effect of this nanoformulation was tested on T-24 cells (urinary bladder carcinoma), HIT-78 cells and MJ cell (T-cell lymphoma) [30]. To the best of our knowledge, no CBD niosome formulations have been specifically developed or reported for the treatment of breast cancer. The main aim of this work is to design, develop and characterize niosomes loaded with CBD as a strategy to enhance its activity in TNBC.

2. MATERIALS AND METHODS

2.1 Materials

Tween 85, cholesterol, pyrene (Pyr), and HEPES buffer were supplied by Sigma-Aldrich (Milan, Italy). Cannabidiol was purchased from THC Pharm (Frankfurt, Germany). MTT reagent was obtained from Acros Organics (Geel, Belgium). Dialysis membranes (MWCO 8000 Da), ethanol, methanol, and chloroform were provided by Carlo Erba Reagents Srl, (Milan, Italy). RPMI 1640 medium, Trypsin-EDTA (0.25%), phenol red, mitomycin C, phosphate buffer solution (PBS), Fetal Bovine Serum (FBS), and Geltrex Matrix were supplied by Fisher Scientific (Madrid, Spain). Milli-Q® demineralized obtained by Millipore® system water was used.

2.2 Niosome preparation

CBD-loaded niosomes (CBD-Nio) were obtained using the thin film hydration method [31]. Briefly, 29mg of cholesterol, 32mg of Tween 85 and 5mg of CBD were dissolved in 2 ml of a mixture of chloroform/methanol (3:1, v/v). Then, the solvents were evaporated using a rotary vacuum evaporator at room temperature for 2 hours (Rotavapor® R-210, Büchi, Milan, Italy), and a thin film was obtained on the wall of the tube. The dried film was then hydrated

with 5mL of HEPES buffer (10 mM, pH 7.4) and mixed using a vortex stirrer (Vortex-Genie 2, Scientific Industries, Inc., USA). The result suspension was sonicated at room temperature (25ºC) for 10 minutes at 30% amplitude using a 20 kHz probe sonicator (VibraCell-VCX 600-Sonics, Massachusetts, USA). Finally, niosomal suspension was purified by centrifugation at 14,000 rpm and 4ºC for 1 hour. Then, the supernatant was collected and filtered with a 0.22 μm membrane filter (MF-Millipore®, Ireland, E.U.) and used for the following tests [32]. Unloaded niosomes (B-Nio) were also prepared following the same protocol without the addition of CBD.

2.3. Morphology

The morphology of CBD-loaded-niosomes was examined by scanning electron microscopy (SEM) using a JSM-IT700HR scanning electron microscope (Jeol, Tokyo, Japan). Niosome suspension was placed onto aluminium stubs and placed in a desiccator at room temperature for 48 hours. Then, the samples were coated with graphene using a Q150TE evaporator (QUORUM) [33] and examined by SEM.

2.4 Physical-chemical characterization of niosomes

2.4.1 Particle size, zeta potential, and polydispersity index

Hydrodynamic diameter and zeta potential were measured by dynamic light scattering using a Zetasizer Nano ZS 90 (Malvern Instruments, Worcestershire, UK). The Zetasizer used had a scattering angle of 90.0° and was equipped with a 5 mW HeNe laser (λ = 632.8 nm).

2.4.2 Anisotropy

The anisotropy of the formulations was studied using 1,6-Diphenyl-1,3,5-hexatriene (DPH) probe [34]. For instance, B-Nio and CBD-Nio loaded with DPH were prepared using the protocol in section 2.2. DPH was included in the organic mixture with the other lipophilic compounds at a concentration of 2×10^{-4} M. DPH was excited with polarized light and with excitation λ ex = 350 nm and detecting the fluorescence intensity at λ em = 428 nm using a luminescence spectrometer (LS5013, PerkinElmer, Waltham, MA, USA) [35]. The spectrofluorometer was set to measure vertical-vertical angle light at 00VV, horizontal-vertical (HV) angle light at 900HV, horizontal-horizontal (HH) angle light at 990HH and vertical-horizontal (VH) angle light at 090HV. The anisotropy (α) was calculated using the following equations:

$$\alpha = \frac{IVV-(G*IVH)}{IVV+(2G*IVH)} \quad \text{(Eq.1)}$$

$$G = \frac{IHH}{IHH} \text{ (Eq.2)}$$

where I_{VV}, I_{VH}, I_{HV} and I_{HH} are the fluorescent intensities at vertical-vertical, vertical-horizontal, horizontal -vertical and horizontal-horizontal measurements. G refers to the ratio of sensitivity for vertically and horizontally polarized light.

2.4.3 CBD loading and entrapment efficiency

The amount of encapsulated CBD was obtained using a UV-visible spectrophotometer (Lambda 25, PerkinElmer, Waltham, MA, USA). CBD-Nio were dissolved in a mixture of isopropanol: HEPES at a ratio of 3:1 (v/v). Then, the solution was filtered (with a 0.22 µm membrane filter, MF-Millipore®, Ireland, E.U.) and analysed spectrophotometrically at 274 nm. The quantification method was previously optimized. A calibration curve of CBD dissolved in isopropanol/HEPES mixture (3:1 v/v) at concentrations ranging 0.03 and 0.5 mg/ml were prepared. The following equation was obtained and used for CBD quantification: y=0,552x+0,0137 with R^2=0.997.

The entrapment efficiency (EE), was calculated using the following equation:

$$EE\ (\%) = \frac{\text{Amount of CBD encapsulated}}{\text{Amount of initial CBD added}}\ x\ 100 \quad \text{(Eq. 3)}$$

2.5 CBD release

The release profile of CBD from CBD-Nio was evaluated in HEPES buffer (10 mM, pH: 7.4) using the dialysis membrane method [32]. Briefly, 550 µL of CBD-Nio suspension and 450 µL of HEPES buffer were placed in a dialysis bag (MWCO 8 kDa, Spectra/Por®). Then, it was immersed into 4 ml of release medium (HEPES/Ethanol 1:1, v/v). The release system was maintained at a temperature of 37°C and gently stirred at 100 rpm using a magnetic stirrer. At specified intervals (2h, 4h, 6h, 8h, and 24h) 1mL of the outer compartment was taken, analyzed by UV-visible spectrophotometer (Lambda 25, PerkinElmer, Waltham, MA, USA) and replaced with fresh medium. Release experiments were conducted in triplicate.

2.6 Stability studies

The physical stability of the B-Nio and CBD-Nio formulations was assessed at 4°C and 25°C over a period of 3 months. At specific time points (0, 30, 60, and 90 days), samples of both niosomal suspensions were analyzed by DLS to determine particle size, polydispersity index (PDI), and ζ-potential [36] as outlined in section 2.4.1.

2.7 Evaluation of the anti-antiangiogenic effect

The effect of CBD-Nio on angiogenesis was evaluated using the yolk sac model (YSM) assay [37]. Fertilized chicken eggs (purchased from Granja Santa Isabel, Cordoba, Spain) were incubated at 37°C and 47% humidity in a rotary incubator until embryonic development day 2 (EDD 2). At this point, viable eggs were selected, and a small hole was carefully made in the eggshell of each egg using a 19-G needle. The hole was sealed, and the eggs were returned to the incubator in a stationary position. On EDD 3.5, the hole was enlarged, and 2-3 mL of albumen were removed. After one hour of stabilization, the chorioallantoic membrane (CAM) of the eggs was treated with 200 µL of CBD solution at a concentration of 100 µM or the equivalent amount of CBD-Nio (calculated based on CBD loading). The effect of B-Nio and NaCl 0.9% solution (used as negative control) was also studied. At EDD 5.5 (48 hours after the treatment), the CAM of each egg was photographed using a Ninyoon 4K microscope (APEX CE Specialists Limited, Manchester, UK) [38]. The total number of blood vessels and vascular density were then quantified using Image J software [39]. A minimum of 5 eggs were studied per treatment.

2.8. Evaluation of the anticancer activity in TNBC

2.8.1 Cell lines

Murine 4T1 (CRL-2539) and human MDA-MB231 (HTB-26 ATCC) cell lines were used as model of TNBC. Both cell types were grown in RPMI-1640 containing 10% (v/v) of FBS and 1% (v/v) of penicillin-streptomycin at 37ºC and 5% CO_2 [40].

2.8.2 Antiproliferative effect

4-T1 and MDA-MB-231 cells were seeded at a cell density of 3000 and 5000 cells/per well respectively in a 96-well plate (CorningTM, Thermo Fisher Scientific, Madrid) [41]. 24 hours after seeding, the growth medium was removed, and the cells were treated with free CBD and CBD-Nio at different CBD concentrations (1-15 µM in 4T1 cells and 5-40 µM in MDA-MB-231 cells). 24 and 48 hours after treatment, cell viability was evaluated using an MTT

assay. Briefly, 200 µL of MTT solution (0.5 mg/mL in complete RPMI medium) was added to each well and incubated at 37ºC and 5% CO_2 for 3 hours. Then, the supernatant was gently removed and 200 µL of DMSO was added to each well to dissolve the formazan crystals. The plate was then stirred at 100 rpm for 10 minutes and the absorbance was measured at 570 nm using a spectrophotometer (Varioskan 3020-2023 LUX, Thermo Fisher Scientific, Madrid, Spain). The effect on cell viability of B-Nio formulation was also analyzed. These studies were carried out in quadruplicate.

2.8.3 Inhibition of cell migration

The effect of CBD-Nio on the migration of 4T1 and MDA-MB231 cells was analyzed using scratch assay [42]. 4T1 and MDA-MB231 cells were seeded into 8-well plates (µ-slide 8, Thermo Fisher Scientific, Madrid, Spain) at cell densities of 10000 and 25000 and cells/well respectively. They were allowed to grow to 90-100% confluence before treatment. After reaching confluence, the cells were incubated with mitomycin C (10 µg/ml) for 30 minutes to inhibit cell proliferation. Then, each well was scratched with a 200 µL pipette tip, the mitomycin C solution was removed and the cells were gently washed twice with PBS (pH 7.4). Cells were then treated with free CBD or CBD-Nio at different concentrations (5 µM and 7.5 µM in 4T1 cells and 10 µM and 20 µM in MDA-MB-231 cells). The scratch produced in each well was photographed before (T_0) and 4, 6, and 24 hours ($T_{4\text{-}24}$) after the treatment using a Primotech 500X microscope (Zeiss, Jena, Germany). Cells treated with a complete RPMI-1640 medium served as control of cell migration.

The images were processed using Image-J software and the percentages of wound closure at each time were calculated using the following equation:

$$\text{Wound closure } (\%) = \frac{\text{Area T0} - \text{Area Tx}}{\text{Area T0}} \text{ x } 100 \quad \text{(Eq. 4)}$$

where wound area at T0 indicates the area of the scratch wound before the treatment and Tx the area after 2,4,6, or 24 hours of treatment.

Migration ratios were also calculated as follows:

$$\text{Migration ratio} = \frac{\text{Wound clousure CBD treatment}}{\text{Wound clousure control}} \qquad \text{(Eq. 5)}$$

2.8.4. *In ovo* antitumor efficacy

The antitumor efficacy of CBD-Nio was also investigated *in ovo* using the chorioallantoic membrane (CAM) assay [43]. Chicken embryos, purchased from Granja Santa Isabel (Córdoba, Spain), were incubated at 37°C and 47% of humidity with automatic rotation until EDD4. That day the rotation was stopped, and the eggshell of the eggs was carefully drilled and sealed with tape to prevent desiccation. On EDD7, the hole was expanded, and CAM was gently scratched using a 25G-needle. Then, 2 x 10^6 of MDA-MB231 cells suspended in Geltrex® extracellular matrix were added. On EDD9, the tumors formed were outlined with a 1 cm diameter silicone O-ring and photographed using a Ninyoon 4K microscope (APEX CE Specialists Limited, Manchester, UK). Then, the eggs were treated topically with free CBD, CBD-Nio or PBS at pH 7.4 (these eggs served as control). CBD treatments were administered at a CBD concentration of 100µM. On EDD 11 (48 hours after the treatment) the tumors were photographed using a Ninyoon 4K microscope (APEX CE Specialists Limited, Manchester, UK) and the area of each tumor was calculated using ImageJ software. Then, tumor growth was calculated as follows:

$$Tumour\ growth\ (\%) = \frac{\text{Area 48h}}{Area\ \ 0h}\ x\ 100 \quad \text{(Eq. 6)}$$

2.9. Statistical data analysis

Statistical analyses were conducted using IBM SPSS Statistics 28 software (IBM, Chicago, Illinois). Data are presented as the mean ± standard deviation (SD) from at least three experiments (n=3). Student's t-test was applied to assess the significance of the differences detected between the two groups. The graphics were elaborated using Origin19 software (OriginLab, Massachusetts, USA).

3. RESULTS AND DISCUSSION

3.1 Morphology, particle size, PDI and zeta potential

Niosomes represent the new generation of vesicular nanocarriers and have emerged as an alternative to liposomes due to their enhanced stability and cost-effective manufacturing, becoming interesting drug delivery systems for a wide range of disorders, including cancer disease [32]. The formation of niosomes typically requires the addition of a nonionic surfactant and a bilayer-inducting agent (usually cholesterol). In this study, niosomes composed of Tween 85 and cholesterol and loaded with CBD were successfully developed

using thin hydration method, a well-established technique for preparing this type of nanoformulation [44].

Figure 1 illustrates the morphology of developed niosomal formulations. Both B-Nio and CBD-Nio nanoparticles exhibited a pseudospherical shape. The incorporation of CBD did not modify the morphology of niosomes. However, it did affect the particle size of the vesicles, as CBD-Nio nanoparticles were smaller. This observation was confirmed by DLS. While B-Nio showed a particle size of approximately 137nm and CBD-Nio exhibited a significantly smaller size (p-value<0.05) around 113 nm (Table 1). Given that CBD is a lipophilic compound (Log P = 6.3), it is incorporated within the lipid bilayer, which may increase the bilayer's hydrophobicity, leading to a reduction in surface free energy and, consequently, a smaller particle size [44–46]. According to the literature, nanoparticles with sizes ranging from 100 to 300 nm tend to accumulate in tumors after i.v administration by EPR effect, and therefore, the CBD-loaded niosomes fall within this size range, making them suitable for achieving selective CBD release in TNBC [47].

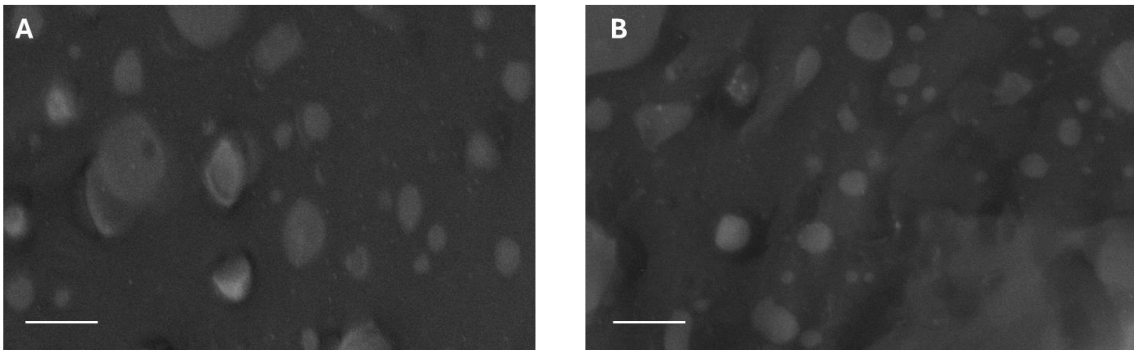


Figure 1: Images obtained by SEM of B-Nio (A) and CBD-Nio (B). Scale bar: 0.3 µm

	Particle size (nm)± sd	**PDI ± sd**	**Zeta Potential (mV) ± sd**
B-Nio	137 ± 1	0.25 ± 0.008	-37.3 ± 0,77
CBD-Nio	113 ± 2	0.20 ± 0.007	-33.8 ± 1,6

Table 1: Particle size, zeta potential, and polydispersity index (PDI) value of B-Nio and CBD-Nio obtained by DLS.

CBD-Nio also exhibited a statistically significant lower PDI value (p-value < 0.05) compared to B-Nio (0.20 vs 0.25 respectively) (Table 1). These values indicate low polydispersity of the developed niosomes, as PDI values below 0.3 generally suggest a size distribution that is nearly monodisperse [48]. Additionally, both formulations exhibited a negative zeta potential with CBD-Nio exhibiting a statistically significant slightly higher value (p-value < 0.05) than B-Nio (-33 mV vs -37 mV respectively). According to the literature, zeta potential values higher than ± 20 mV guarantee sample stability as prevent particle aggregation [49].

In a previous study, Gugleva and co-workers developed niosomes loaded with CBD. These formulations were prepared using Span20 (HLB= 8.6, S20-Nio), Span60 (HLB= 4.7, S60-Nio) or Span80 (HLB= 4.3, S80-Nio) as non-ionic surfactants [30]. In the present study, CBD-Nio was prepared using Tween85, which has a higher HLB value (HLB=11). The literature reports that the higher the HLB value of non-ionic surfactants, the larger the particle size of the niosomes [50]. Therefore, larger particle sizes could be expected in the niosomes prepared with Tween85 compared with the formulations prepared with spans. However, S20-Nio, S60-Nio and S80-Nio formulations exhibited larger particle sizes ranging from 186 to 230 nm, depending on the formulation. Interestingly, when CBD-loaded niosomes were prepared with Tween 60 (HLB = 14.9), Span60, and cholesterol (T60-S60-Nio), the particle size decreased to 150 nm. These results suggest that the incorporation of Tweens can lead to the formation of smaller CBD niosomes. The larger particle sizes observed in the S20-Nio, S60-Nio, S80-Nio, and T60-S60-Nio formulations may also be influenced by the choice of hydration buffer (these formulation were prepared using PBS as hydration buffer), as it has been reported to affect the niosome particle size [51], and also by the sonication time. While these formulations were sonicated for 2-5 minutes[30], CBD-Nio of this study were sonicated for 10 minutes, and in the development of nanoformulation, the sonication time is one of the most influential factors affecting the particle size. Typically, the higher the sonication time, the smaller the particle size [38].

3.2. Anisotropy

The structure of the vesicular membrane of B-Nio and CBD-Nio was also investigated by determining anisotropy using niosomes loaded with DPH. CBD-Nio exhibited a slightly higher anisotropy value (0.185) compared to B-Nio (0.168). Anisotropy is a measure of membrane fluidity, with higher values indicating less fluidity. CBD-Nio has a more rigid bilayer than B-Nio, supporting the localization of CBD within the vesicle membrane [52].

3.3 CBD loading, entrapment efficiency and CBD release profile

CBD-Nio formulation exhibited a drug loading of 0.92±0,025 mg of CBD per 1 ml of formulation and a high entrapment efficiency (92±2.09%). CBD is highly lipophilic and tends to be rapidly entrapped into the lipid bilayer during niosome formation. Notably, the formulation developed in this study (CBD-Nio prepared with Tween 85 and cholesterol) showed a higher entrapment efficiency compared to niosomes from the previous study of Gugleva and co-workers, prepared using Spans and cholesterol (S20-Nio, S60-Nio, and S80-Nio). These formulations exhibited EE values ranging from 65 to 81%. Interestingly, the formulation prepared with Tween 60 and span 60 as non-ionic surfactants (T60-S60-Nio) showed a higher EE (≈ 93%) [30], which is similar to the EE of the CBD-Nio formulation in the present study. The higher drug content in niosomes containing Tweens than Spans may be related to the smaller particle size of Tween based niosomes. As mentioned earlier, CBD is very lipophilic and may increase bilayer hydrophobicity, and consequently decrease the particle size of the vesicles.

3.4 CBD release

As depicted in Figure 2, CBD-Nio exhibited controlled CBD release for 24 hours, with more than 88%±4.07 of CBD released by this time. A relatively high burst effect occurred, with approximately 30% of CBD released within the first 2 hours, and around 60% released within 6 hours. Comparing to our niosomes, T60-S60-Nio formulation exhibited a slower CBD release with about 20% of CBD released after 6 hours and approximately 35% after 24 hours [30]. These differences may be attributed to the type of surfactant used in each formulation and especially to their HLB value [53]. The higher the HLB value of the non-ionic surfactant the faster the drug release [54, 55].

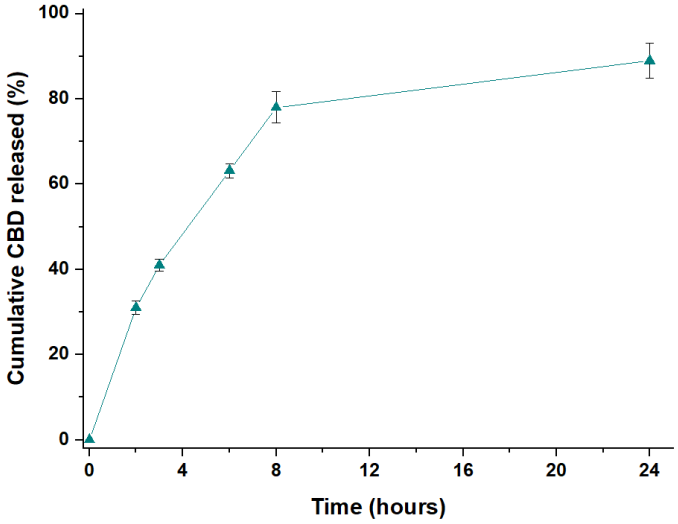


Figure 2**:** CBD release profile of CBD-Nio.

3.5 Stability studies

The physical stability of B-Nio and CBD-Nio formulations was evaluated over a three-month period at 25ºC and 4ºC by analyzing particle size, PDI, and zeta potential (Figure 3). When stored at 4ºC, the particle size of B-Nio remained stable for the first two months, with no significant changes in the mean particle size (p-value > 0.05). However, after three months the particle size significantly increased (p-value<0.05), reaching approximately 160 nm, compared to the initial size of around 136.6 nm. At 25 ºC, the particle size was stable only for one month. After this period, a particle size of 150nm was appreciated. The increase of the particle size can likely be attributed to the fusion of the niosomal vesicles [45]. In contrast, CBD-Nio demonstrated higher physical stability, with no significant changes in particle size observed over the entire three-month period. Although there was a slight increase in the particle size, from approximately 113 nm to around 115–120 nm at both 4ºC and 25ºC, this change was not statistically significant (p-value > 0.05) (Figure 3A). The differences observed between empty and CBD-loaded niosomes can be attributed to the presence of the cannabinoid in the lipid bilayer, which may exert a stabilizing effect. This is related to the ability of CBD to increase membrane rigidity, as evidenced by the higher anisotropy of CBD-Nio compared to B-Nio.

Regarding the PDI values (Figure 3B), no major changes in both B-Nio and CBD-Nio formulations were observed for the first two months when they were stored at both 4 ºC and 25 ºC. However, after 3 months, both B-Nio and CBD-Nio formulations exhibited a higher polydispersity, reaching PDI values of around 0,37±1,2 and 0,35±1,3, respectively.

Nevertheless, statistically significant differences were just achieved ($p<0.057$). Finally, regarding the zeta potential (Figure 3C), no significant changes were observed in either B-Nio or CBD-Nio when stored at 4ºC or 25ºC.

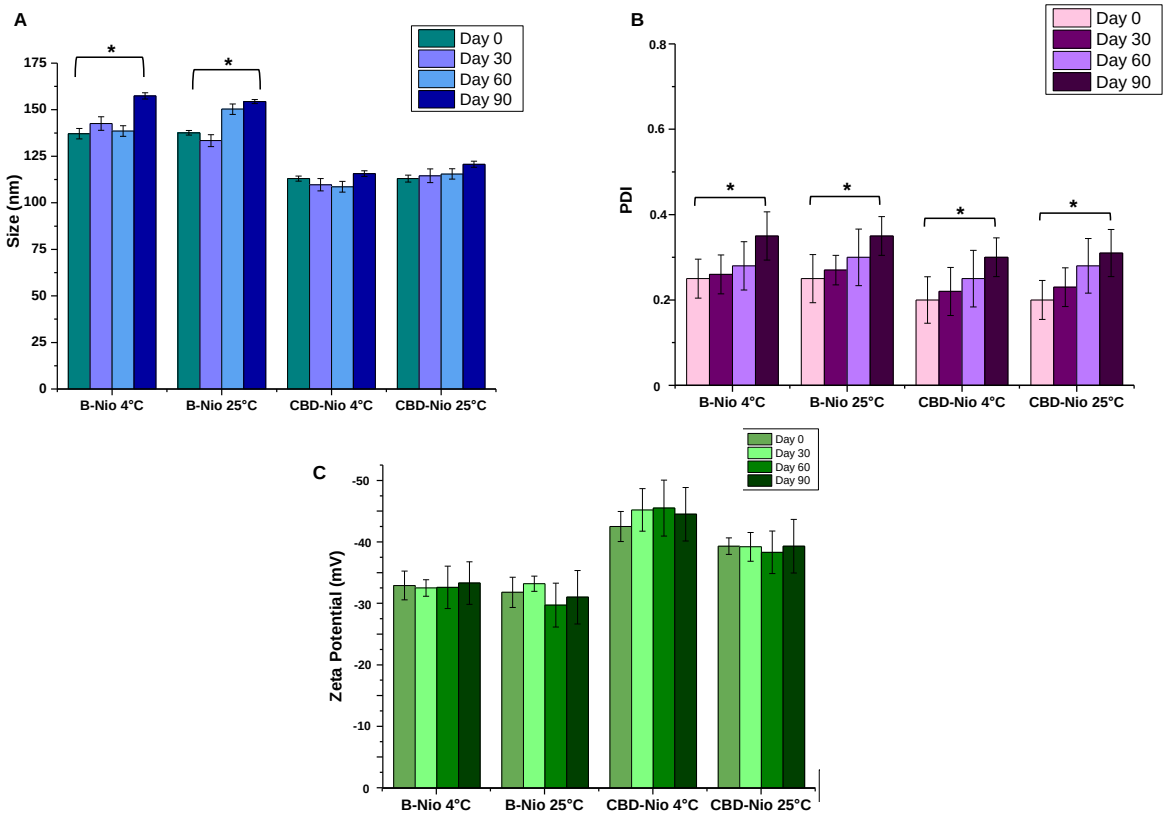


Figure 3: Particle size (A), PDI values (B) and zeta potential (C) of B-Nio and CBD-Nio stored at 4ºC and 25ºC for three months.

3.7 Evaluation of the anti-antiangiogenic effect

As shown in Figure 4A both CBD in solution and CBD-Nio reduced the vascularization of CAM membrane. Notably, niosomes loaded with CBD demonstrated a stronger effect compared to free CBD. While free CBD resulted in a blood vessel inhibition percentages of 72.7 ± 8.5%, CBD-Nio showed a higher reduction of 82.8 ± 4.1%. Additionally, vascular density was assessed, with free CBD exhibiting a density of 12.7 ± 1.8 and CBD-Nio of 11.5 ± 0.93, which represents a 1.2-fold decrease compared to the free drug. However, statistically significant differences between these treatments were not appreciated. B-Nio did not exert any effect on the vascularization of the chorioallantoic membrane. No signs of toxicity of both B-Nio and CBD-Nio on CAM membrane were observed.

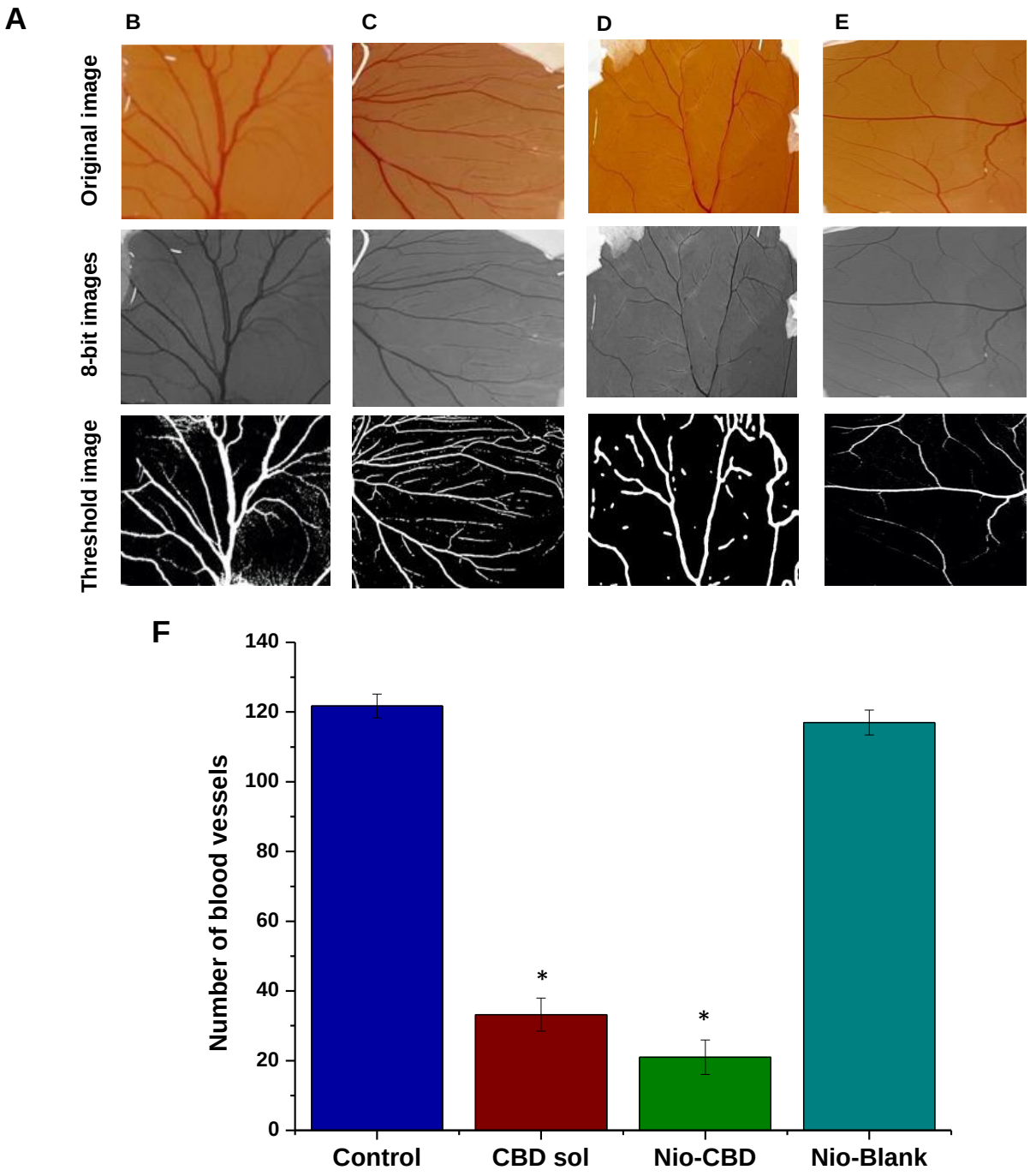


Figure 4: Original and Image J processed images of the CAM of eggs treated with NaCl 0.9% solution (Control, A), B-Nio (B), free CBD (C) and CBD-Nio (D). CBD. Treatments were administered at a drug concentration of 100µM. At least 5 eggs/per treatment were analyzed. * Indicates statistically significant differences between control group and each treatment.

3.8 Evaluation of the anticancer effect in TNBC

3.8.1 Antiproliferative studies

Several studies have demonstrated that CBD reduces the proliferation of TNBC cells [56]. In the present work, the antiproliferative effect of CBD-Nio was evaluated in two triple-negative cell line models: MDA-MB-231(human origin) and 4T1 (murine origin) cells. The empty niosomes showed no signs of cytotoxicity at the highest concentrations (40 μM and 15 μM in MDA-MB-231 and 4T1 cells respectively) with viability percentages greater than 97% and 92% in these cells respectively, after 48 hours of incubation. However, CBD-loaded niosomes reduced the viability of both cell lines, with a more pronounced effect observed in the 4T1 cells.

Table 2 displays the IC_{50} values of both CBD in solution and CBD-Nio in MDA-MB-231 and 4T1 cells after 24 and 48 hours of treatment. Notably, the values obtained for free CBD in both cell lines are consistent with those reported in other studies [17, 57].

	CBD in solution		**CBD-Nio**	
	24h	**48h**	**24h**	**48h**
MDA-MB231	27.70 ± 3.21 μM	13.26 ± 1.08 μM	14.48 ± 2.75 μM	10.79 ± 1.34 μM
4T1	13.04 ± 1.62 μM	7.07 ± 0.68 μM	8.99 ± 0.99 μM	6.26± 1.35 μM

Table 2: IC_{50} values of free CBD and CBD-Nio after 24 and 48 hours of treatment in MDA-MB231 and 4T1 cells.

In MDA-MB-231 cells, CBD-Nio demonstrated a significantly greater antiproliferative effect compared to non-encapsulated CBD (Figures 5A and 5B), exhibiting statistically lower IC_{50} values (p-values < 0.05). Specifically, the IC_{50} values for CBD-Nio were 1.9 and 1.2 times lower than those for CBD in solution after 24 and 48 hours of treatment, respectively. In 4T1 cells, CBD-Nio also displayed a higher effect on cell viability compared with free CBD (Figures 5C and 5D). Nevertheless, statistically significant differences in IC_{50} values (p-values <0.05) were only observed after 24 hours of incubation. At this time, CBD-Nio showed an IC_{50} 1.5-fold lower than free CBD.

The enhanced effect of CBD-Nio on cell viability can be attributed to the internalization of these formulations, which leads to an improved intracellular delivery of CBD. CBD-Nio shows a particle size around 113 nm which is suitable for cell internalization [58]. Similar findings have been reported in other studies involving niosomes loaded with various anticancer agents, such as sunitinib or a combination of doxorubicin and cisplatin. In these formulations, drug loaded niosomes demonstrated a significantly greater antiproliferative effect in cancer cells compared to the administration of non-encapsulated drugs at equivalent concentrations [59, 60].

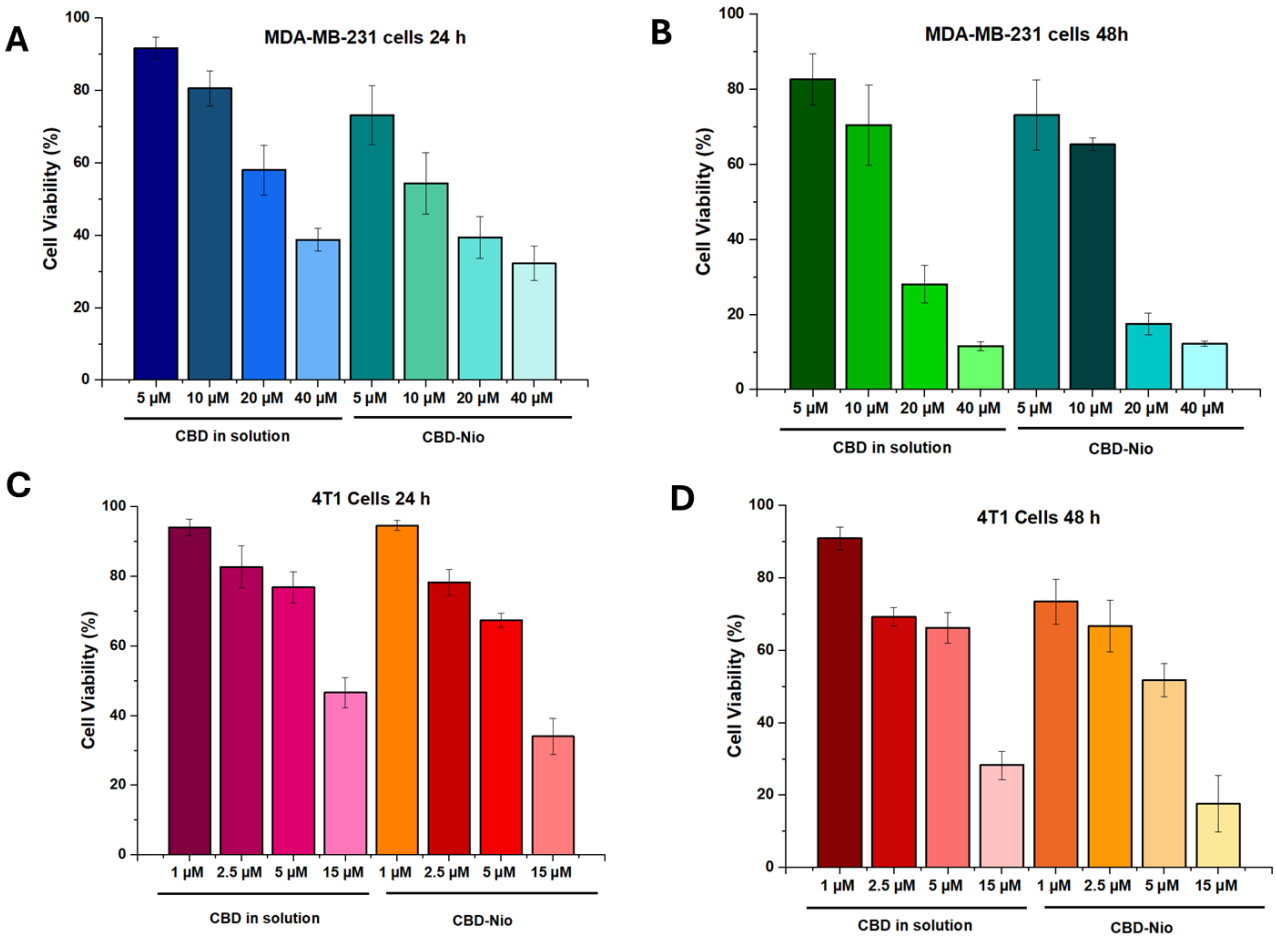


Figure 5: The effect of free CBD and CBD-Nio on the viability of TNBC cells. Results of MDA-MB-231 cells after 24 (A) and 48 (B) hours of incubation. Results of 4T1 cells after 24 (C) and 48 (D) hours of incubation.

3.8.2 Inhibition of cell migration

TNBC is an aggressive carcinoma more likely to spread to distant organs compared to other breast cancer subtypes. Inhibition of cell migration is a critical therapeutic strategy for preventing the spread of TNBC cells. Literature has reported that CBD inhibits the migration of TNBC cells, including MDA-MB-231 and 4T1 cells [3, 61]. For this reason, the ability of CBD-Nio to inhibit the migration of these cells has been assessed using scratch assay. In this study, CBD concentrations below its IC_{50} values of each cell line (Table 2) were used.

Similar to what was observed with cell viability, empty niosomes had no effect on the migration of MDA-MB-231 and 4T1 cells, exhibiting migration ratios around 1 (Supplementary Material Table 1). However, both CBD in solution and CBD-loaded niosomes were effective in reducing cell migration (Figures 6 and 7).

In MDA-MB-231 CBD-loaded niosomes at a CBD concentration of 10µM were more effective than the administration of CBD in solution, with reduction wound closure percentages of approximately 28 and 62% respectively after 24 hours of incubation, with statistically significant differences between both treatments (p-value <0.05). At 20µM, the effect of CBD-Nio was slightly higher compared to CBD in solution, with reduction percentages around 66% and 59%. However, in this case statistically significant differences were not achieved (p-values >0.05). These results are in accordance with the migration ratios obtained in these cells (Table S1, Supplementary Material). After 24 hours of treatment and at CBD concentration of 10µM, CBD-Nio formulation showed a lower migration than CBD in solution with values around 0.37 and 0.71 respectively. However, at a concentration of 20 µM similar migration ratios of 0.40 and 0.33 were observed respectively.

In 4T1 cells, CBD in solution and the CBD-Nio formulation exhibited similar effects on cell migration. After 24 hours of treatment, at a CBD concentration of 5 µM, both non-encapsulated and encapsulated CBD resulted in a wound closure reduction of approximately 35%. At a higher concentration of CBD 7.5 µM, the reduction in wound closure was more pronounced, reaching around 50%. Similar migration ratios after 24 of treatments were also detected with both CBD in solution and CBD-Nio, showing values of around 0.7 and 0.5 at concentrations of 5 and 7.5 µM respectively (Table S1, Supplementary Material).

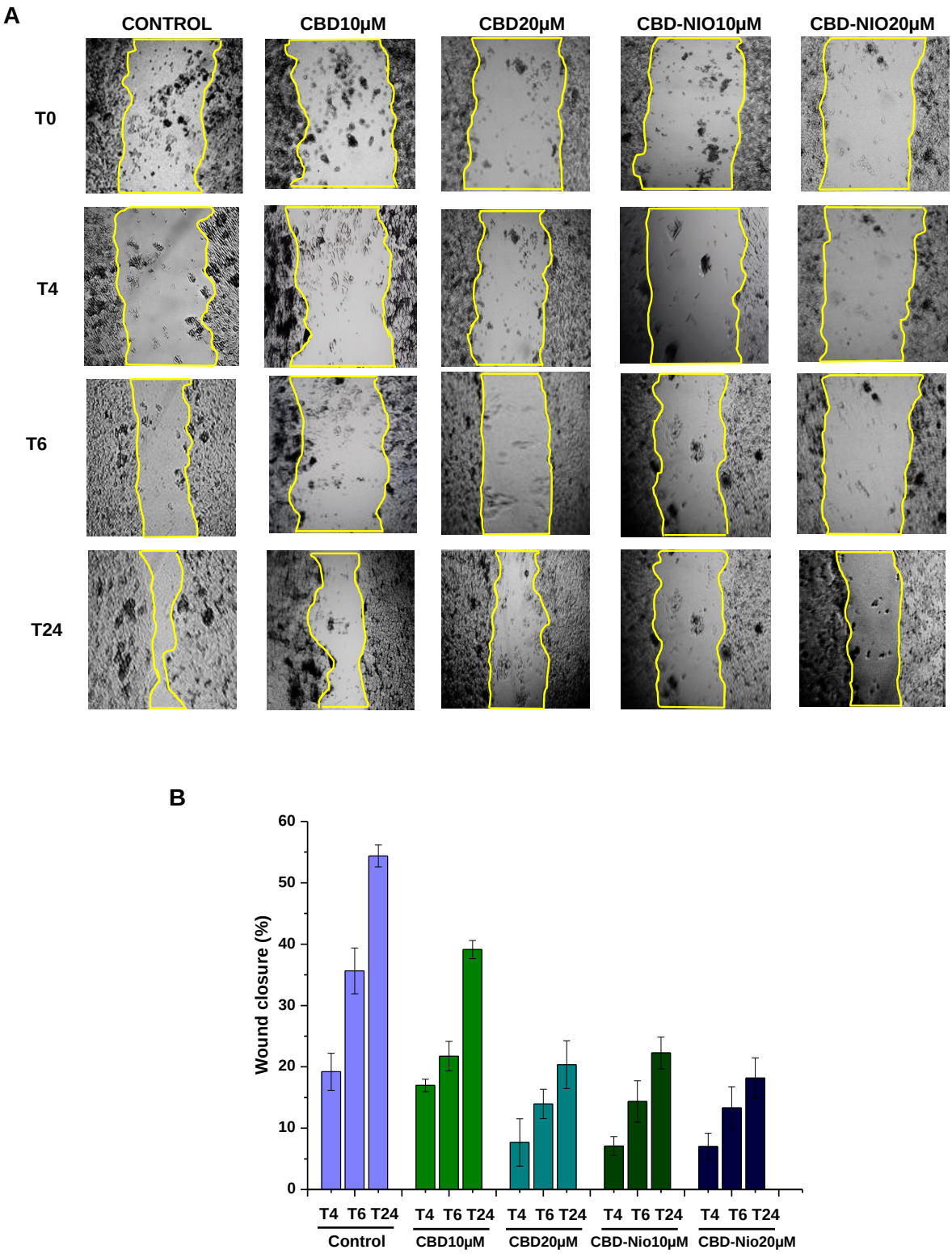


Figure 6: Results of in vitro scratch wound healing assay in MDA-MB-231 cells. A) Images at time 0 (T0h) and after 4 (T4), 6 (T6) and 24 hours (T24) of treatment (A). Wound closure percentages at each time.

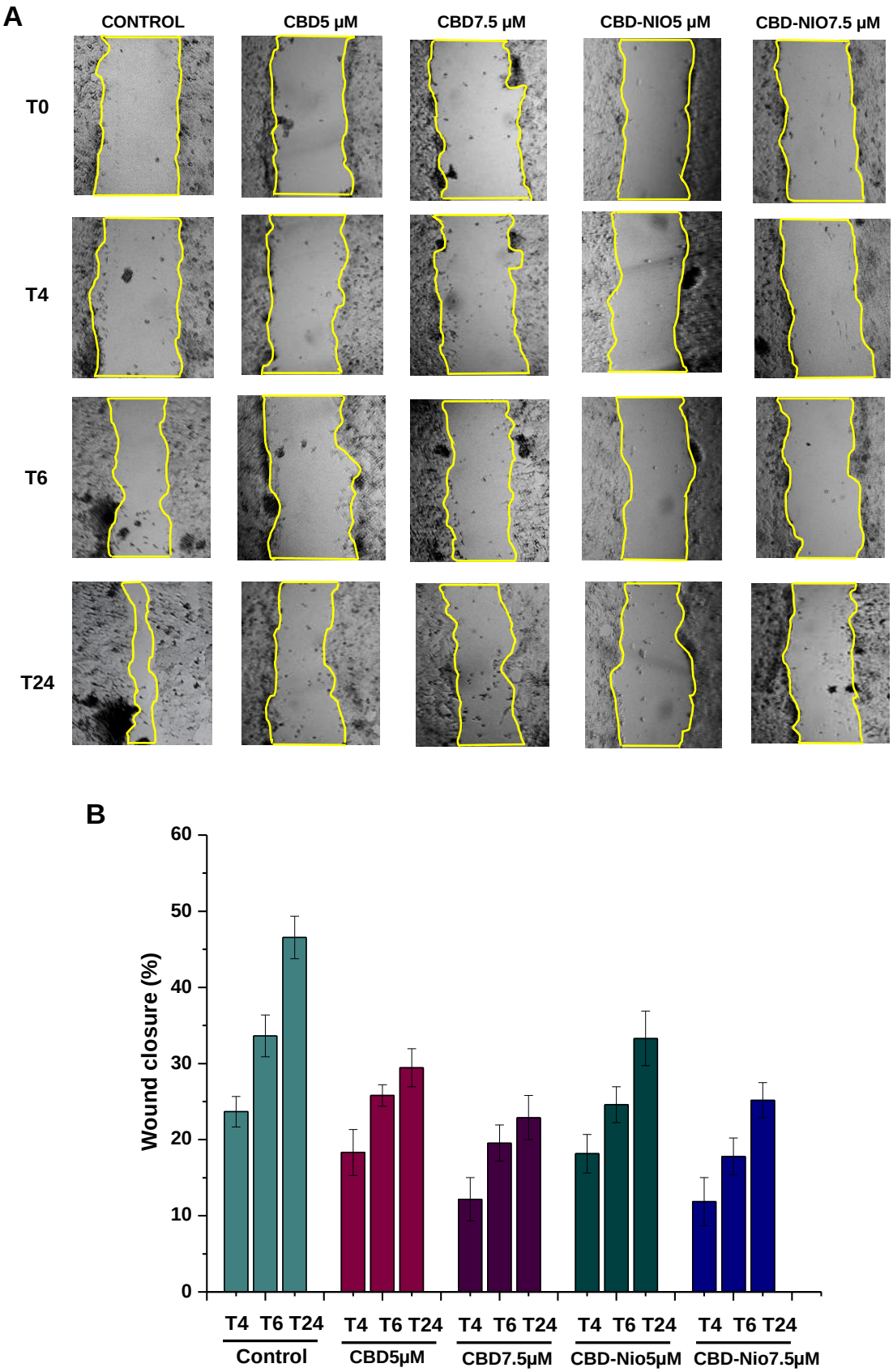


Figure 7: Results of in vitro scratch wound healing assay in 4T1 cells. A) Images at time 0 (T0h) and after 4 (T4), 6 (T6) and 24 hours (T24) of treatment (A). Wound closure percentages at each time.

3.8.3 *In ovo* antitumor efficacy

In this study, the effect of the CBD-Nio formulation on the growth of tumors derived from MDA-MB-231 cells was also evaluated. These cells were selected over 4T1 cells due to their human origin and because niosomes showed a greater effect in the overexplained cell culture studies. The tumors were developed on the chorioallantoic membrane of chicken embryos.

As displayed in Figure 8, both CBD in solution and CBD-Nio (administered at CBD concentration of 100µM) slow the growth and reduce the size of MDA-MB-231 derived tumors after 48 hours of incubation. Interestingly, CBD niosomal formulation exhibited a higher effect. While non-encapsulated CBD reduced the size of these tumors by around 55%, CBD-Nio showed a reduction of approximately 62% compared to the control. However, statistically significant differences between both treatments were not detected.

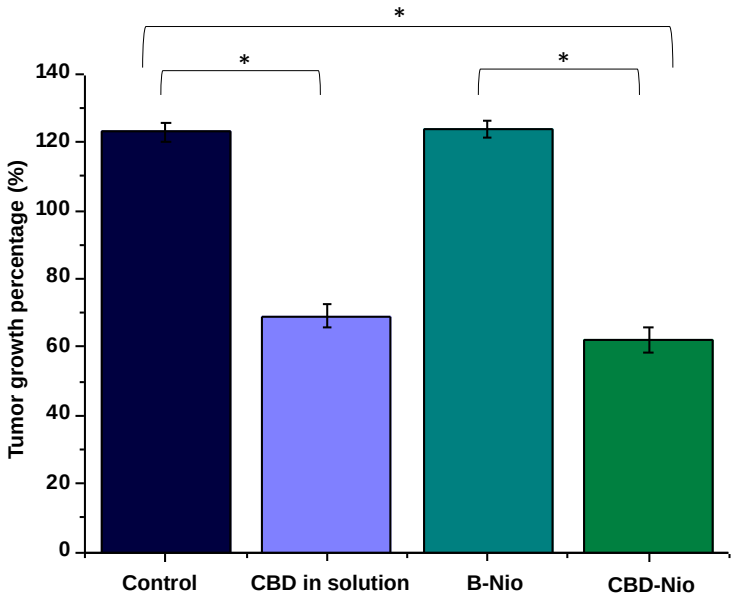


Figure 8: Percentage of tumor growth of MDA-MB-231 derived tumors developed in CAM model. * Indicates statistical significant differences with the control or empty niosomes.

4. CONCLUSIONS

The main aim of the present study was to assess whether niosomes serve as an effective carrier for CBD and enhance its efficacy in the treatment of triple-negative breast cancer.

Noteworthy, niosomes composed of Tween 85 and cholesterol appeared to be a suitable nanocarrier for the delivery of CBD, and the thin-film hydration technique a good method for their elaboration. During the formation of these nanosystems, CBD, due to its high lipophilicity, becomes trapped within the niosome membrane, resulting in an increase in membrane rigidity, which seems to improve the physical stability of the formulation. Moreover, the presence of CBD further reduces the particle size which would favor the internalization by the cancer cells. In fact, the nanoencapsulation of CBD in niosomes increased its antitumor efficacy. Firstly, the niosomes exhibited a stronger antiangiogenic effect. Additionally, in general, there was an enhanced capacity to inhibit the proliferation and migration TNBC cells, particularly the MDA-MB-231 cells, which are of human origin. Finally, the antitumor efficacy in ovo was also enhanced. The CBD niosomes demonstrated a greater inhibition of the growth of tumors derived from MDA-MB-231 cells. However, this increase was slight and not statistically significant.

Although further in vivo studies are needed for a more comprehensive biological characterization, CBD niosomes present an intriguing potential for the treatment of TNBC. Additionally, its potential utility could be extended to other carcinomas where CBD may exhibit activity.

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SUPPLEMENTARY MATERIAL

	MDA-MB231 cells				4T1 cells			
Time	**CBD sol**		**CBD-Nio**		**CBD sol**		**CBD-Nio**	
(h)	**10µM**	**20µM**	**10µM**	**20µM**	**5µM**	**7.5µM**	**5µM**	**7.5µM**
4	0,88±0,33	0,39±0,14	0,36±0,05	0,36±0,15	0,77±0,36	0,95±0,12	0,76±0,13	0,50±0,34
6	0,60±0,05	0,39±0,08	0,40±0,26	0,37±0,12	0,76±0,22	0,58±0,29	0,73±0,18	0,52±0,27
24	0,71±0,26	0,37±0,29	0,40±0,14	0,33±0,17	0,73±0,14	0,54±0,21	0,71±0,13	0,48±0,15

Table S1: Migration ratios of CBD in solution and CBD-Nio in MDA-MB-231 and 4T1.

DISCUSSION

Breast cancer represents a major health problem, especially in the female population. It is the most frequently diagnosed carcinoma, with 1 in 10 new cancer diagnoses each year, and the leading cause of cancer death in women worldwide [1]. Among all breast carcinomas, triple-negative breast cancer (TNBC) is the most aggressive subtype, accounting for 15-20% of all breast cancers. It is frequently diagnosed in women under the age of 50 and is very difficult to treat. Approximately 45% of TNBC patients show metastases. TNBC is characterized by a lack of estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2, and treatments targeted to these receptors are not effective. Chemotherapy remains the main therapeutic approach of this neoplasm [2]. However, conventional antineoplastics show a high toxicity due to their non-selective biodistribution. For these reasons, new therapeutic agents with lower overall toxicity that potentiate the effect of conventional antineoplastics and allow the reduction of their doses without impairing their antitumoral efficacy are needed.

In the last decades, cannabinoids have attracted a great deal of interest in cancer disease. On the one hand, they are useful as palliative agents as they reduce vomiting and nausea related to chemotherapy (several cannabinoid-based medications are approved for this purpose). On the other hand, they show anticancer activity per se, as they have demonstrated to reduce the growth and metastases of many types of tumors, such as prostate, brain, lung and breast carcinomas, and to potentiate the effect and reduce the toxicity of conventional antineoplastics like paclitaxel or doxorubicin [3, 4].

Among all cannabinoids, cannabidiol (CBD), which lacks psychoactive effects, is one of the most promising cannabinoids. CBD shows a promising anticancer effect in TNBC, as it induces apoptosis and reduces the migration and invasion of TNBC cells. Moreover, it shows an antiangiogenic activity. Moreover, CBD is useful in combination therapy. Preclinical studies have reported that CBD enhances the anticancer activity of paclitaxel and doxorubicin in TNBC and also reduces the main toxicities (peripheral neuropathy and cardiotoxicity, respectively) of these agents [5]. Despite all these advantages, the low aqueous solubility and poor oral bioavailability of CBD limit its administration. The use of drug delivery systems may be a good alternative to resolve these limitations and optimize the anticancer efficacy of this cannabinoid.

On the other hand, the development of in situ forming implants (ISFIs) could be highly interesting. These systems would enable the formation of a depot of CBD directly within the tumor area, allowing for a localized controlled release of this cannabinoid. This approach

would provide a long-term therapeutic effect with a single administration, minimizing the need for frequent dosing while ensuring sustained drug delivery at the targeted site. Polymeric implants based on the solvent exchange method have been prepared. These formulations are administered in liquid form by injection. At the injection site, the solvent rapidly diffuses into the surrounding aqueous environment, and the polymer solidifies and forms a depot in which the drug is entrapped [6].

These implants are composed of biodegradable and biocompatible polymers, typically poly-lactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA), that are not soluble in water and are administered dissolved in a water-miscible solvent such as N-methyl-2-pyrrolidone (NMP) and dimethylsulfoxide (DMSO) [7]. Polycaprolactone (PCL) is another biodegradable polyester that also exhibits high biocompatibility and can be used in the development of in situ forming implants. **In this doctoral thesis, in situ forming implants of CBD** (**chapters 2 and 3) made of PLGA, PLA, or PCL dissolved in NMP and/or DMSO have been developed with the objective of obtaining a formulation that would allow a controlled and sustained release of this cannabinoid for at least 1 month.** Table 1 displays the characteristics of the polymers used in this work. PLGA polymers with an average molecular weight (MW) of ≈12 kDa and ester (PLGA-502) or acid (PLGA-502H) end group and an average MW of ≈31kDa and an ester end group (PLGA-503) were used. PLA polymers had an average MW of ≈14 kDa (PLA-202) or ≈23 kDa (PLA-203) and an ester end group.

Polymer	End Group	Molecular Weight Range (KDa)	Inherent viscosity (dl/g)	Degradation timeframe	Tm (°C)	Tg (°C)
PLA-202	Ester	10–18	0.16-0.24	< 9 months	Amorphous	38–42
PLA-203	Ester	18–28	0.25-0.35	< 12 months	Amorphous	46–50
PLGA-502	Ester	7–17	0.16–0.24	< 3 months	Amorphous	42–46
PLGA-502-H	Acid	7-17	0.16–0.24	< 3 months	Amorphous	42–46
PLGA-503	Ester	24–38	0.32–0.44	< 3 months	Amorphous	44–48
PCL-C-203	Ester	-	0.35-0.43	Not measured	Semicrystalline	-60

Table 1: Characteristics of the polymers used in the development of CBD-loaded ISFIs. Data was obtained from the supplier (Evonik®).

The polymeric solution of ISFI formulations must be easily injected using conventional needles. For this reason, the injectability of different polymeric solutions dissolved in NMP and/or DMSO at concentrations of 0.25, 0.33 and 0.66 mg/μL were evaluated using panel test. In this test, volunteers were asked to score the ease of injection and flow through 23G and 25G needles. The solution was considered to have good injectability properties when all participants provided scores of 3 or 4 (moderate to easy injection and continuous flow) through both needles.

Except for PLA-203 dissolved in NMP and PLGA-503 dissolved in NMP or DMSO, all polymers showed adequate injectability through 23G and 25G needles at concentrations of 0.25 mg/μL due to their lower viscosity (<100 cP) (Table 2), without statistically significant differences in the injectability between both needles (p-value > 0.05). Therefore, polymeric solutions of PLGA-502, PLGA-502-H, and PLA 202 in NMP and DMSO, solutions of PLA-203 in DMSO and solutions of PCL in NMP at 0.25 mg/μL were selected for preparing ISFIs loaded with CBD. Formulations with two different drug: polymer ratios 2.5:100 (CBD-ISFI-2.5) and 5:100 (CBD-ISFI-5), were developed.

		NMP			DMSO		
	Polymer concentration (mg/μL)	Injectability		Viscosity (cP)	Injectability		Viscosity (cP)
		23G*	25G*		23G*	25G*	
PLA-202	0.66	3-4(3)	2-3 (2)	297.78 ± 2.98	2 (2)	1-2(1)	304.58 ± 5.00
	0.33	3-4(3)	2-3 (3)	70.62 ± 0.99	3-4 (3)	2-3(2)	126.86 ± 0.59
	0.25	3-4 (4)	3-4 (4)	35.97 ± 0.35	3-4 (4)	3-4(4)	53.15 ± 5.12
PLA-203	0.66	1-3 (1)	1-2 (1)	948.00 ± 26.85	1-2 (1)	1-2 (1)	838.95 ± 21.09
	0.33	2-3 (2)	2-3 (2)	249.60 ± 3.70	3-4 (3)	2-3 (2)	156.13 ± 1.13
	0.25	2-4 (3)	2-4 (2)	205.57 ± 1.16	3-4 (4)	3-4 (3)	98.06 ± 1.75
PLGA-502	0.66	1-2 (2)	1-2 (2)	589.88 ± 6.62	1-2 (2)	1-2 (1)	485.58 ± 14.23
	0.33	3-4 (3)	2-3 (2)	92.57 ± 0.91	3-4 (3)	1-2 (1)	158.93 ± 10.71
	0.25	3-4 (3)	3-4 (3)	40.38 ± 3.38	3-4 (4)	3-4 (3)	94.69 ± 6.48
PLGA-502H	0.66	1-3 (2)	1-2 (1)	594.33 ± 5.88	2-3 (2)	1-2 (2)	485.58 ± 14.23
	0.33	3-4 (4)	2-3 (2)	119.40 ± 2.25	2-4 (3)	2-4 (3)	113. 65 ± 2.59
	0.25	4 (4)	3-4 (4)	37.98 ± 1.25	3-4 (4)	3-4 (4)	98.04 ± 1.49
PLGA-503	0.66	1-2 (1)	1-2 (1)	8249.60 ± 3.70	1-2 (1)	1 (1)	7000.50 ± 8.66
	0.33	2-3 (2)	1-2 (2)	5473.63 ± 5.14	2-3 (2)	1-2 (1)	1119.75 ± 26.32
	0.25	2-3 (3)	2-3 (2)	205.57 ± 1.16	2-3 (2)	1-2 (1)	98.06 ± 1.75
PCL	0.66	1-2 (1)	1-2 (1)	582.23±2.54	-	-	
	0.33	2-3(3)	2-3 (3)	134.05±3.85	-	-	
	0.25	3-4(4)	3-4 (4)	65.6±1.10	-	-	

Table 2: Results of injectability panel test and viscosity studies. *Ranges of scores provided by the volunteers (median value).

All these implants were prepared using the direct injection technique. Upon contact with the simulated physiological medium (PBS pH 7.4), the solvent rapidly diffused, and a quick precipitation of the polymer occurred, triggering the formation of soft implants. CBD was entrapped in the polymeric matrix. No differences in the formation of the implants prepared with the different polymers (PLGA, PLA or PCL) or solvent (NMP or DMSO) and with the different CBD: polymer ratios were appreciated. All the implants exhibited an irregular shape (Figure 1), which is typical of the implants prepared with this technique [8]. Nevertheless, when the surface of the implants was examined by scanning electron microscopy (Figure 2), some differences were observed. The implants prepared with PLGA and PLA dissolved in NMP showed a rough surface, whereas the implants prepared with these polymers

dissolved in DMSO exhibited a smooth surface. The PCL implants also had a smooth surface. It should be mentioned that some pores were appreciated on the surface of the implants. The presence of these pores is attributed to the rapid diffusion into the aqueous medium of NMP and DMSO when the polymeric solution is injected [9]. It is more evident in the formulations prepared with PCL, especially in unloaded implants. It has been demonstrated that the faster solvent removal, the higher the porosity of the polymeric implant. Unloaded PCL-ISFIs showed a more porous surface than CBD-loaded formulations, probably due to the plasticizer effect of CBD on PCL [10, 11].

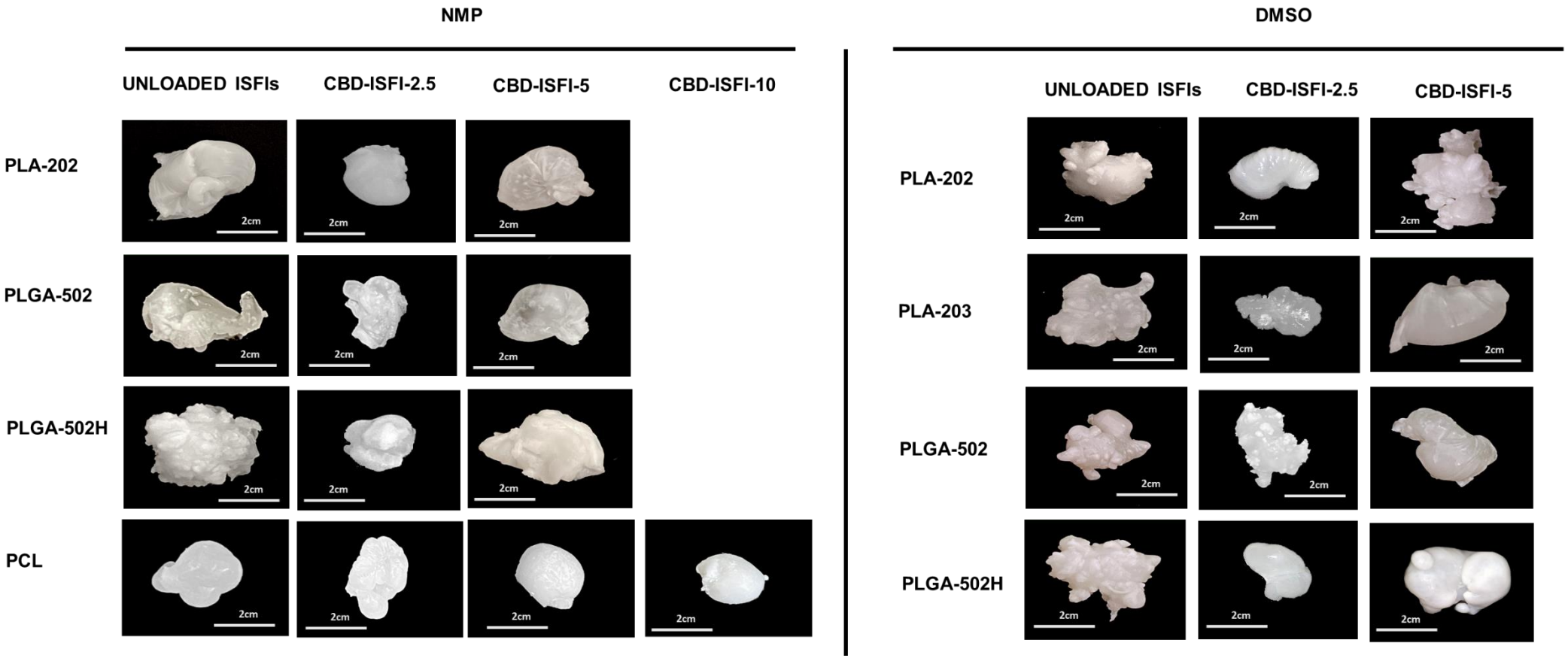

Figure 1: Images of recently prepared ISFIs obtained by optical microscopy.

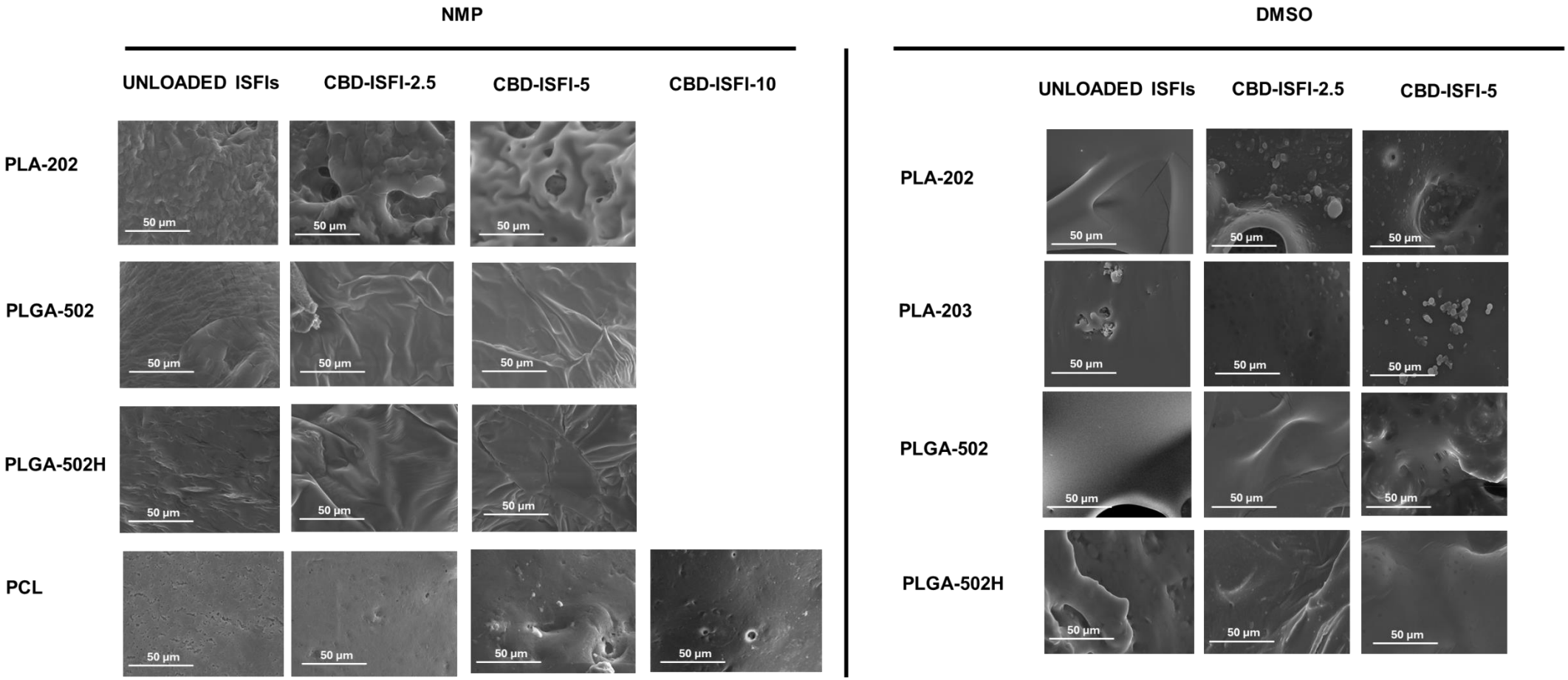

Figure 2: Images of the surface of recently prepared ISFIs obtained by scanning electron microscopy.

As shown in Figure 3, all the formulations exhibited a controlled CBD release. However, the drug release profiles varied significantly with polymer type, solvent and drug: polymer ratio, demonstrating their influence on the release rate.

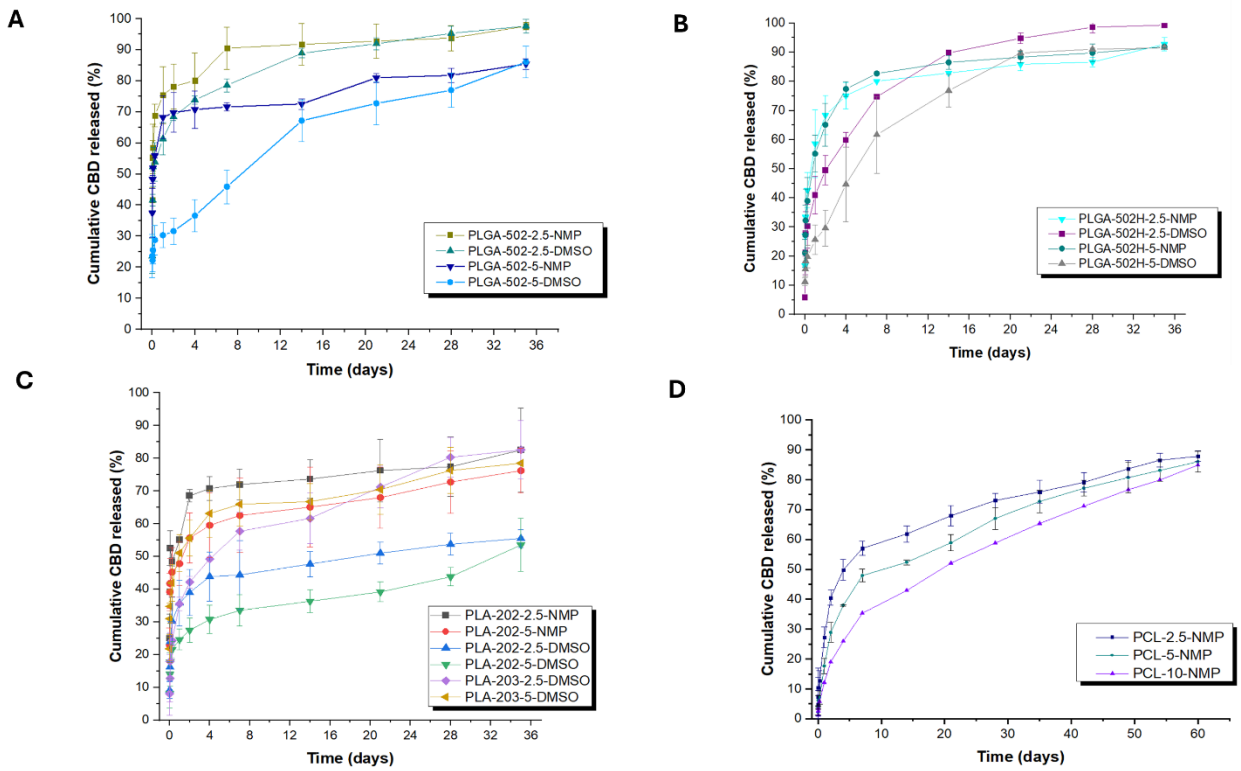

Figure 3: CBD release profile from PLGA-502 ISFIs (A), PLGA-502H-ISFIs (B), PLA-202-ISFIs, PLA-203-ISFIs (C) and PCL-ISFIs (D).

PLGA-based implants, particularly those prepared with NMP, exhibited a rapid release, especially PLGA-502-2.5-NMP. This formulation released over 40% of CBD in the first 15 minutes, 75% in 1 day, and nearly 91% after 14 days. PLGA-502-5-NMP showed a slightly slower release, with around 35 % of CBD released in 15 minutes, 71 % in 1 day, 77 % within 14 days, and approximately 85 % in 21 days. In comparison, formulations prepared with DMSO showed a slower release. PLGA-502-2.5-DMSO and PLGA-502-5-DMSO implants released around 24% and 18% of CBD in 15 minutes, 61 % and 26 % in 1 day, and 90 % and 70 % in 14 days, respectively.

A slightly faster release but with a slightly lower burst effect was appreciated in PLGA-502-H formulations. PLGA-502-H-2.5-NMP and PLGA-502-H-5-NMP released around 15% of the CBD in 15 minutes, 68 % in 2 days, and ≈77% and ≈85% in 7 days respectively. As occurred in PLGA-502-ISFIs, PLGA-502-H-DMSO formulations exhibited a slightly slower

release. PLGA-502-H-2.5-DMSO formulation showed a CBD release of around 6 % in 15 minutes, 40 % in 1 day, and around 90 % in 14 days, while PLGA-502-H-5-DMSO showed release percentages of around 3 %, 20 %, and around 85 % at these times respectively.

PLA-based implants exhibited a slower CBD release than PLGA formulations. It is well known that PLGA exhibits a less hydrophobic nature than PLA due to the presence of glycolic acid and, consequently, a faster degradation rate (Table 1). While D,L-PLA polymers show a degradation time lower than 9–12 months depending on PLA molecular weight, the degradation time of 50:50 PLGA polymers is lower than 3 months [12]. In PLA-202–2.5-NMP formulation, around 25 % of CBD was released in 15 minutes, around 65 % in 1 day, and around 45 % in 14 days. PLA-202–5-NMP showed a slightly faster release, with around 25 % released in 15 minutes, around 65 % in 1 day, and around 70 % in 14 days. As occurred in PLGA implants, the formulations elaborated with DMSO exhibited a slower release compared to the implants prepared with NMP. PLA-202–2.5-DMSO exhibited around 9 % of the drug released in 15 minutes, around 35 % in 1 day, and around 45% in 14 days and PLA-202–5-DMSO around 9 %, 24 %, and 36 % at these times, respectively.

When comparing PLA-202 and PLA-203 implants, a lower initial CBD release is appreciated in this latter formulation. In PLA-203–2.5-DMSO formulation, 8 % of the CBD was released in 15 minutes, around 35 % in 1 day, and around 60 % in 14 days. PLA-203–5-DMSO exhibited drug release percentages of 21 % in 15 minutes, 50 % in 1 day and around 66 % in 14 days. The slower initial CBD release from PLA-203 formulation can be related to the higher molecular weight of this polymer. Polymers with higher molecular weights lead to the formation of more compact polymeric networks, thereby hampering drug diffusion during the solidification of the implant. Furthermore, an increase in the polymer's molecular weight results in an increase in the viscosity of the formulation, which may also contribute to decreasing drug escape [12, 13].

The high initial drug release detected in some of these ISFIs, especially in those developed with NMP, can be related to a CBD lost during implant solidification. When the polymeric solution is injected into an aqueous environment, the solvent rapidly diffuses, and the polymer precipitates, forming a deposit that entraps the drug. However, complete polymer solidification is not immediate, and part of the drug can be lost in this process. CBD is a small lipophilic molecule (MW=314.47 g/mol) that can be transferred to the aqueous environment with the solvent before being completely entrapped into the polymeric matrix. DMSO (ε=46.83) shows a higher hydrophily than NMP (ε=32.2) and a lower affinity for PLGA

and PLA polymers, diffusing more rapidly to an aqueous environment [16]. In this context, the initial drug release should be higher in the formulations prepared with DMSO than in NMP-based systems. Nevertheless, opposite results were found. The higher CBD release appreciated in the formulations prepared with NMP could be attributed to the higher solubility of CBD in NMP (logP=-0.38) than in DMSO (logP=-1.35). The partition of this drug (logP=6.1) between each polymer and each solvent is more favorable for NMP. Similar results were also found by Li and collaborators, who reported that the release of leuprorelin acetate was higher in PLGA ISFIs prepared with NMP than with DMSO, with drug release percentages of around 60% and 52% after 4 days [17]. In general, the higher the CBD content, the slower the drug release.

Among all PLGA and PLA ISFIs, CBD-PLGA-502–5-DMSO formulation showed the most suitable CBD release. PLGA-502–2.5-DMSO, PLGA-502-NMP, and PLGA-502H-NMP formulations showed significantly faster drug release with more than 70% of CBD released in 4 days and were discarded. PLGA-502-H-DMSO, PLA-202-NMP formulations and PLA-203-5-DMSO showed a slower drug release compared to all these formulations, but also relatively fast as nearly or above 60% of CBD was released in 7 days. On the contrary, PLA-202-DMSO ISFIs exhibited a very slow drug release from day 4 and were also discarded. Finally, PLGA-502-5-DMSO and PLA-203-2.5-DMSO ISFIs showed a controlled drug release from one month, exhibiting this later a faster release during the first times. After 4 days, CBD release percentages around 30% and 50% were appreciated. For this reason, PLA-203-2.5-DMSO were also discarded. However, it should be noted that although PLGA-502-5-DMSO formulation meets the stated objectives of achieving a controlled release of CBD for at least a month, the profile is not optimal because the initial release is still high, and PCL ISFIs were developed.

PCL-based implants were prepared using NMP as solvent (PCL shows poor solubility in DMSO) and exhibited the most controllable CBD release profile, with the lowest burst effect. As occurred in most PLGA and PLA CBD-loaded ISFIs, the higher the drug content, the slower the CBD release. Several authors have pointed out that the increase in drug-to-polymer ratio leads to higher polymer-drug interactions, which slows down drug release [14]. PCL-2.5-NMP formulation released around 5% of CBD in 15 min, 30% in 1 day and around 70% in 28 days. In PCL-5-NMP formulation, release percentages of approximately 2%, 18% and 70% were appreciated at these times. Due to this good release profile and the ability to deliver as much CBD as possible with the smallest amount of polymer, implants with a CBD:

PCL ratio of 10:100 (PCL-10-NMP) were also developed. This formulation exhibited the most controllable drug release, with 1% of CBD released in 15 minutes, 12% in 1 day and around 60% in 28 days. It showed a constant release phase from day 2 to day 60 fitted to zero-order release kinetics (K_0=53 µg/day, R^2=0.967). The slower CBD release of PCL formulations compared to PLA and PLGA formulations can be attributed to the semicrystalline structure of PCL, which limits water penetration and bulk erosion, thereby slowing down the overall release of this cannabinoid [15].

Among all CBD-PCL-ISFIs, PCL-10-NMP formulation exhibited the most controlled and suitable release profile. This formulation exhibited the lowest initial CBD release (12% in one day) and a controlled zero-order release for around 2 months and was selected for anticancer efficacy studies.

The Hen's egg test on chorioallantoic membrane (HET-CAM) is a method widely used to evaluate the local (such as ocular or skin) toxicity of new molecules and formulations [18]. The toxicity of CBD-ISFIs was evaluated using this technique. The toxicity of the solvents was first assessed. As displayed in Figure 4, while the eggs treated with NMP exhibited signs of toxicity (the appearance of haemorrhage, lysis, and coagulation of the blood vessels was observed), the eggs treated with DMSO did not exhibit signs of toxicity with irritation scores (IS) of 6.74± 0.77 and 0 respectively. This is a point to bear in mind, as the vast majority of implants currently on the market have NMP as a solvent. The toxicity of PLGA-502-5-DMSO and PCL-10-NMP ISFIs was also evaluated in the HET-CAM assay without detecting signs of damage on the CAM (IS=0) (Figure 4). The absence of toxicity from PCL implants compared to NMP can be related to the progressive diffusion of the solvent from the polymeric matrix.

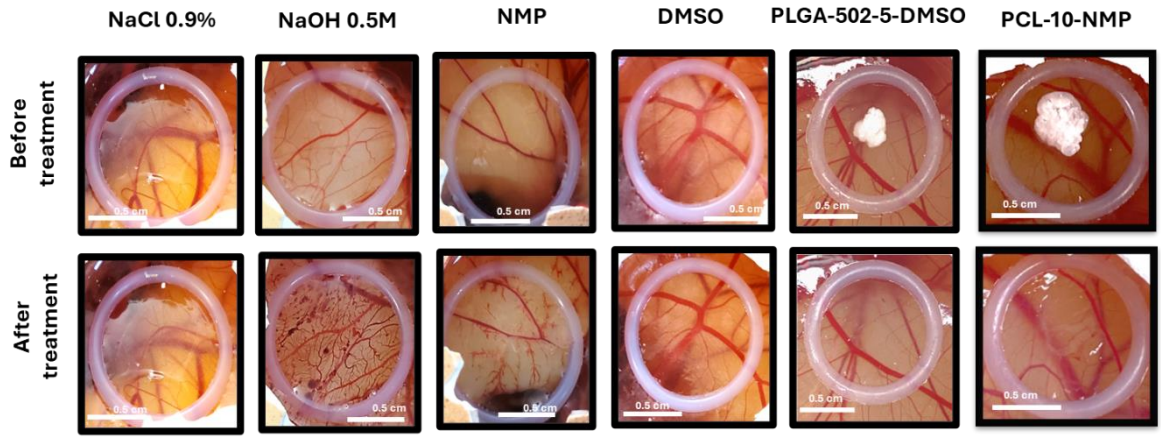

Figure 4: Images obtained of the HET-CAM toxicity assay before and 5 min after each treatment. CBD-ISFIs were successfully formed directly on the CAM.

Once the absence of toxicity of these formulations was evaluated, the anti-angiogenic effect of these CBD implants (PLGA-502-5-DMSO and PCL-10-NMP) was studied. Angiogenesis is essential in cancer because it enables the formation of new blood vessels, which supply oxygen and nutrients to the growing tumor. This process facilitates tumor expansion and metastasis, allowing cancer cells to invade surrounding tissues and spread to distant organs. A statistically significant (p-value<0.001) lower vascularization was appreciated in the eggs treated with both free CBD and CBD-ISFIs compared to the control group (eggs treated with PBS pH 7.4) (Figure 5A). Interestingly, administered at the same CBD concentration (100 μM), CBD-ISFIs exhibited a higher antiangiogenic effect than free CBD. While free CBD showed vascular inhibition in the range of 60-65%, PLGA-502-5-DMSO and PCL-10-NMP exhibited reduction percentages of around 75% and 80%, respectively (Figure 5B). Nevertheless, statistically significant differences between free CBD and each CBD-ISFI formulation were not observed (p-value > 0.05).

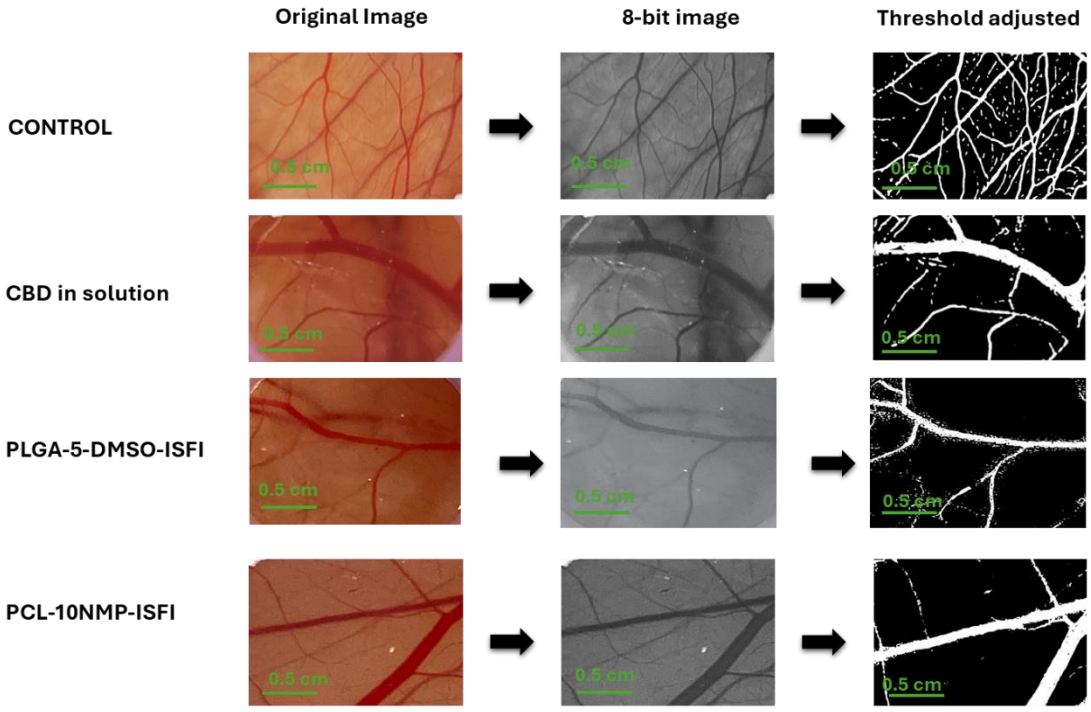

Figure 5: Images of the eggs treated with free CBD and CBD-loaded-ISFIs for 48 hours during the angiogenesis study. Image processing was carried out using ImageJ-Fiji software.

Due to their better release profile, PCL-10-NMP formulation was selected to evaluate the anticancer effect against TNBC. Firstly, the antiproliferative effect was evaluated in MDA-MB231 and 4T1 cells. These cells were treated with free CBD or PCL-CBD-10-NMP implants at a CBD concentration of 15μM and 7.5μM, respectively. These concentrations are equivalent to the IC50 values of free CBD after 48 hours of incubation in MDA-MB-231 (16.5 μM ± 5.2) and 4T1 (7.4 μM ± 3.4) cells. While unloaded implants did not show any effect on the viability of these cells, PCL-CBD-10-NMP provided a long-lasting and constant antiproliferative activity for at least 8 days (Figure 6). Compared with free CBD, CBD-loaded ISFIs exhibited a lower antiproliferative effect, detecting cell death percentages after 48 hours of incubation of around 25% and 30% in MDA-MB-231 and 4T1 cells, respectively. This lower antiproliferative effect can be attributed to the controlled CBD from ISFIs during 48 hours of incubation with the cells.

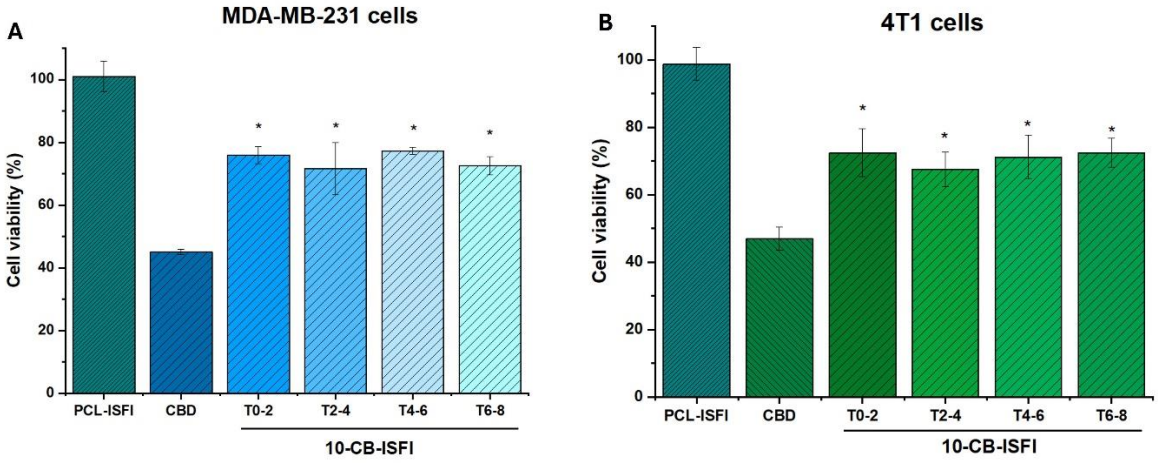

Figure 6: Effect of PCL-CBD-10-NMP ISFI on the proliferation of MDA-MB-231 (A) and 4T1 (B) cells.

Several studies have reported that CBD also inhibits the migration of TNBC cells [21, 22]. For this reason, the effect of PCL-CBD-10-NMP implants on the migration of MDA-MB-231 and 4T1 cells was also evaluated using the scratch assay. In this study, the two different cell lines were treated with both free CBD and PCL-CBD-10-NMP implants at different CBD concentrations (MDA-MB-231 cells: CBD at 10 and 20μM, 4T1 cells: CBD at 5 and 7.5μM) and the migration activity was evaluated after 24 hours. As displayed in Figure 7, both CBD and PCL-CBD-10-NMP formulations reduced the migration of TNBC cells. In this case, no differences between free CBD and CBD-loaded implants were appreciated. After 24 hours of incubation, the wound closure percentage of the untreated cells was approximately 50% and 95% in MDA-MB-231 and 4T1, respectively, while the cells treated with either free CBD or PCL-CBD-10-NMP implants exhibited wound closure percentages of around 20% and 17% in MDA-MB-231 cells when administered at 10 and 20μM respectively, and around 65% and 57% in 4T1 cells at concentrations of 5 and 7.5μM respectively. The migration ratios were also calculated, detecting values below 1 in all cases (Table 3). This indicates an anti-migration effect of CBD treatments in both cell lines.

	MDA-MB231		4T1	
	10 µM	20 µM	5 µM	7.5 µM
CBD	0.40±0.02	0.35±0.01	0.74±0.11	0.63±0.10
PCL-CBD-10-NMP	0.40±0.02	0.34±0.01	0.58±0.12	0.73±0.20

Table 3: Migration ratios of CBD treatments in MDA-MB-231 and 4T1 cells.

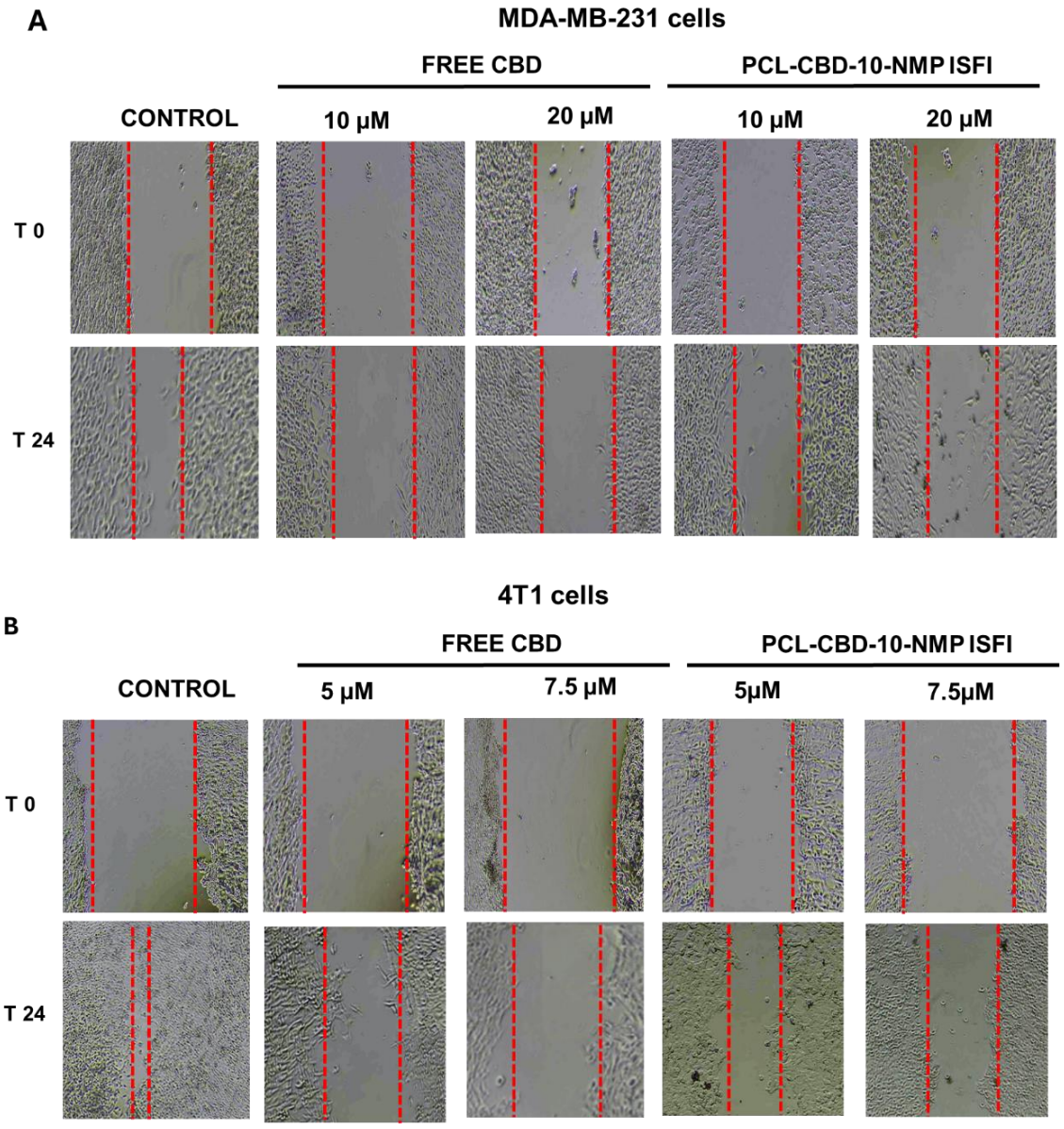

Figure 7: Images of the scratch assay in MDA-MB-231 (A) and 4T1 (B) cells.

Finally, the anticancer activity of PCL-CBD-10-NMP formulations was also evaluated in ovo in MDA-MB-231-derived tumours (Figure 8). After 48 hours of treatments, the control group (eggs treated with PBS pH 7.4) exhibited a tumour growth of around 21%, while the eggs treated with free CBD (daily administration of 7.7µg of this cannabinoid) or PCL-CBD-10-NMP formulations (single administration of equivalent doses of CBD) not only the tumour did not grow but their size was reduced by around 60%. In this case, statistically significant differences (p-value > 0.05) between free CBD and CBD implants were not achieved either. All these results highlight the promising utility of the developed CBD-loaded implants for treating TNBC.

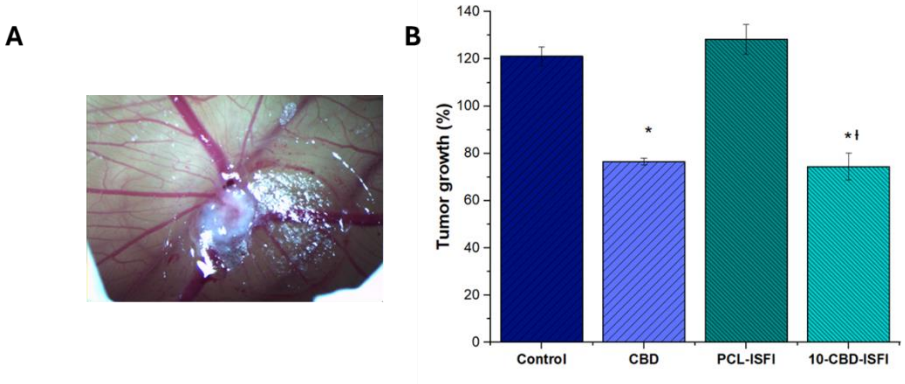

Figure 8: Image of an MDA-MB-231 derived tumor formed on CAM membrane (A) and tumor growth percentages (B).

Nanomedicine holds great promise for enhancing anticancer therapies, as it can selectively accumulate in tumor tissues due to the enhanced permeability and retention (EPR) effect. This phenomenon occurs in solid tumors due to the altered tumor vasculature and poor lymphatic drainage, which allow nanoparticles to penetrate and remain in the tumor area, minimizing their biodistribution in healthy tissues [23]. Liposomes are one of the most widely used nanocarriers in clinical practice, particularly for the delivery of antitumor drugs[24]. In recent years, niosomes, vesicles composed of a double membrane made of lipids (mainly cholesterol) and non-ionic surfactants (as the principal constituent) surrounding an aqueous core, have emerged as a promising alternative to liposomes due to their greater stability and more cost-effective manufacturing. At preclinical level, these systems have demonstrated their usefulness in the administration of antitumor agents [25, 26]. In this doctoral thesis, niosomes loaded with CBD have also been developed as a strategy to enhance its antitumor activity in TNBC (**Chapter4)**. This work has been carried out in collaboration with the Department of Drug Chemistry and Technology of the University of 'Sapienza', Rome.

The CBD-loaded niosomes (CBD-Nio) were prepared using the thin-film hydration method using polysorbate 85 (Tween 85) as non-ionic surfactant and cholesterol. Both the CBD-loaded and unloaded niosomes (B-Nio) exhibited a pseudospherical shape (Figure 9). As displayed in Table 4, CBD-Nio exhibited a significantly smaller particle size (expressed as hydrodynamic diameter) than B-Nio (113 and 137 nm respectively). This size difference can be attributed to the presence of CBD, a lipophilic and liposoluble compound (Log P= 6.3), which is entrapped in the lipid membrane. Its presence in the membrane can increase its hydrophobicity, leading to a reduction in the particle size [27–29]. Furthermore, the presence of CBD also increases the rigidity of the membrane (as the anisotropy of the B-Nio is 0.168, while that of the CBD-Nio is 0.185). This increased rigidity may reduce the tendency of the niosomes to aggregate, which is therefore associated with a smaller particle[30]. In fact, CBD-Nio exhibited a statistically lower PDI value than B-Nio (0.2 vs 0.25). Nevertheless, both values are acceptable and indicate low polydispersity. Regarding the zeta potential, both B-Nio and CBD-Nio displayed negative zeta potential values of approximately -34 mV and -37 mV, respectively. CBD-Nio exhibited a loading of 0.92 ±0.025mg of CBD per 1 ml of formulation and a high entrapment efficiency (92 ± 2.09%).

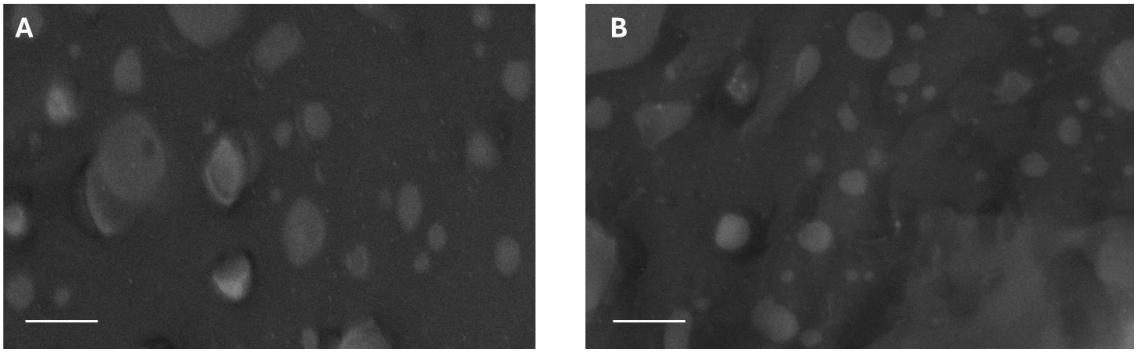

Figure 9: Images obtained by SEM of B-Nio (A) and CBD-Nio (B). Scale bar: 0.3 µm

Sample	Hydrodynamic diameter (nm)±SD	PDI ±SD	Zeta Potential (mV) ±SD	Entrapment Efficacy (EE %)
B-Nio	137±1	0,25±0,008	-34±0,77	-
CBD-Nio	113±2	0,20±0,007	-37±0,67	92 ± 2.09

Table 4: particle size, zeta potential, polydispersity index and entrapment efficacy of no loaded and CBD loaded niosomes at pH 7,4 in Hepes buffer.

It is worth noting that in a previous study, Gugleva and collaborators developed CBD-loaded niosomes using different sorbitan esthers (Spans), specifically Span 20, Span 60 and Span80, as non-ionic surfactants[31]. These niosomes exhibited a much larger particle size (ranging from 186 to 230 nm) compared to the niosomes prepared in this doctoral thesis with Tween 85 (with a size of 113 nm). This seems contradictory, as Tween 85 has a higher HLB value (HLB=11) than the Spans (ranging 4.3-8.6), and generally, a higher HLB value results in a larger particle size. However, it is important to highlight that the use of Tween 60 (HLB= 14.9) together with Span 60 to prepare these niosomes resulted in smaller formulations (150 nm), indicating that the use of Tweens in the development of CBD niosomes leads to smaller formulations. This could be due to the presence of a higher amount of CBD in the membrane, as formulations made with Tween have a higher encapsulation efficiency. As aforementioned, the presence of CBD in the membrane triggers a reduction of the particle size. The presence of CBD in the membrane also increased the physical stability of the niosomes (Figure 10). While B-Nio exhibited an increase in particle size after 1 month of storage at 25°C and 2 months at 4°C, CBD-Nio remained stable at both temperatures for at least 3 months. This increased stability can be correlated with the higher rigidity of the membrane due to the presence of CBD, which reduces the tendency of the vesicles to aggregate.

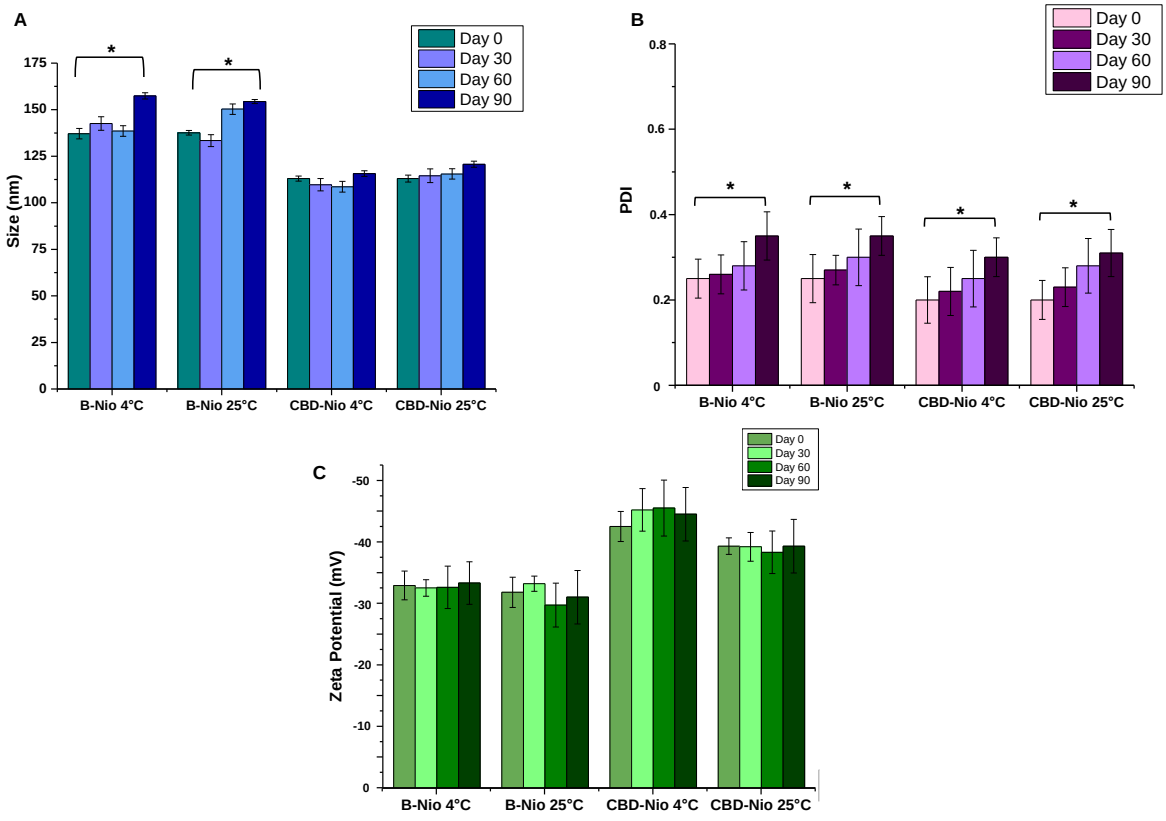

Figure 10: Size, PDI and Zeta potential of blank niosomes and CBD loaded niosomes stored for 3 months at 4°C and 25°C. * indicate statistical significant differences.

Regarding the release profile, it is worth highlighting that CBD-Nio developed in this thesis exhibited a controlled drug release over 24 hours, with approximately 90% of the CBD released by this time. A significant burst effect was observed, as around 30% of the CBD was released within the first 2 hours (Figure 11).

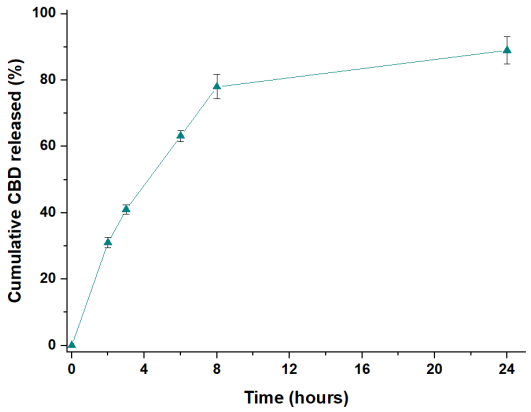

Figure 11: Release profile of CBD-Nio.

As with the CBD-ISFIs, the ability of the niosomes to inhibit the formation of new blood vessels was evaluated. In this case, yolk sac model (YSM) assay was used. In this model, CBD in solution administered at 100µM showed an inhibition of blood vessel formation of around 73% (slightly higher than what was observed in the CAM model). The niosomes exhibited a superior effect with an inhibition percentage of approximately 83% (Figure 12). This value is similar to those detected with PCL-10-NMP ISFI.

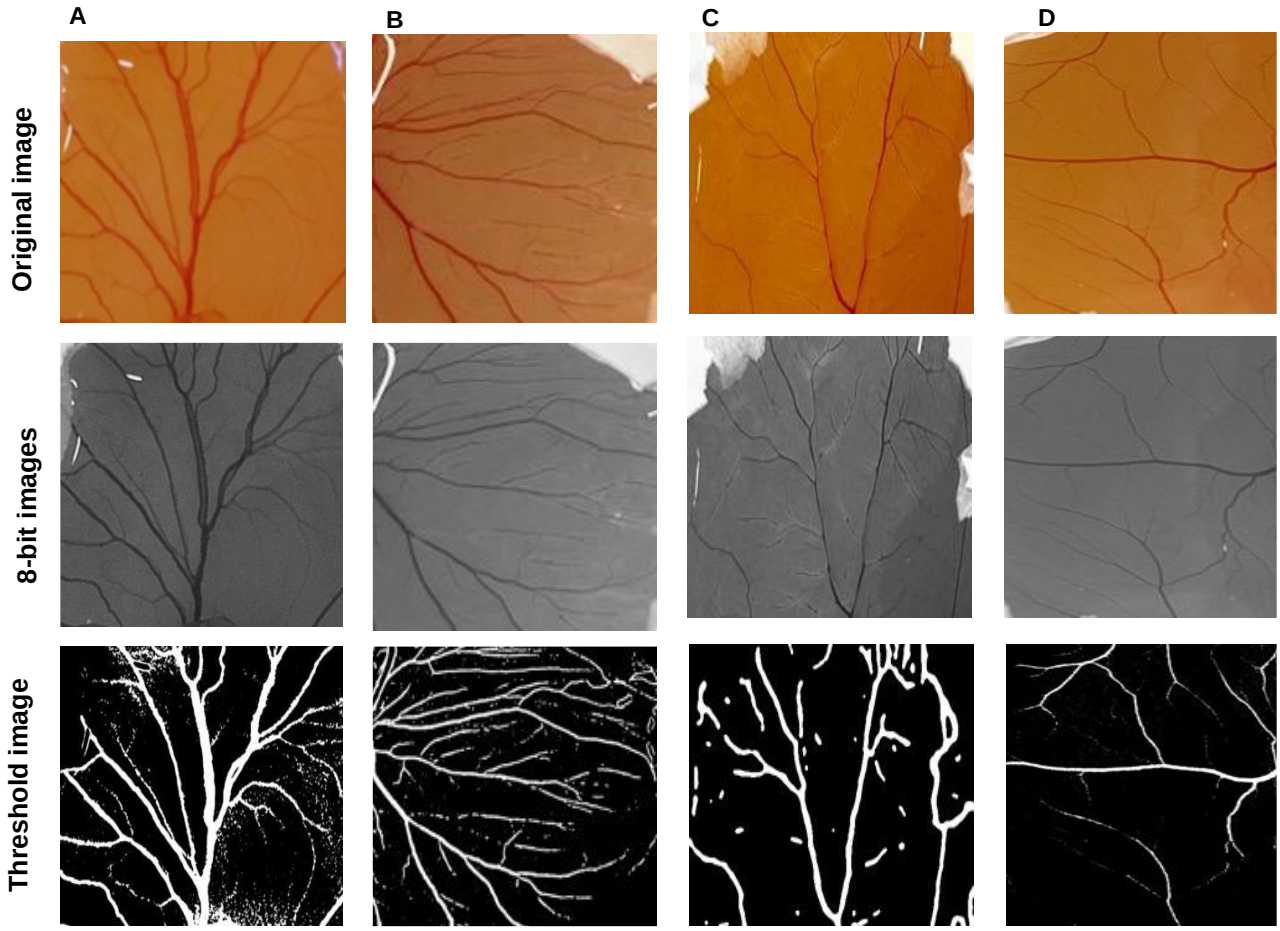

Figure 12: Original and Image J processed images of fertilized chicken eggs treated with NaCl 0.9% solution (Control, A), B-Nio (B), free CBD (C) and CBD-Nio (D) in YSM evaluation.

CBD-Nio also exhibited a stronger antiproliferative effect on MDA-MB-231 and 4T1 cells, which were used as models for TNBC, with IC_{50} values lower than CBD in solution in both cell lines after 24 and 48 hours of incubation. In fact, except in 4T1 cells at 48 hours of treatment, statistically significant differences between CBD in solution and the niosomes were observed (Figure 13). The enhanced effect of CBD-Nio on cell viability can be attributed to the internalization of these formulations, which leads to an improved

intracellular delivery of CBD. CBD-Nio shows a particle size around 113 nm which is suitable for cell internalization [32].

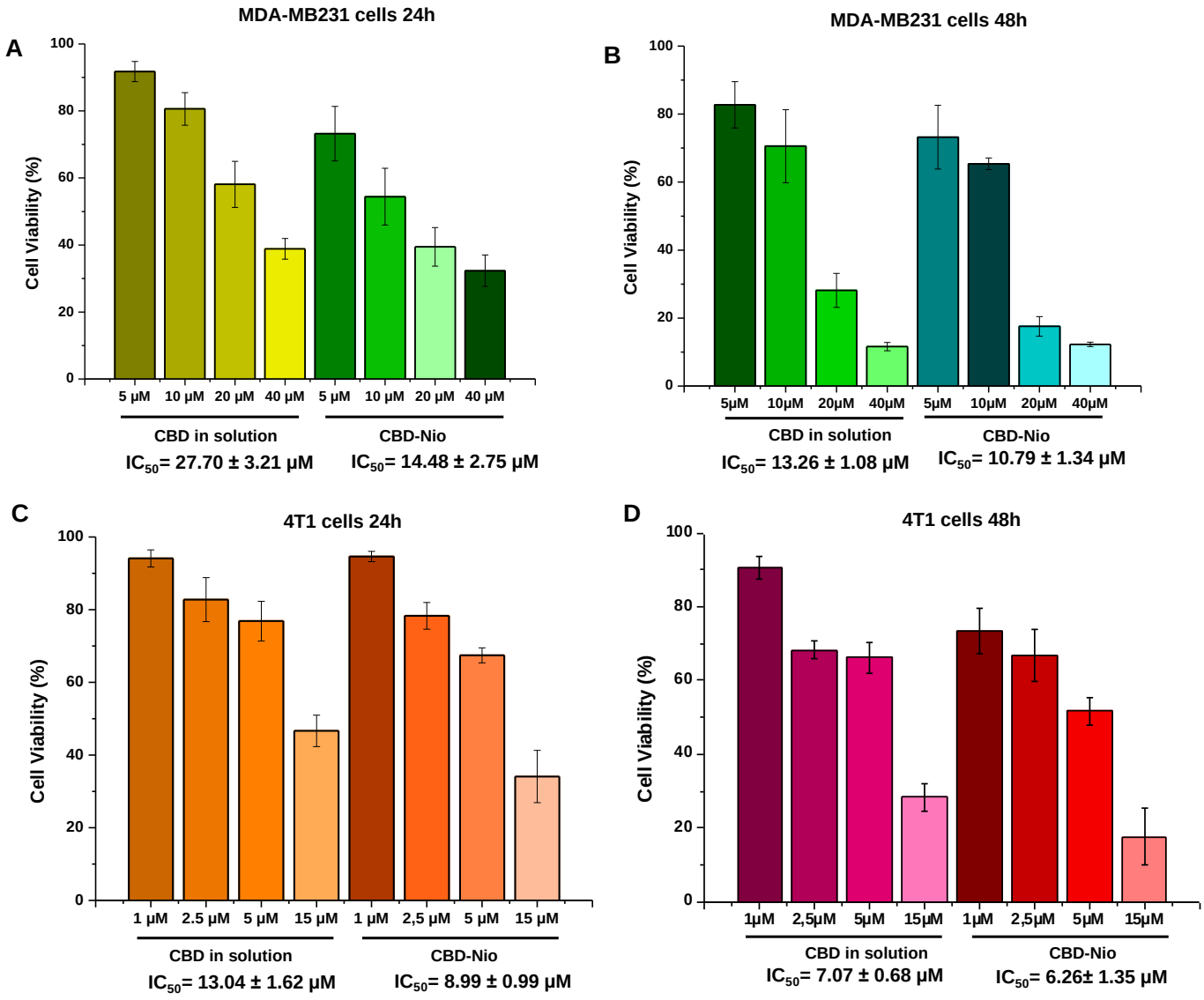

Figure 13: Viability assay on TNBC cell line tumor model. Results of free CBD and CBD-Nio administered on MDA-MB231 after 24hours (A) and 48hours (B), and on 4T1 cells after 24 hours (C) and 48 hours (D).

The CBD-loaded niosomes were also effective in reducing the migration of TNBC cells (Figure 14) when administered at CBD concentrations of 10 and 20μM in MDA-MB-231 cells and 5 and 7.5μM in 4T1 cells, respectively.

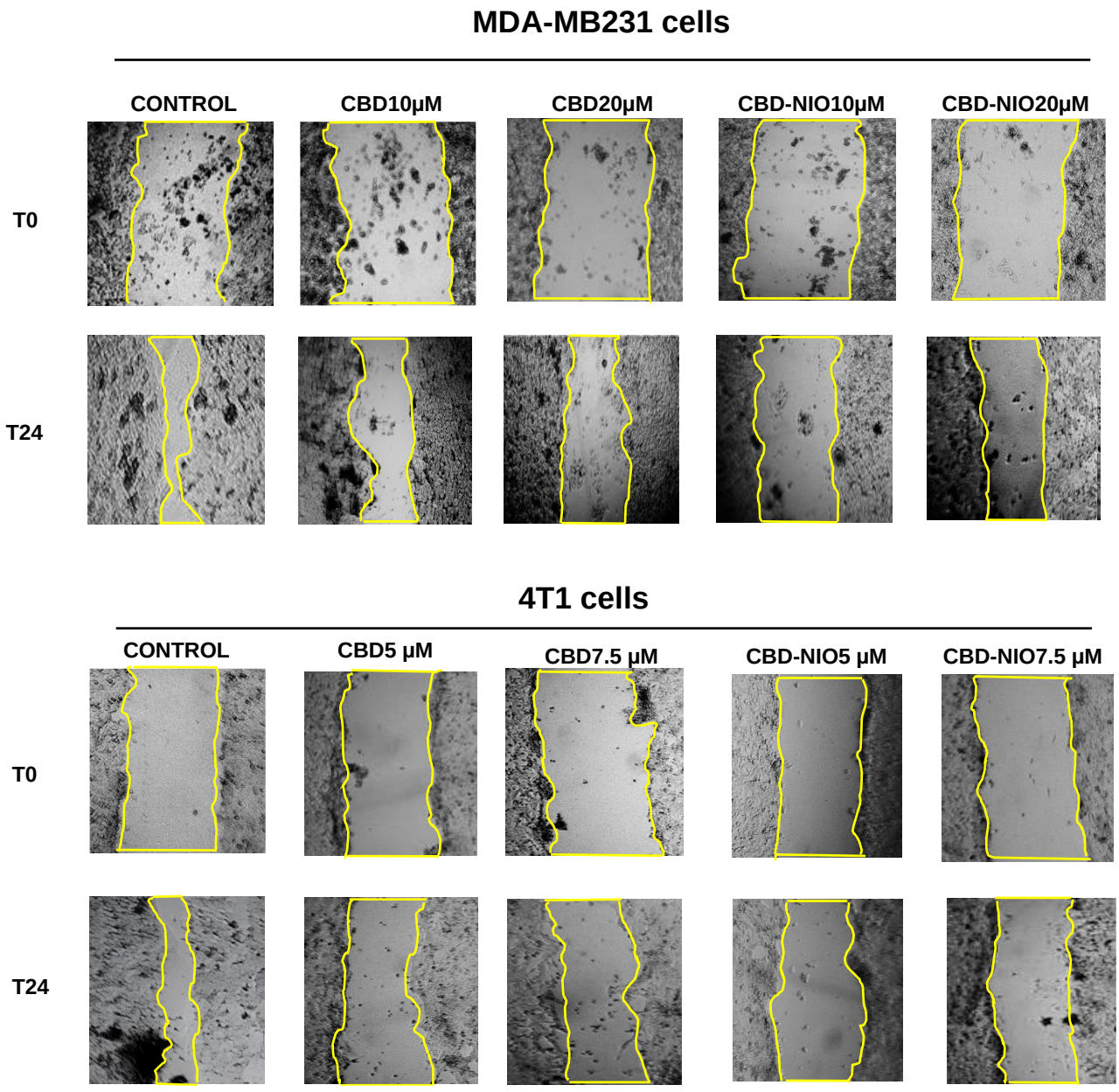

Figure 14: Images of wound closure of MDA-MB231 and 4T1 before (T0) and 24 hours (T24) after the treatment with CBD in solution or CBD-Nio. Untreated cells were used as control.

After 24 hours of incubation, in MDA-MB-231 cells, CBD-Niosomes showed a greater and statistically significant (p-value <0.05) inhibition in wound closure at 10 μM than CBD in solution with reduction percentages of 28 and 62%, respectively. At 20 μM, the difference between both treatments was smaller and not statistically significant, with reduction percentages of 59 and 66%. In 4T1 cells, both CBD in solution and CBD-Nio showed similar effects, reducing wound closure by approximately 35% at 5 μM and 50% at 7.5 μM. These results are in accordance with the calculated migration ratios. Table 5 displays the ratios after 24 hours of treatment.

MDA-MB231 cells				4T1 cells			
CBD sol		CBD-Nio		CBD sol		CBD-Nio	
10µM	20µM	10µM	20µM	5µM	7.5µM	5µM	7.5µM
0,71±0,26	0,40±0,29	0,40±0,14	0,33±0,17	0,73±0,14	0,54±0,21	0,71±0,13	0,48±0,15

Table 5: Migration ratios of CBD in solution and CBD-Nio in MDA-MB-231 and 4T1 cells after 24 hours of treatment. Data are expressed as the mean±standard deviation.

Finally, the anticancer efficacy of CBD-Nio was also evaluated in ovo in tumors derived from MDA-MB-231 cells. Similar to the implant studies, this cell line was chosen due to its human origin. In this model, both CBD in solution and CBD-Nio slowed the growth and reduced the size of these tumors. CBD-Nio exhibited a slightly higher tumor reduction (approximately 50.3%) than CBD in solution (approximately 44%). However, statistically significant differences were not detected (Figure 15).

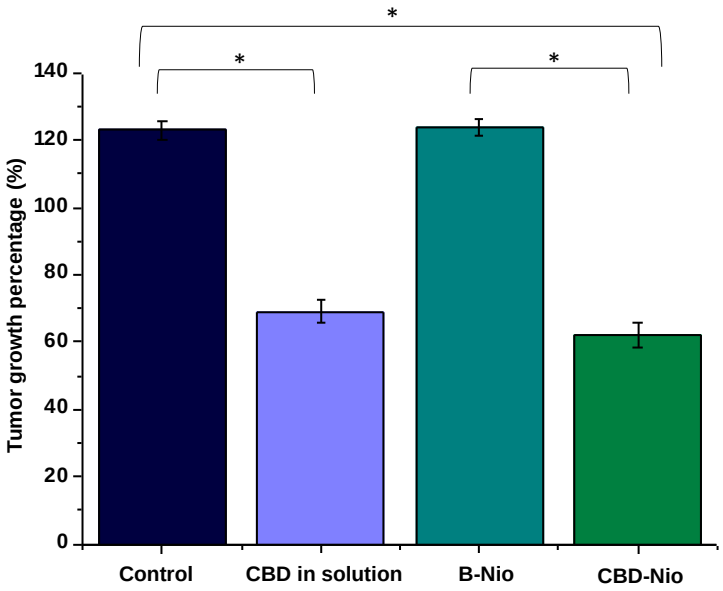

Figure 15: Tumor growth inhibition of MDA-MB-231 derived tumors developed using CAM model assay. Percentage of tumor growth expressed as volume percentage of free CBD, CBD-Nio and Blank-Nio after 48 hours of incubation compared to the control group. * Symbol displays statistically significant values between the treatment groups.

The results obtained in this doctoral thesis indicate that both the development of CBD-loaded ISFIs and CBD-loaded niosomes represent effective strategies for formulating this

cannabinoid. The development of these systems not only maintained but enhanced the antitumor efficacy of CBD. The optimized in situ forming implants (ISFIs) prove to be an excellent formulation for achieving long-lasting effects with a single administration, which is especially useful given the long nature of chemotherapy treatments. On the other hand, the development of niosomes offers a promising technological tool for administering CBD without the need for solvents and for improve its delivery in the tumor area. Although further studies are still needed, both formulations show promising potential in the treatment of TNBC.

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CONCLUSIONES/CONCLUSIONS

CONCLUSIONES

1. Las soluciones poliméricas de PLGA 502, PLGA 502H, PLA 202 o PLA 203 en DMSO, y de PLGA 502, PLGA 502H, PLA 202 o PCL en NMP, a una concentración de 0.25 mg/µL, mostraron buenas propiedades de inyectabilidad a través de agujas de calibre 23G y 25G, por lo que son adecuadas para la elaboración de ISFIs cargados con CBD.

2. Se han desarrollado ISFIs de PLGA y PLA con dos diferentes ratios de CBD:polímero (2.5:100 y 5:100), y formulaciones de PCL con tres ratios (2.5:100, 5:100 y 10:100). Todos los implantes mostraron una liberación controlada de CBD durante al menos una semana. Los perfiles de liberación del fármaco variaron significativamente en función del tipo de polímero, el solvente y el ratio fármaco:polímero. De todos los ISFIs de PLGA y PLA, la formulación de PLGA-502 y DMSO con un ratio fármaco:polímero de 5:100 mostró una liberación controlada de un mes, pero con un efecto burst del 30%.

3. Los ISFIs de PCL preparados con una proporción de fármaco:polímero de 10:100 mostraron una liberación controlada del CBD durante dos meses y un bajo efecto burst del 12%. Esta formulación no presentó signos de toxicidad en el ensayo HET-CAM y mostró efectos antiproliferativos y anti-migración en células de TNBC tanto humanas (MDA-MB-231) como murinas (4T1), así como una reducción del tamaño de los tumores derivados de células MDA-MB-231 generados in ovo. Además, esta formulación redujo la angiogénesis en estudios in ovo.

4. Se han obtenido niosomas de CBD compuestos de Tween 85 y colesterol mediante el método de “evaporación y rehidratación” con un tamaño de 137 nm y una carga de 0.92 mg de CBD por ml de formulación. En el proceso de nanoencapsulación, el CBD queda atrapado en la membrana vesicular, lo que aumenta su hidrofobicidad y conduce a una disminución del tamaño de partícula y a un aumento de la rigidez de la membrana que mejora la estabilidad física de los niosomas.

5. Los niosomas mejoran el efecto antiangiogénico del CBD y su capacidad para reducir la proliferación de células MDA-MB-231 y 4T1. Además, disminuyen el tamaño de los tumores derivados de estas mismas células generados in ovo.

6. Aunque se requieren más estudios in vivo para una caracterización biológica más completa de los sistemas desarrollados en esta tesis doctoral, tanto los ISFIs como los niosomas representan recursos tecnológicos prometedores para la administración de CBD y la optimización de su actividad anticancerígena en TNBC.

CONCLUSIONS

1. Polymeric solutions of PLGA 502, PLGA 502H, PLA 202, or PLA 203 in DMSO, as well as PLGA 502, PLGA 502H, PLA 202, or PCL in NMP, at a concentration of 0.25 mg/µL, exhibited good injectability properties through 23G and 25G needles, being suitable for the development of CBD-loaded ISFIs.
2. ISFIs based on PLGA and PLA have been developed using two different CBD:polymer ratios (2.5:100 and 5:100), while PCL formulations were prepared with three ratios (2.5:100, 5:100, and 10:100). All implants exhibited controlled CBD release for at least one week. Drug release profiles varied significantly depending on the type of polymer, solvent, and drug:polymer ratio. Among all PLGA- and PLA-based ISFIs, the formulation prepared with PLGA-502 and DMSO at a drug:polymer ratio of 5:100 exhibited a controlled release for one month, albeit exhibiting a Burst effect of 30%.
3. PCL-based ISFIs with a 10:100 drug:polymer ratio showed a controlled CBD release over two months and a low burst effect of 12%. This formulation exhibited no signs of toxicity in the HET-CAM assay and antiproliferative and anti-migratory effects in both human (MDA-MB-231) and murine (4T1) TNBC cells, as well as a reduction in the size of tumors derived from MDA-MB-231 cells developed in ovo. Moreover, this formulation reduced angiogenesis in ovo studies.
4. CBD niosomes, composed of Tween 85 and cholesterol, were obtained using the "thin film " method. They exhibited a particle size of 137 nm and a CBD loading of 0.92 mg per mL of formulation. During the nanoencapsulation process, CBD is entrapped within the vesicular membrane increasing its hydrophobicity, which in turn leads to a reduction in particle size. An increase in membrane rigidity is also triggered. This enhances the physical stability of the niosomes.
5. Niosomes enhance the anti-angiogenic effects of CBD and its ability to reduce the proliferation of MDA-MB-231 and 4T1 cells and to reduce the size of tumors derived from these cells developed in ovo.
6. Although further in vivo studies are required for a more comprehensive biological characterization of the systems developed in this doctoral thesis, both ISFIs and niosomes represent promising technological platforms for CBD delivery and the optimization of its anticancer activity in TNBC.