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***Physicochemical and bioengineering studies of drug-macromolecules
interactions for therapeutic applications***

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Abstract

Cancer is a leading global cause of mortality, pushing continuous efforts in scientific research toward the development of new therapeutic approaches. Polymeric nanoparticles are pivotal in this perspective due to possibility of obtaining drug targeting and a preferential accumulation in tumors. The main objective of this thesis was to engineer nanoparticles based on poly(lactic-co-glycolic acid) (PLGA) as advanced systems to overcome the pharmacokinetic limitations of conventional chemotherapeutics.

The research was divided into three main phases. In the first part, the impact of different surface properties on the *in vivo* biodistribution of nanoparticles was studied. To this aim, nanoparticles composed of only PLGA (P NPs) and a blend of PLGA and Poloxamers (PP NPs) were produced and loaded with fluorescent dye. The presence of Poloxamers resulted in smaller mean diameters and only partial crystallinity. Biodistribution studies in mouse models showed that, while both formulations had comparable blood concentrations, only PP NPs exhibited a relevant sequestration in lungs and, to a lesser extent, in the kidneys, whereas P NPs were undetected in the analysed organs.

A second part of the study was focused on the encapsulation of silibinin (SLB), which is the active component of silymarin, a flavonoid derived from the seeds of milk thistle. SLB has many interesting properties which make it attractive in tumor treatment. Indeed, SLB possesses inhibitory effects on tumor invasion, and metastasis and can reverse multidrug resistance. Nonetheless, the bioavailability of SLB is strongly hindered by its low aqueous solubility, which causes the need for high doses to elicit adequate plasma levels. SLB have been loaded in polymeric NPs. The resulting nanoparticles showed encapsulation efficiency greater than 84%, with the SLB present in an amorphous state. *In vitro* release mechanism was found to be dependent on drug loading. Crucially, bioactivity tests on several lung cancer cell

lines (H1299, H1975, and H358) demonstrated that encapsulated SLB has significantly greater efficacy than the free drug.

Finally, the NPs were endowed with active targeting ability by coating with hyaluronic acid (HA) through physical interaction. The main focus of this research was to determine the combined effect of active targeting and X-ray on NPs internalization by breast cancer cells. The results showed that high-dose irradiation induces a significant upregulation of CD44 receptor, consequently leading to a marked increase in the internalization of HA coated NPs *via* a receptor mediated mechanism. This contributes to paving the way for a combined therapeutic strategy.

Overall, in this work a versatile, surface-tunable biodegradable nanocarrier platform capable of overcoming critical pharmacokinetic barriers, developed through a one-step procedure based on physical blending. The main outcomes of this work indicate that rational surface engineering can provide NPs with different targeting abilities, potentially contribute to improved therapeutic outcomes in oncology.

Chapter 1

Background and aims

1.1 Epidemiological Distribution of Cancer Worldwide.

Cancer is one of the leading causes of mortality worldwide, second only to cardiovascular disease. According to the latest data from the Global Cancer Observatory (GLOBOCAN), approximately 20 million new cases of cancer and 9.7 million cancer-related deaths were recorded worldwide in 2022.

Cancers, or neoplasia, are a group of diseases characterised by the uncontrolled proliferation of abnormal cells that evade the normal regulatory mechanisms governing cell division and growth. Among the most common cancers in terms of incidence are:

- Lung cancer, the most frequently diagnosed malignancy, with approximately 2.5 million new cases. It also represents the leading cause of cancer-related death.
- Breast cancer, the second most common cancer, with approximately 2.3 million cases, and the most prevalent cancer among women.
- Colorectal cancer, with over 1.9 million new cases, it ranks third in global incidence.
- Prostate cancer, the fourth most diagnosed cancer, with an estimated 1.5 million cases.
- Stomach cancer, responsible for approximately 1.2 million new cases worldwide.

Absolute numbers, Incidence, Both sexes, in 2022
 Continents
 (Top 13 cancer sites)

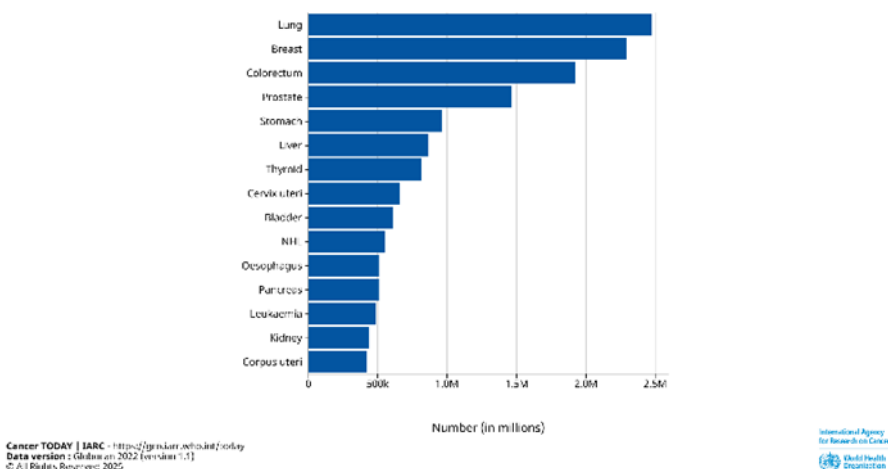


Figure 1: Incidence of tumors worldwide

The geographical distribution of cancer types shows significant inhomogeneities. In high-income countries, lifestyle-related cancers, such as lung, breast and colorectal cancer, are more prevalent (Fig. 2). In contrast, in low- and middle-income countries, infection-related cancers. Notably, cervical cancer (associated with human papillomavirus, HPV) and liver cancer (associated with hepatitis B and C viruses), account for a substantial proportion of cancer burden in these regions (Fig. 3)¹.

Absolute numbers, Incidence, Both sexes, in 2022
High income

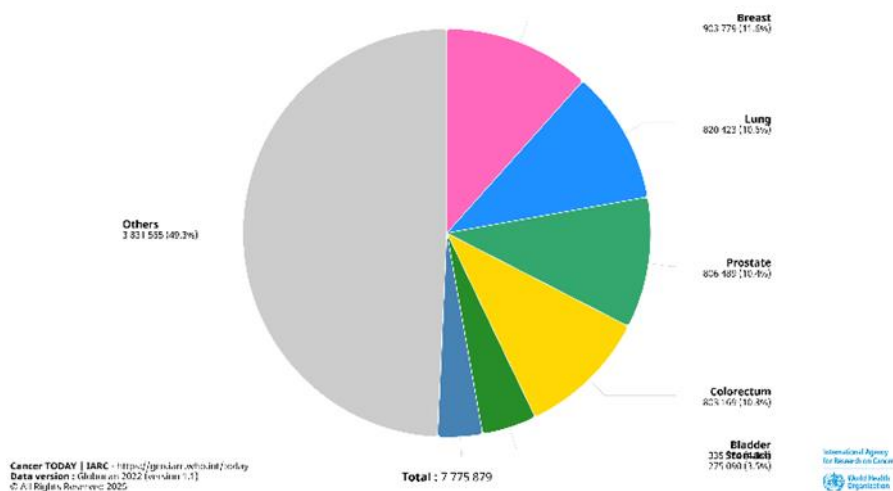


Figure 2: Incidence of tumors in high-income countries.

Absolute numbers, Incidence, Both sexes, in 2022
Low income

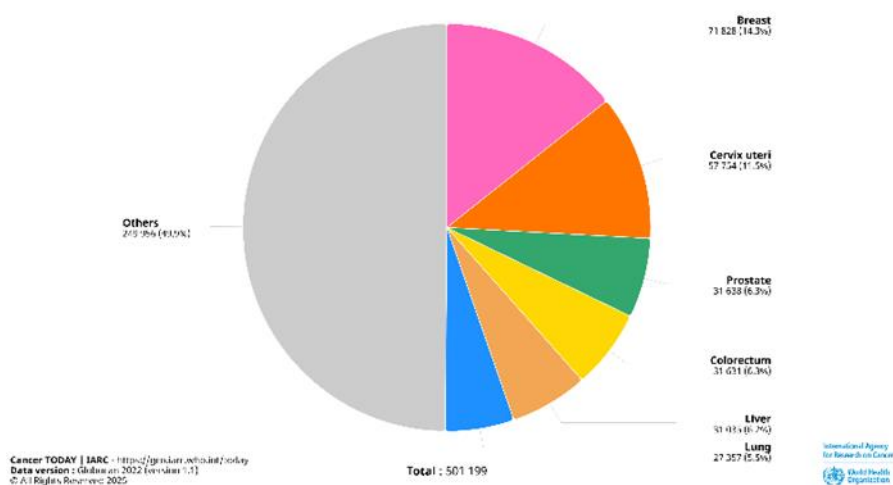


Figure 3: Incidence of tumors in low- and middle-income countries.

1.2 Physiopathology of tumors

In a healthy organism, the regulation of the cell cycle, cell growth, division and death, is a process strictly controlled by a complex system of molecular signals. When these control mechanisms fail, cells can begin to proliferate abnormally and uncontrollably, accumulating genetic mutations that give them advantages in growth and survival over normal cells, leading to the formation of tumors.

The term “tumor” derives from the Latin *tumor*, meaning “swelling”, and refers to the mass of abnormal tissue that forms. It is precisely the genesis of this tissue with its characteristics that has allowed the pathology to be identified with the term “neoplasia”, from the Greek *neo-* (new) and *plásis* (formation). Another synonym used to describe tumors is “cancer”, historically used by the ancient Greeks and Latins to describe the appearance of the swollen veins surrounding the tumor, which resembled the shape of a crab, (the central body is the mass of tumor and the veins appeared as the legs of the "crab"; etymologically, this word derives from the Greek *karkinos* and the Latin *cancer*, both meaning “crab”). In fact, cancer, in the strict sense, is a malignant tumor that has acquired the ability to infiltrate and destroy surrounding tissues and spread to distant sites².

The formation of a tumor, known as carcinogenesis, is based on a series of complex and multi-stage phenomena. It is generally the result of the accumulation of mutations affecting key genes that regulate cell growth and differentiation. These mutations can be caused by external agents (carcinogens) or by internal errors during the DNA replication process.

The main types of genes involved in carcinogenesis are:

Oncogenes originate from proto-oncogenes, genes that, under normal conditions, stimulate cell growth and division. When a proto-oncogene undergoes a mutation, it transforms into an oncogene, which promotes uncontrolled cell proliferation independent of external growth signals. A classic example is the RAS oncogene³.

Tumor suppressor genes, by contrast function to inhibit cell division and maintain genomic integrity through DNA repair mechanisms. Mutations in these genes lead to the loss of their control function, allowing cells to proliferate unchecked. The p53 gene is a crucial tumor suppressor, known as the “guardian of the genome”⁴.

DNA repair genes play a critical role in repairing the damage that occurs continuously in our DNA. Mutations in these genes increase the frequency of other mutations, accelerating the process of carcinogenesis. Examples are the BRCA1 and BRCA2 genes, whose mutations are associated with an increased risk of breast and ovarian cancer⁵.

The carcinogenesis process typically consists of several stages:

- Initiation, a cell undergoes an initial mutation in a critical gene, often following exposure to a carcinogen (e.g. tobacco smoke, UV rays).
- Promotion, the mutated cells begin to proliferate in response to growth-promoting stimuli, leading to the formation of a mass of pre-cancerous cells.
- Progression, further mutations accumulate, making the cell mass more aggressive, able to invade neighbouring tissues and, ultimately, metastasise⁶.

1.3 Classification of Tumors: Benign, Malignant, Solid and Less Common

Tumors can be classified according to their biological nature and the type of tissue from which they originate. During histopathologic diagnosis, the lesion of suspected tissue is first classified as neoplastic or non-neoplastic and subsequently as epithelial neoplasm or a mesenchymal neoplasm. The next step is the determination of the behavioural nature of the tumor as benign or malignant.

Benign Tumors grow slowly and are localised in a specific area, surrounded by a capsule of connective tissue. They do not invade surrounding tissues or spread to

distant sites (they do not metastasise). Examples include fibroids, lipomas and adenomas⁷. They are not usually life-threatening, unless they compromise vital structures and the physiology of surrounding organs. Tumor masses can compress surrounding organs and tissues, leading to secondary symptoms: for example, a benign tumor in the brain near the optic nerves may compress them, thereby altering vision.

In contrast, malignant tumors are characterized by a rapid cellular proliferation and the ability for local tissue invasion, ultimately leading to the destruction of adjacent tissues and organs. A defining feature of malignancy is the ability to metastasize, which consists in the dissemination of neoplastic cells to distant sites from the primary tumor location, typically *via* the circulatory or lymphatic system. The metastatic potential confers the clinical danger and the general poor prognosis associated with malignant tumors⁸⁻¹⁰.

Malignant tumors are broadly categorized into two major groups based on their physical form and tissue of origin: solid tumors and hematologic malignancies. Solid tumors are the most common type of cancer and are characterized by the formation of a distinct mass or lump of tissue. These are further classified based on the cell type from which they originate. Specifically, carcinomas are the most prevalent type of solid tumor, which originate from the epithelial cells that line organs, glands, and the skin. Common examples of carcinomas include cancers of breast, lung, prostate, and colon, just to name a few. Conversely, sarcomas are less common and arise from connective (mesenchymal) tissues, which serve for body structural support. This category includes tumors of bone, cartilage, fat, muscle, and blood vessel, with examples such as osteosarcomas and liposarcomas. Other, less common solid tumors may originate from other tissues, such as the central nervous system.

Hematologic cancers are tumors that do not form localized, solid masses but primarily affect the blood, bone marrow, and lymphatic system¹¹. One major example

of these cancers includes leukemias, that are malignant tumors of the blood and bone marrow, characterized by the uncontrolled proliferation of abnormal white blood cells systemically disseminated by the bloodstream¹². Another key group of hematological cancers are lymphomas, which originate in lymphoid tissues, specifically affecting the cells of the immune system like lymphocytes, often presenting at the start as swollen lymph nodes.

1.3.1 Lung cancer

Lung cancer is currently recognized as the most frequently diagnosed malignant tumor and the leading cause of cancer-related deaths worldwide. Among the main etiological factors that determine the onset of lung tumors, tobacco smoking clearly plays a pivotal role^{13,14}, although familial predisposition and genetic susceptibility have also been widely recognized as concurrent causes¹⁵.

Primary lung cancer originates from epithelial cells, and the histological classification divides it into two major categories, namely Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). NSCLC constitutes approximately 80-85 % of cases. This form typically shows a slower progression rate and is more often confined to the primary site after detection¹⁶. SCLC roughly accounts for 15-20% of cases and is a highly aggressive cancer characterized by a swift growth, and early metastatic dissemination, along with a rapid onset of drug resistance. These features lead to poor prognosis and severely limit survival¹⁷.

This categorization is fundamental because it guides the therapeutic approach. In NSCLC exist three typical clinical protocols determined by the stage of tumor. In the patients with the tumor in stage I-II (early stage), the surgical resection of pulmonary parenchyma is the standard procedure so far. In contrast, in patients at stage II-III (locally advanced) the therapy commonly consists in three to four cycles of chemotherapy using cisplatin and vinorelbine. For the clinical cases with metastasis,

in stage IV, a palliative treatment regimen is normally used, typically with a combination of cisplatin and gemcitabine, vinorelbine, paclitaxel or docetaxel¹⁸.

Unlike NSCLC, SCLC had seen limited therapeutic progress for decades. In fact, a combination of platinum-based chemotherapy and etoposide was the first-line treatment which remained unchanged for over 30 years. More recently, the introduction of combination chemo-immunotherapy, utilizing agents such as atezolizumab and durvalumab, has led to a significant improvement of survival expectancy for patients in extensive stage disease¹⁷.

1.4 Therapeutic Approaches: Surgery, Drugs and Radiotherapy

Cancer treatment is a constantly evolving field based on a multidisciplinary approach, often combining different therapeutic strategies to maximise effectiveness and minimise side effects. Conventional therapies encompass surgery, radiotherapy and pharmacological treatment.

Surgery is the primary treatment choice for most solid tumors, particularly when the disease is localized and potentially curable. In addition to its therapeutic role, surgery is fundamental for the diagnosis, subtyping and grading of cancers through biopsy. In the surgical resection, the primary goal is to completely remove the tumor mass with a margin of surrounding healthy tissue to prevent local recurrence. In many cases, surgery is used in combination with other therapies. If a tumor is too large or has already metastasised, surgery may not be the first option, but it can be used for debulking, the reduction of the tumor volume, or for palliative surgery, to relieve symptoms¹⁹. For instance, debulking has demonstrated a significant survival benefit in advanced ovarian cancer, even when curative resection is not achievable²⁰.

Drug therapy acts systemically and is essential for the treatment of metastatised or haematological tumors. Among drug-based approaches, chemotherapy uses cytotoxic drugs that kill rapidly dividing cells, including cancer cells. Unfortunately, chemotherapy is generally non-specific and can damage also healthy, rapidly

replicating cells, such as those in the bone marrow, hair follicles and intestinal mucosa, thereby causing severe side effects such as nausea, hair loss and immunosuppression. In this context, targeted therapy offers a more selective approach that acts on specific molecular targets in cancer cells, such as abnormal proteins or signalling pathways. Because of the focus on specific mechanisms, targeted therapy is often less toxic than traditional chemotherapy. Some relevant examples of targeted therapy rely upon tyrosine kinase inhibitors (e.g. imatinib for chronic myeloid leukaemia) and monoclonal antibodies (e.g. trastuzumab for HER2-positive breast cancer). A third major therapeutic approach is immunotherapy, which is aimed at stimulating the immune system of the patient to recognise and attack cancer cells. This approach has revolutionised cancer treatment, exemplified by the use of immune checkpoint inhibitors (e.g. pembrolizumab), which block the inhibitory signals that cancer cells exert against the immune system, allowing it to activate²¹.

While in systemic treatments drugs travel through the bloodstream to attack cancer cells throughout the body, radiotherapy employs high-energy radiation beams ionising radiation (e.g. X-rays or gamma rays) targeted at specific tumor sites to kill cancer cells locally. Radiotherapy induces damage the DNA of cancer cells, thereby inducing cells death, and can be used alone for localised tumors, or in combination with surgery (such as neoadjuvant radiotherapy, before surgery; or adjuvant, after surgery) and chemotherapy²².

1.4.1 Naturally occurring substances as potential chemotherapeutic drugs.

Medicinal plants and their metabolites represent a valuable and promising resource in cancer treatment. Plant secondary metabolites, mainly involved in the defence mechanisms of plants and interspecies communication, exhibit an interesting pharmacological profile on multiple metabolic pathways and cellular signalling pathways. This activity can enable the arrest or slowing down of cancer cells proliferation^{23,24}. Furthermore, the structural complexity of these natural products,

which is often difficult to reproduce through synthetic lab processes, offers the possibility of identifying scaffold structures that can be suitably modified to improve their pharmacokinetic profiles and pave the way for the identification of novel active compounds as nature-inspired anticancer agents ²⁵.

Among the main classes we find:

- Alkaloids, nitrogen compounds, often with complex structures. Well-known examples: Vinca alkaloids (vincristine, vinblastine) which block microtubules;²⁶
- Flavonoids and polyphenols, these compounds include quercetin, resveratrol, curcumin, silibinin etc. They act as antioxidants, but also through induction of apoptosis, modulation of signalling pathways and cell cycle arrest^{27,28}
- Terpenoids/terpenes, molecules derived from the mevalonate or non-mevalonate biosynthetic pathways, often lipophilic. An example is β -elemene, which induces autophagy and alters pathways such as PI3K/Akt/mTOR and MAPK/ERK²⁹.
- Saponins and steroid compounds, found in many plants such as Paris polyphylla. Saponins can have direct cytotoxic activity, stimulate immunity, alter cell permeability or membrane interactions³⁰.
- Carotenoids, such as lycopene, which has been shown to have antitumor activity in various studies. They also act as antioxidants, modulating oxidative stress, but also on signalling pathways³¹.

1.4.1.1 Silibinin: therapeutic potential in cancer therapy

Among natural compounds with anticancer potential, silibinin stands out as particularly promising. It is the principal bioactive flavonolignan extracted from the seeds of *Silybum marianum*, commonly known as milk thistle. *Silybum marianum* is a biennial, spiny plant that belongs to the Asteraceae family. It was originally grown

in southern Europe and Asia but now can be found throughout the world. For years, in many cultures it has been widely used in traditional medicine for its therapeutic properties³² such as antioxidant, antineoplastic and hepatoprotective, along with anti-cancer effects *in vitro* demonstrated in literature^{33–35}. The phytocomplex responsible for its active properties consists of a mixture of flavonolignans, including silibinin, isosilibinin, silicristin and silidianin. However, silibinin, which exists as a combination of two diastereomers, silibinin A and B, is the primary active ingredient extracted from the seeds.

In fact, as reported in the recent review of Anupam Sharma et al, silibinin shows a strong potential in both cancer prevention and treatment. This efficacy is attributed to its ability to target multiple key processes involved in cancer development and progression, summarized in the following:

- Inhibition of cancer cell growth and survival by increasing intracellular levels of Ca and downregulating key signals pathways as PI3K/AKT, MAPK and STAT3;
- Induction of apoptosis triggering ROS production, which activates MAPKs, enhancing the levels of proapoptotic, such as Bax, p53 etc, and suppressing anti-apoptotic protein Bcl-2 proteins;
- Autophagy by promoting markers such as LC3-1 to LC3-II, Beclin-1 ATG5, ATG12;
- Anti-angiogenesis reducing proteins MMP-2, MMP-9, COX2, iNOS, VEGF, Flt-1;
- Antimetastatic effects reducing MMP-2, MMP-9, EGFR and lysyl oxidase;
- Cell cycle arrest inducing arrest in G0/G1 and G2/M and upregulating Cip1/p21 and Kip1/p27³⁶.

However, similarly to many plant-derived active molecules, the *in vivo* bioavailability of silibinin is very low, mainly because of its intrinsically poor solubility, limited absorption, rapid elimination and non-selective toxicity. Altogether, these features severely limit the use of silibinin in clinical settings. To address these pharmacokinetic limitations nanotechnology-based delivery systems offer a promising solution^{33,37–39}.

1.5 Nanotechnology

Nanotechnology-based therapeutics typically include nanosized particles with a mean diameter of 1–1000 nm, composed of different substances such as lipids, polymers, and inorganic materials, to name a few. Among the various platforms, polymer nanoparticles have the advantages of ensuring greater stability over time and higher encapsulation efficiency.

Polymer particles can be classified according to their structure and divided into two main groups⁴⁰:

- Nanospheres, consisting of a more or less compact polymer matrix in which the drug is dispersed.
- Nanocapsules, consisting of a polymer membrane surrounding an internal cavity where the drug is encapsulated.

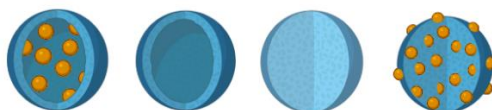


Figure 4: Examples of polymeric nanoparticles (Created in <https://BioRender.com>).

Different techniques and approaches can be used for their production according to the characteristics of the polymers and drugs, site of action and release time⁴¹.

Among the materials used to produce nanoparticles, those approved by Food and Drug Administration (FDA) play a prominent role due to their established safety profiles and clinical applicability. One of the most commonly used degradable polymer for the production of nanoparticles is poly-lactic co-glycolic acid (PLGA), which belongs to the category of polyesters⁴². These polymers are characterized by monomeric units joined by ester bonds, which confer hydrolytic degradability. The main release mechanisms involved in PLGA-based drug delivery systems are diffusion and erosion.

Water absorption leads to the formation of pores in the polymer matrix and swelling, until pores large enough for drug transport are created.

The hydrolysis of ester bonds occurs immediately after contact with water and causes the production of glycolic and lactic acid, the monomeric byproducts of PLGA degradation. These acidic compounds progressively lower the pH within the polymeric matrix, thereby accelerating the hydrolytic process through autocatalysis.

In fact, in PLGA-based systems, degradation is closely linked to water absorption, which in turn is dictated by manifold variables, such as⁴³:

- the molecular weight of the polymer (typically between 10 and 150 kDa in the case of PLGA);
- the molar ratio between lactic acid and glycolic acid; a low L:G ratio promotes water absorption and therefore hydrolysis and erosion rates;
- the nature of the terminal group (ester or acid);
- the nature of the release medium;
- the pH and osmolarity;
- the nature of the drug loaded.

In principle, a wide array of active molecules can be encapsulated in nanoparticles. These nanodevices can act as cargo systems protecting the loaded compound(s) from the harsh biological environment and can also play a role in dictating the release time frame. In the last decades, research efforts have been increasingly focused on developing new strategies in anticancer treatment to overcome the limitation of traditional therapy, such as systemic toxicity, poor selectivity, and multidrug resistance. A key advancement in this field is the targeted delivery of therapeutic agents directly to tumor sites, which enhances efficacy while minimizing adverse effects. Drug delivery to tumors can be achieved through two main mechanisms: passive targeting and active targeting^{44,45}.

In passive targeting, the accumulation of nanoparticles in tumor cells is favored by taking advantage of Enhanced permeability and retention (EPR) effect, which is due to a set of anatomical and physiological features of solid tumor tissues. Specifically, the endothelial lining of immature vessels feeding the tumor is looser compared to healthy tissues due to the presence of larger fenestrations. This results in an increased vascular permeability. When combined with a less efficient lymphatic drainage, the promoted tumor permeability results into leads to an inadequate circulatory recovery of extravasated fluid. Therefore, nanoparticles exposing hydrophilic moieties on the surface can elude the reticuloendothelial system and possess prolonged circulation (stealth) properties. This can encourage the preferential, spontaneous accumulation of nanoparticles within tumor tissues thus allowing to exert a partial control over their distribution *in vivo*.

In active targeting, the accumulation of nanoparticles in tumor cells is prompted through the recognition of ligands present on the surface of the particles by specific receptors overexpressed on the surface of tumor cells. The ligands used include small molecules, antibodies, growth factors, and polysaccharides such as hyaluronic acid. For this strategy to be effective, the neoplastic tissue must overexpress the receptor

compared to healthy cells, ensuring selective uptake, and the selected ligand must have high affinity, minimizing off-target interactions.

1.6 Aim of the work

The overarching goal of this thesis was to develop and optimize polymer-based nanoparticles (NPs) for the targeted delivery of anticancer agents. The nanocarrier platform was engineered using an amphiphilic polymeric blend of poly(lactic-co-glycolic acid) (PLGA) and poloxamers, designed to spontaneously self-assemble into a core-shell structure in aqueous environments. This architecture features a hydrophobic PLGA core encapsulated by a hydrophilic poloxamer shell. Multiple formulation techniques were explored to refine and stabilize this structure.

The study was structured into three main phases:

- **Platform Characterization and Biodistribution (Chapter 2):**

The initial phase focused on evaluating the physicochemical properties and biodistribution of the developed NPs in healthy murine models, establishing a baseline for systemic behavior and organ-specific accumulation.

- **Encapsulation and Therapeutic Evaluation (Chapter 3):**

In the second phase, the nanoparticle platform was employed to encapsulate silibinin, a natural flavonolignan with established anticancer properties. Its efficacy was assessed in vitro against lung cancer cell lines, with particular attention to cytotoxicity and cellular uptake.

- **Surface Functionalization and Synergistic Therapy (Chapter 4):**

To enhance active targeting capabilities, the NP surface was further functionalized with hyaluronic acid (HA), a polysaccharide known to bind CD44 receptors, commonly overexpressed in various solid tumors.

Additionally, the potential synergy between NP-mediated delivery and radiotherapy was investigated. Specifically, the study examined whether irradiation could promote NP internalization in breast cancer cells, thereby amplifying therapeutic outcomes through a combined treatment approach.

Chapter 2

Investigating the effect of surface hydrophilicity on the destiny of PLGA-poloxamers nanoparticles in an *in vivo* animal model*

2.1 Introduction

The advent of nanoparticle-based systems has prompted a significant paradigm shift in cancer treatment and drug delivery⁴⁶. Still to date, poly(lactide-co-glycolic acid) (PLGA) has garnered a significant deal of attention as a material of choice for the production of nanoparticles (NPs). Indeed, as highlighted in a recent review, among the various biodegradable synthetic polymers such as polyanhydrides, polyorthoesters, polyphosphazenes, polyamidoesters (heterochain polymers) and polycyanoacrylates (homochain polymers), thermoplastic aliphatic polyesters have always dominated in the field of controlled drug release, due to their wide range of favourable physicochemical and biological properties⁴⁷. More in detail, PLGA has been approved by the US FDA for various therapeutic applications due to its biocompatibility, complete biodegradability, and tunable properties^{47,48}. Furthermore, an important property of block copolymers is that, by changing the content of the two monomers in the copolymer, it is possible to modulate their physicochemical properties. For example, the rate of biodegradation of PLGA can be suitably tailored according to the application purposes, being the *in vivo* resorption period of PLA (100% LA) between 12 and 24 months, while that of PGA (100% GA) only 6–12 months. This variation in the biodegradation rate is due to the poorer hydrophilicity and slower degradation rate of PLA and the relatively higher hydrophilicity and faster degradation rate of PGA. With an LA:GA ratio of 50:50,

* Silvestri T, Grumetto L, Neri I, De Falco M, Graziano SF, Damiano S, Giaquinto D, Maruccio L, de Girolamo P, Villapiano F, Ciarcia R, Mayol L, Biondi M. Investigating the Effect of Surface Hydrophilicity on the Destiny of PLGA-Poloxamer Nanoparticles in an *In Vivo* Animal Model. *Int J Mol Sci*. 2023 Sep 25;24(19):14523. doi: 10.3390/ijms241914523. PMID: 37833971; PMCID: PMC10572154.

PLGA copolymers have a high rate of degradation that slows down as the proportion of LA increases from 50 to 100 and that of GA decreases from 50 to zero ⁴⁷.

However, the clinical translation of PLGA-based NPs necessitates a thorough understanding of their behavior in biological systems, including their biodistribution, clearance, and potential accumulation in off-target organs ⁴⁹.

To overcome limitations associated with the rapid clearance and non-specific uptake of NPs by the reticuloendothelial system (RES), surface modification strategies have been employed ⁵⁰. Among these, the surface coating of NPs with hydrophilic moieties has emerged as an effective means to discourage their rapid phagocytosis in vivo, therefore enhancing their stealth properties and prolonging their circulation time ⁵¹.

In this context, the incorporation of a hydrophilic polymer such as polyethylene glycol (PEG) on NP's surface is expected to hinder the adsorption of opsonins, hence enhancing NP stability in physiological conditions. This fosters the extended circulation of NPs, thereby increasing their passive accumulation to tumors ⁵².

To this aim, we have recently formulated NPs made up of an equiponderal mixture of PLGA and poloxamers. Poloxamers, also known by the brand name Pluronic, are triblock copolymers made up of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO), displaying amphiphilic properties taking advantage of the presence of hydrophilic EO and hydrophobic PO segments on polymer backbone. There are many types of poloxamers on the market that differ in the number of units of EO and PO, however in previous studies we have adjusted NP formulation, in terms of both the type of poloxamers used and the relative ratio of the various polymers, in order to obtain NP optimized in terms of size, polydispersity and zeta potential ^{53,54}. The production of NPs was based on the formation of an amphiphilic polymeric blend of PLGA and poloxamers by means of emulsion techniques followed by solvent evaporation, all with an external aqueous phase.

Under these experimental conditions, the hydrophilic segments of the poloxamers spontaneously orient toward the aqueous phase, therefore allowing an increase of NP surface hydrophilicity. In two recent publications, we have shown, in an *in vitro* cellular experiment on mesothelioma cells, that NPs were not toxic and curcumin encapsulated in such NPs was released for days and this allowed a block of mesothelioma cancer cells amplification in the G₀/G₁ phase of the cell cycle for up to 72 h^{53,54}.

Despite the lasting interest in PLGA-based NPs, there remains a critical knowledge gap regarding their biodistribution and organ-specific targeting in healthy organisms⁵⁵. Investigating the *in vivo* distribution of NPs in healthy mice is important for evaluating their safety, optimizing therapeutic dosing regimens, and minimizing potential off-target effects⁵⁶.

Hence, in this work we intended to validate our previous encouraging results also *in vivo*, by carrying out biodistribution tests of NPs in healthy mice. Specifically, the aim was to verify whether the presence of poloxamers in the formulation of PLGA NPs, leads to a different *in vivo* biodistribution. For this purpose, fluorescent NPs were formulated by loading diphenylhexatriene (DPH) as a dye to the organic phase of the primary emulsion used to formulate NPs, allowing for NP visualization *in vivo*. The obtained NPs underwent comprehensive characterization, encompassing their morphology, technological attributes, DPH release rate, and thermodynamic properties. Then, NPs were administered by intraperitoneal injection, and, at scheduled time points, the animals were euthanized, and serum was withdrawn, while liver, lungs, kidney, were removed, divided in aliquots and frozen at -20 °C or fixed in paraformaldehyde. Organs were processed to be analysed by immunofluorescence studies. The histological analyses of the excised organs were carried out to qualitatively evaluate the *in vivo* biodistribution of both fluorescent bare PLGA NPs and those obtained by adding poloxamer(s) to the formulation.

2.2 Materials and methods

2.2.1 Materials

Equimolar PLGA (Resomer® RG504H, poly(D,L-lactide-co-glycolide) (ester terminated; lactide:glycolide ratio 50:50; Mw = 38–54 kDa), KCl, NaCl, Na₂HPO₄, Ethanol (EtOH), 1,6-diphenyl-1,3,5-hexatriene (DPH; CAS number 1720-32-7), Poloxamer 407 and 188 (also known as Pluronic F127 and Pluronic F68, respectively) were obtained from Sigma-Aldrich (Milan, Italy). MilliQ water was produced in-house (conductivity 0.055 $\mu\text{S}\cdot\text{cm}^{-1}$ at 25 °C, resistivity equals 18.2 M $\Omega\cdot\text{cm}$). Acetone and Acetonitrile, HPLC-analytical grade, were both from Sigma Aldrich (Milan, Italy). The wild type mice of the CD1 strain supplier was Charles River Laboratories (Milan, Italy).

2.2.2 Methods

2.2.2.1 Nanoparticle preparation

Fluorescent NPs were fabricated by nanoprecipitation technique followed by organic solvent evaporation, as described in our previous works^{53,54}. Briefly, NPs were produced without any chemical reaction, by merely exploiting a lipophilic gradient between an aqueous phase and an organic phase. In detail, a primary water-in-oil emulsion was obtained by sonicating (Misonix Sonicator 3000; 5 min, 4 W; Microtip 419) 500 μl of an ethanol internal aqueous phase within an organic phase composed of a solution of either PLGA (namely P NPs) or PLGA:F68:F127 (1:0.5:0.5 weight ratio) in acetone (namely PP NPs). The overall polymer concentration was 3% w/v in all cases and 1 mg of the fluorescent dye 1,6-diphenyl-1,3,5-hexatriene (DPH). Thereafter, the obtained W₀/O emulsion was poured through a syringe needle (22 G) by a syringe pump ($Q = 333.3 \mu\text{l}/\text{min}$; $d = 11.99 \text{ mm}$) in 40 ml of an aqueous phase, containing F127 and F68 as surfactants (1:1 weight ratio; 0.0375 mg/mL each). Acetone was evaporated overnight by magnetic stirring (700 rpm) at room temperature and the resulting suspension was washed three times with double

distilled water by centrifugation to withdraw the non-encapsulated DPH (10,000 rpm, 10 min; MKRO 300).

2.2.2.2 Nanoparticle mean diameter, polydispersity index and ζ potential

Mean diameters and ζ potentials of NPs were determined through dynamic light scattering (DLS) analysis performed by Zetasizer Ultra (Malvern Instruments, Malvern, UK). For particle size measurements, NPs were suspended in ultrapure water. Five runs were performed for each experiment.

2.2.2.3 Morphological analyses of nanoparticles

Morphological investigation on P and PP NPs with or without DPH were performed by means of a Field Emission Scanning Electron Microscope (SEM) equipped with an Energy Dispersive Spectrometer (FESEM/EDS; Zeiss Merlin VP Compact coupled with Oxford Instruments Microanalysis Unit; Carl-Zeiss Strasse, Oberkochen, Germany). Samples were glued by a carbon tape film on a sample holder and then gold-metalized, using an automatic sputter coater (Agar Scientific ltd-Parsonage Lane, Stansed-Essex, UK), with an automatically controlled complete sequence of flush, leak, coat, and vent.

The INCA (Oberkochen, Germany) X-stream pulse processor and the INCA Energy software 5.05 were used to obtain datasets. Operative conditions were reported on each acquisition and were chosen to be consistent with the performed measurement.

2.2.2.4 Thermal analyses

Analyses by differential scanning calorimeter (DSC) were carried out to investigate the interactions between the different polymers in NP formulations. Specifically, the tests were conducted on freeze-dried P and PP NPs, in the presence/absence of DPH using by DSC Q20 (TA Instruments, USA). The samples were loaded in aluminium pans sealed with Tzero lid and underwent a single scan, from 10 °C to 80 °C with a

5 °C/min heating rate. Measurements were performed under an inert nitrogen atmosphere, purged at a flowrate of 50.0 ml/min.

2.2.2.5 Entrapment Efficiency of DPH

The DPH entrapment efficiency was determined by an indirect method by evaluating the difference between the initial DPH amount (DPH_{tot}) and the untrapped DPH in the supernatant (DPH_{sur}), with respect to the total DPH mass used for NP preparation⁵⁷. The following equation was used:

$$EE\% = \frac{DPH_{tot} - DPH_{sur}}{DPH_{tot}}$$

In detail, after organic solvent evaporation, NP suspensions were centrifuged three times (10,000 rpm, 10 min) and the recovered supernatant analysed via HPLC to detect the non-entrapped dye content after each centrifugation step. Stock standard solution of DPH (2 mg·ml⁻¹) was dissolved in acetone and used for dilutions required for calibration curve. Calibration curve was performed using dilution 25.0, 50.0, 100.0, 200.0, and 400 ng·ml⁻¹. All solutions were stored at 4 °C and kept in the dark until experiments.

HPLC analyses were performed using LC-20 AD apparatus (Shimadzu Corp., Kyoto, Japan) equipped with a fluorescence detector (model RF-20, Shimadzu Corp., Kyoto, Japan) set at the excitation wavelength (λ_{Ex}) of 350 nm and at the emission wavelength (λ_{Em}) of 452 nm. The isocratic mobile phase utilized consists of acetonitrile:water MilliQ (85:15, v/v) and the flow rate was set to 0.8 ml·min⁻¹. All mobile phases were vacuum filtered through 0.45 μ m nylon membranes (Millipore, Burlington, MA USA). The stainless-steel column was a Supelco, Ascentis®, C18, (250 $\times$ 4.6 mm, 5.0 μ m particle size) with an Ascentis® C18 Supelguard™ Guard Cartridge (20 $\times$ 4 mm, 5 μ m particle size), and three times the loop volume, i.e., 60 μ l, were injected at room temperature (i.e., 22 $\pm$ 2 °C). Data acquisition and integration were accomplished by Cromatoplus software. The linearity of the

response was assessed in DPH concentration ranging from 25.0 to 400.0 ng·ml⁻¹, with an $R^2 = 0.9949$. Limit of detection (LOD) and limit of quantification (LOQ) of the methods resulted as 5.10 ng·ml⁻¹ and 25.78 ng·ml⁻¹ respectively.

2.2.2.6 Release kinetics of DPH in phosphate buffer solution (PBS)

The release kinetics of DPH were determined in phosphate buffer solution (PBS), which emulates physiological conditions. Specifically, the buffer was prepared by dissolving 1.420 g of Na₂HPO₄, 0.201 g of KCl and 7 g of NaCl in 500 ml of double distilled water; after complete dissolution under magnetic stirring at room temperature, the pH was adjusted to 7.4 and the solution further diluted to 1 l final volume. The prepared buffer was filtered through a 0.45 µm membrane (RC). Afterwards, 200 µl of NP suspension were diluted in 1 ml of PBS and the samples were placed at 37 °C in a thermostatic bath under mild magnetic stirring (100 rpm). After 1 and 4 h, the suspensions were centrifuged (13,000 rpm, 15 min; MKRO 300) and the supernatant was analysed by HPLC for DPH quantification.

2.2.2.7 Animal studies

The use and care of the animals in this work was approved by the Institutional Animal Care and Ethics Committee (Approval Number: 476/2018-PR) and carried out in accordance with the associated guidelines of the national law D.L. 26/2014 on the use of animals for research based on EU Directive 2010/63/EU.

Eight-week-old female wild type mice of the CD1 strain were randomly distributed into two experimental groups (8 mice for each group) and housed in cages for at least 8 days under standard conditions (temperature 20 ± 2 °C and 12 h day/night cycles; relative humidity: 45–65%). The animals received a standard diet ad libitum.

At the time of experiment, 0.15 ml of fluorescent nanoparticles suspensions (0.15% w/v) were administered by intraperitoneal injection. At scheduled time points (1 and 4 h after injection), the mice were anesthetized with 2% isoflurane (Isotec 4, Palermo, Italy) and, after complete sedation, euthanized by cervical dislocation.

After euthanasia, blood samples were withdrawn through the intracardiac route, collected in heparinized Vacu-test® tubes and promptly centrifuged to separate the serum from other whole-blood components. Thereafter, the supernatant was transferred to a clean glass vial, frozen and immediately cryopreserved in refrigerator at $-20\text{ }^{\circ}\text{C}$. For analytical tests, $475\text{ }\mu\text{l}$ of each sample were added to 1 ml of acetonitrile and maintained in ice at $4\text{ }^{\circ}\text{C}$ for 5 min to allow proteins precipitation. Then, each sample was vortexed for 1 min and ultra-sounded for 30 sec and finally centrifuged at 9500 rpm for 10 min . The supernatant was collected and injected into the HPLC; the results are the averages of at least three determinations.

In addition, liver, lungs, and kidneys were removed, weighed, and homogenized in isotonic KCl. An aliquot (0.490 g) of the homogenized solution was mixed with 2 ml of an acetone: formic acid ($9:1\text{ v/v}$) solution, and the resulting mixture was immediately vortexed for 10 min . The samples were then centrifuged at 6000 rpm for 10 min at $4\text{ }^{\circ}\text{C}$ and the supernatant was collected and frozen at $-20\text{ }^{\circ}\text{C}$. Before the analysis, samples were thawed and centrifuged at $12,000\text{ rpm}$ for 15 min ; $20\text{ }\mu\text{l}$ of supernatants were collected and analysed by the HPLC system. Recovery extraction from sera and organs were assessed by spiking both, sera and organ homogenates, with known concentrations of the DPH, before the extraction procedure.

2.2.2.8.1 Histological Analyses

To evaluate distribution of P and PP NPs, liver, lungs, and kidneys were fixed in Bouin for 24 h at room temperature, dehydrated in ascending alcohols, paraffin embedded and cut into $5\text{ }\mu\text{m}$ serial sections using a rotative microtome. Subsequently, the sections were subjected to routine histological analysis and stained with haematoxylin and eosin (H&E)⁵⁸. Serial sections were observed with Axioshop epifluorescence microscope using a $40\times$ objective and the acquisition software Axiovision 4.7 (Carl Zeiss, Milano, Italy).

2.2.2.9 Statistics

Results are reported as the mean of at least three replicates $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) and t-test were conducted to perform statistical analyses, and p values < 0.05 were considered statistically significant.

2.3 Results and Discussion

This study focused on the effect of surface properties of PLGA-based NPs on their biodistribution in healthy mice. As described beforehand, the surface composition of the NPs is influenced by the presence of hydrophilic units in the organic phase of the primary emulsion^{53,54}. In particular, in previous publications we have demonstrated that the addition of amphiphilic poloxamers to the organic phase of NPs results into the spontaneous arrangement of hydrophilic ethylene oxide (EO) units on NPs, and this is expected to discourage the adsorption of serum proteins^{53,54}. Thus, in this work we aimed to assess whether the different surface properties of P and PP NPs can affect their in vivo biodistribution in healthy mice. The production technique in emulsion followed by nanoprecipitation allows an effective control of the size and size distribution of the nanocarriers⁵⁹. Indeed, in this study NPs with a mean diameter below 200 nm were obtained in all cases (Table 1).

Table 1: Mean diameter, Polydispersity index (Pdl), Zeta Potential (ZP) and Entrapment Efficiency (E.E.) of DPH-loaded PLGA (P) and PLGA-Poloxamers (PP) NPs. Results are expressed as mean values $\pm$ SD ($n = 3$).

Formulation	Mean diameter $\pm$ SD [nm]	PdI $\pm$ SD	ZP $\pm$ SD [mV]	E.E.[%]
P NP	182 $\pm$ 7.1	0.124 $\pm$ 0.07	-43.8 $\pm$ 2.3	96.8 $\pm$ 0.04
PP NP	120 $\pm$ 4.2	0.117 $\pm$ 0.02	-39.1 $\pm$ 2.0	96.2 $\pm$ 0.03

Specifically, PP NPs present a lower mean diameter than P NPs, and this is ascribable to the presence of amphiphilic poloxamers in the organic phase which is associated to a reduction of the interfacial tension between the continuous and the dispersed phases in the emulsion used to prepare the NPs. In turn, this results into the formation

of smaller droplets and, consequently, of NPs with smaller mean size. It must also be specified that PdI values were low in both formulations. The presence of poloxamers in PP NPs also leads to a slight increase in ζ potential values due to a partial masking of PLGA carboxylic groups on NP surface. We have previously investigated the superficial composition of PLGA-poloxamers microparticles MPs, by means of electron Electron dispersive X-ray spectroscopy (EDS)⁶⁰. More in details, EDS analyses were performed on the raw materials (poloxamers and PLGA), as well as on PLGA (P) and PLGA-Poloxamers (PP) MPs. Results of elemental composition of PLGA powder closely resembled the one of PP MPs. However, a slightly higher carbon percentage was found in this latter case, indicating that some poloxamers were present on MP surface, even if PLGA prevails at the micro-scale. Moreover, in two previous works, we found that, at the nanoscale, the presence of poloxamers did enhance the superficial hydrophilicity of nanoparticles based on PLGA and poloxamers. This indicates that the superficial arrangement of hydrophilic segments of poloxamers is enhanced also at the nanoscale⁵³.

Results reported in Table S1 show that DPH entrapment efficiencies were > 96% for both P and PP NPs.

Table 2: Entrapment efficiency and release results.

	P NPs ng ml⁻¹	PP NPs ng ml⁻¹
entrapment efficiency		
Post first centrifugation	1307.64	1032.44
Post second centrifugation	821.25	574.00
post third centrifugation	819.07	513.46
Release		
Mean 1 h	726.39 ± 375.51	37.14 ± 27.57
Mean 4 h	700.55 ± 304.54	36.39 ± 7.46

SEM micrographs (Figure 1) show that the produced particles were uniformly spherical and well dispersed, with a smooth surface morphology.

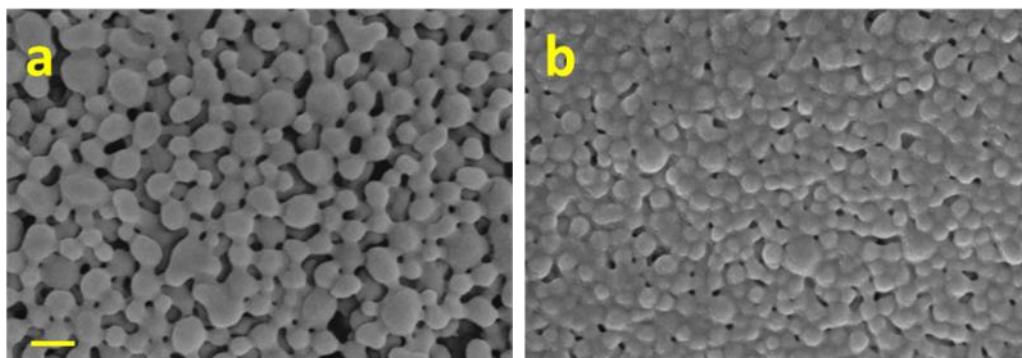


Figure 5: Scanning electron microscopy (SEM) images of: (a) PLGA nanoparticles (P NPs); (b) PLGA-Poloxamers nanoparticles (PP NPs). The bar is 200 nm.

As shown in Figure 2 and summarized in Table 3, the thermograms of blank and DPH-loaded P and PP NPs are reported. Both NP formulations show a glass transition around 49 °C followed by an endothermic hysteresis peak. Due to the amorphousness of PLGA, no endothermic melting peaks were observed. As it can be noted, with regards of DPH-loaded NPs, the peak is shifted on the left compared to unloaded formulations. This can be correlated to a slight plasticizing effect of dye. Besides, the thermo-grams of both PP NPs show a melting peak, ascribed to the fusion of the crystalline phase, around 51.6 °C and 47.8 °C, respectively. Again, the presence of DPH causes a plasticizing effect, thereby reducing the melting temperature.

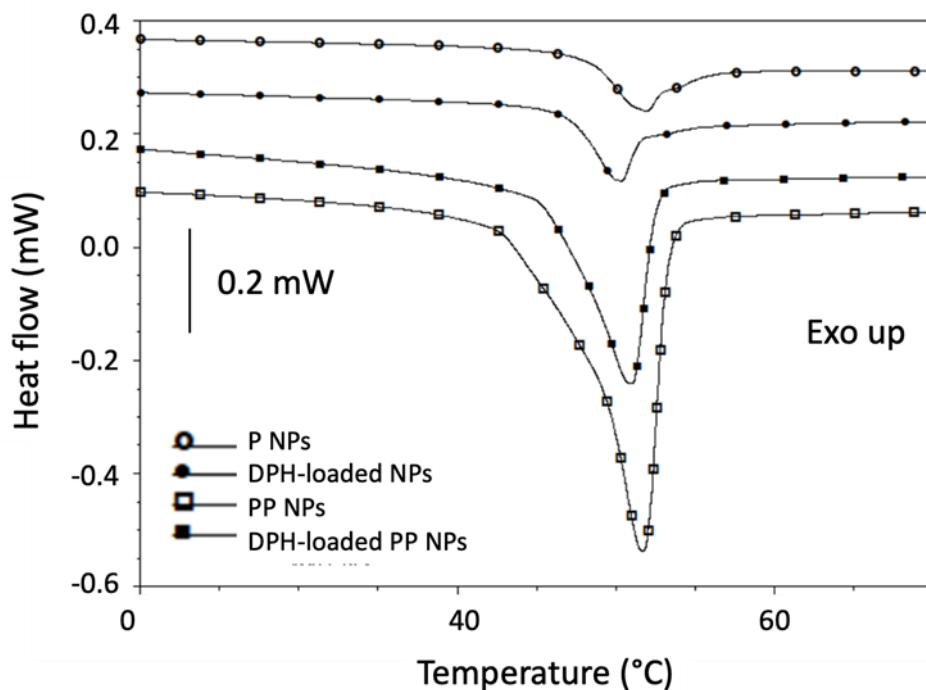


Figure 6: Thermograms of PLGA nanoparticles (P NPs) and PLGA-Poloxamers nanoparticles (PP NPs) with/without DPH. The exotherm is upwards.

Table 3: Glass transition (T_g), peak temperature (T_{peak}), onset temperature (T_{onset}), melting Enthalpy (ΔH_m) of unloaded and DPH loaded nanoparticles.

NP formulation	T_g [°C]	T_{onset} [°C]	T_{peak} [°C]	ΔH_m [J/g]
P	49.4 ± 0.6	-	-	-
PP	-	47.6 ± 0.1	51.6 ± 0.1	33.0 ± 0.6
P (loaded with DPH)	49.3 ± 0.5	-	-	-
PP (loaded with DPH)	-	48.5 ± 3.2	47.8 ± 4.5	20.5 ± 3.4

To assess that the detected fluorescence in vivo can be associated to NP accumulation in the target organs, we have performed in vitro DPH release experiments in buffer solution in the same time frame of in vivo biodistribution experiments. Figure 3 shows the cumulative release of the DPH after four hours in the medium release.

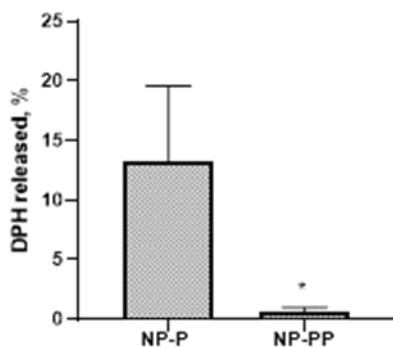


Figure 7: Percentage of DPH released from PLGA nanoparticles (P NPs) and PLGA-Poloxamers nanoparticles (PP NPs). Results are expressed as the mean $\pm$ SD of three replicates. Statistical analysis was performed by an unpaired t-test (* $p < 0.05$).

The percentage of the released DPH is $13.2 \pm 6.3\%$ for P NPs and $0.06 \pm 0.32\%$ for PP NPs. The faster release displayed by P NPs is probably ascribable to the more hydrophobic nature of the PLGA polymeric matrix which enhances the free diffusion of the DPH through the porous architecture of NPs. Anyway, we can state that after four hours most of DPH is still within NPs and therefore the fluorescent signal detected in the excised organs can be associated to NPs. It must also be underlined that DPH fluorescence is higher in a lipophilic milieu ⁶¹. Consequently, it can be assumed that the quantified DPH in different organs (liver, kidney and lung summarized in tables 3,4 and 5, respectively) is indicative of the NP amount.

Table 4: Liver results; PLGA nanoparticles (P NPs) and PLGA-Poloxamers nanoparticles (PP NPs); nd = not detected.

Samples	Time [h]	Concentration [ng·ml ⁻¹]
P NPs	1	nd
PP NPs	1	13.39 ± 15.24
P NPs	4	nd
PP NPs	4	nd

Table 5: Kidney results; PLGA nanoparticles (P NPs) and PLGA-Poloxamers nanoparticles (PP NPs); nd = not detected.

Samples	Time [h]	Concentration [ng·ml ⁻¹]
P NPs	1	nd
PP NPs	1	8.86 ± 2.65
P NPs	4	nd
PP NPs	4	<LOD

Table 6: Lung results; PLGA nanoparticles (P NPs) and PLGA-Poloxamers nanoparticles (PP NPs); nd= not detected.

Samples	Time [h]	Concentration [ng·ml ⁻¹]
P NPs	1	nd
PP NPs	1	10.43 ± 0.74
P NPs	4	nd
PP NPs	4	< LOD

As it can be seen from Table 7, NP accumulation in serum is very similar for both P and PP NPs. Contrariwise, only PP NPs are detectable in the analysed organs. These data were also corroborated by results of histological experiments shown in Figure 4. These outcomes indicated that PP NPs could be detected in the lungs (Figure 4a) and, to a lower extent, within the kidneys (Figure 4b), indicating the prompt elimination of P NPs from these organs. As for PP NPs, the slight uptake in the lungs is clearly detect-able in the intercellular spaces around pneumocytes composing alveolar structures and around blood vessels, whereas in the kidneys, PP NPs are located inside blood vessels and in the intercellular spaces around tubules. Figure 4c shows that in the liver, PP NPs could be detected only inside blood vessels.

Table 7: Serum results; PLGA nanoparticles (P NPs) and PLGA-Poloxamers nanoparticles (PP NPs); nd = not detected.

Samples	Time [h]	Concentration [ng·ml ⁻¹]
P NPs	1	232.40 ± 62.60
PP NPs	1	253.73 ± 31.35
P NPs	4	201.91 ± 11.11
PP NPs	4	208.48 ± 22.53

These biodistribution outcomes can be related to the promoted surface hydrophilicity of PP NPs ⁶² and to the route of administration. Specifically, intraperitoneal administration of NPs is followed by rapid adsorption of plasma proteins after a brief retention within the peritoneal cavity⁶³. The subsequent formation of a protein corona surrounding NPs is expected to affect NP biodistribution. For example, opsonin adsorption is known to enhance NP recognition by the reticuloendothelial system (RES)⁶⁴. The rapid sequestration of NPs by RES can be delayed if non-ionic hydrophilic units such as the EO segments of poloxamers are present on the NPs, which confer steric stabilization to the nanodevices, as well as a partial reduction of surface charges.

According to the biodistribution data, it is possible to assume that both P and PP NPs are promptly sequestered and eliminated by Kupffer cells after administration (Figure 4c). Anyhow, NP charge also slows their elimination from the body, which explains their persistence in the serum 4 h after administration.

The elimination of NPs through the kidneys is less likely, due to their relatively large size and negative zeta potential ⁶⁵. The localization of a small amount of PP NPs in lungs and kidneys is not completely clear, but it is possible to hypothesize that alveolar and kidney macrophages may be involved. This is indicative of the fact that the biodistribution in healthy organs changes due to the different surface hydrophilicity of the formulated NPs. Taken all together, these results encourage us to deepen the results found out in this study by loading a hydrophobic drug in PP

NPs targeted to lungs, liver, or spleen to evaluate both the biodistribution in pathological organs and the effect of the loaded drug.

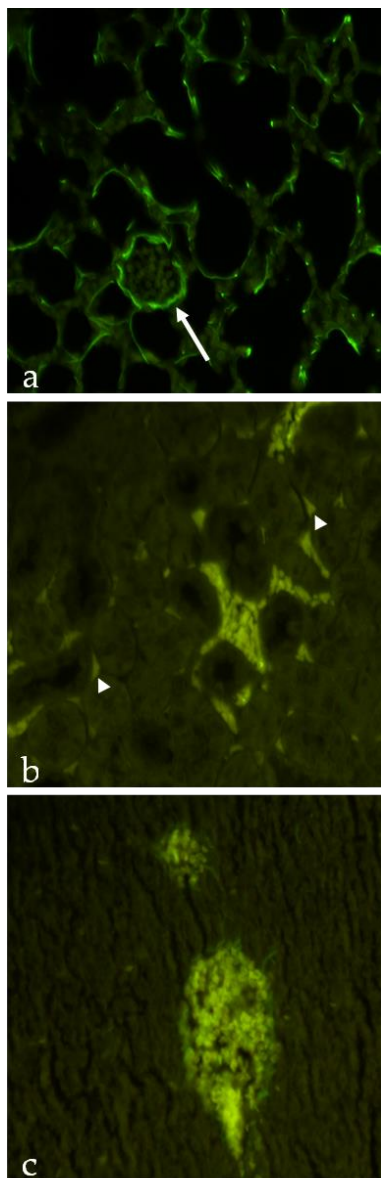


Figure 8: Photomicrograph of PLGA-Poloxamers nanoparticles (PP NPs): (a) in the lungs PP NPs are clearly detectable in the intercellular spaces around pneumocytes composing alveolar structures and around blood vessels (arrow); (b) in the kidneys, PP NPs are located inside blood vessels and in the intercellular spaces around tubules (arrowheads); (c) in the liver, PP NPs can be detected only inside blood vessels. Original magnification was 40 $\times$ in all micrographs.

2.4 Conclusion

In conclusion, this study describes a comparison between the biodistributions of two nanoparticulate formulations after a single intraperitoneal injection. The results showed that both formulations can persist in serum in similar concentrations, but P NPs cannot be determined in the other organs under study. Conversely, PP NPs were sequestered by the lungs and, to a lower extent, by the kidneys. To evaluate the potential of the formulations produced, it will be necessary to select a lipophilic active molecule to be loaded into PP NPs. Future in vivo studies will concern the determination of the toxicity of the free drug and of the drug-loaded NPs for longer times, with a view to their preclinical validation as possible carriers for targeted chemotherapies.

The versatility of these nanoparticles (NPs) extends across various administration routes, opening non-invasive applications beyond cancer treatment. For instance, NPs can be integrated within hydrogels to breach the skin barrier in the treatment of psoriasis⁶⁶ and melanoma⁶⁷. Additionally, NPs can be introduced through the nasal route via sprays or drops, effortlessly traversing the olfactory pathway to reach the brain and treat epilepsy⁶⁸. Inhalation serves as another avenue for NPs, applicable in the management of asthma, chronic obstructive pulmonary disease and cystic fibrosis⁶⁹. Altogether, the implications of this study encompass the design of NPs with finely tuned surface hydrophilicity, unlocking new dimensions in an array of targeted therapeutic approaches.

Chapter 3

Silibinin-loaded amphiphilic PLGA–poloxamer nanoparticles: physicochemical characterization, release kinetics, and bioactivity evaluation in lung cancer cells[†]

3.1 Introduction

Silymarin is a flavonoid derived from the seeds of the medicinal plant *Silybum marianum*, commonly known as milk thistle, and has been widely used in the medical field, especially for the treatment of liver disease (Figure 1). Silymarin is composed of seven flavonolignans (silibinin, isosilibinin, silycristin, isosilycristin, and silydianin) and one flavonoid (taxifolin)⁷⁰. In particular, silibinin (SLB) is the biologically active compound of silymarin and is composed of two diastereoisomers (silybin A and silybin B)^{71,72}. Recent studies have shown that SLB possesses inhibitory effects on multiple cancers, such as prostate, colon, breast, and skin cancer⁷³. SLB also possesses significant antiproliferative activity and has effects on the induction of apoptosis, chemosensitization, growth inhibition, reversal of multidrug resistance (MDR), and inhibition of angiogenesis, tumour invasion, and metastasis^{74–77}. Nonetheless, the efficacy of SLB is drastically hindered by its low aqueous solubility (<0.1 mg/ml) and poor bioavailability after oral administration^{78,79}, which causes the need for high doses to elicit adequate plasma levels. To overcome this issue, a wide array of delivery systems have been developed to increase SLB solubility and bioavailability⁸⁰. To this end, the formulation and use of polymeric nanoparticles (NPs) for drug delivery are promising for the treatment of cancer compared to traditional chemotherapy⁸¹. Moreover, NPs endowed with a hydrophilic surface possess prolonged circulation properties which in turn allow

[†] Villapiano F, Piccioni M, D'Aria F, Crispi S, Rassu G, Giunchedi P, Gavini E, Giancola C, Serri C, Biondi M, Mayol L. Silibinin-Loaded Amphiphilic PLGA-Poloxamer Nanoparticles: Physicochemical Characterization, Release Kinetics, and Bioactivity Evaluation in Lung Cancer Cells. *Materials* (Basel). 2024 Nov 10;17(22):5480. doi: 10.3390/ma17225480. PMID: 39597304; PMCID: PMC11595691.

them to take advantage of the enhanced permeability and retention (EPR) effect of solid tumours⁸². Such NPs can extravasate within tumours through aberrant vasculature resulting from uncontrolled angiogenesis^{82–87} or inflamed or compromised capillary endothelium, and be retained for prolonged periods of time due to the reduced draining of fluids from the tumour⁸¹. Among the polymers used to produce NPs for tumor treatment, poly(lactic-co-glycolic acid) (PLGA) is a material of choice. Indeed, PLGA NPs have several advantageous properties⁸⁸, such as biocompatibility, biodegradability, and the possibility of obtaining targeted drug delivery^{82,89}. These are pivotal features in that they allow the minimization of the systemic toxicity associated with traditional chemotherapy. The functionalization of these carriers with specific ligands can further improve their specificity toward tumor tissues. PLGA particles are quickly phagocytosed after intravenous injection depending on their size, thereby attaining tendentially short circulation times⁸⁹. This problem can be attenuated by preparing NPs using a blend of PLGA and poloxamers. In previous works, we produced NPs based on a physical blend of PLGA and poloxamers F127 and F68 to confer stealth properties to the produced NPs^{82,85}. Stealth NPs are designed to evade immune detection, employing polyethylene glycol (PEG) or poloxamers for improved circulation. PEG forms a hydrated steric barrier around NPs, reducing aggregation and prolonging systemic presence^{82,90}. Poloxamers similarly utilize EO for creating a hydrated protective layer. The unique triblock configuration of poloxamers allows the hydrophobic PO block to anchor effectively to NP surfaces, potentially enhancing stability in complex biological environments. While PEG provides uniform surface coverage, poloxamers offer robust anchoring due to their amphiphilic nature^{82,91,92}. In our previous studies, NPs based on physical blends of PLGA–poloxamers were shown to increase the stability of the produced devices in cell culture media⁸⁹ and promote their accumulation in vivo in the lung in a murine model⁸⁹. Furthermore, the addition of poloxamers has been shown to increase the circulation time of NPs after intravenous administration^{82,83,85,86,93–95}, thus providing NPs with passive targeting

ability⁹⁶. Therefore, the objective of this work was to load SLB within PLGA–poloxamer NPs and assess their bioactivity against different lung tumour cells. To this aim, NPs were produced by the nanoprecipitation technique and fully characterized for their technological and thermodynamic features, along with their release mechanism. Also, the in vitro bioactivity of the produced NPs was assessed against three human lung cancer cell lines, namely H1299, H1975, and H358 cells^{82,83,86}.

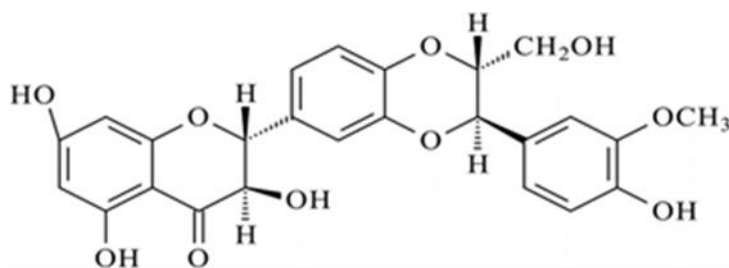


Figure 9: Structure of silibinin

3.2 Materials and methods

3.2.1 Materials

Silibinin (SLB), 2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-6-(3,5,7-trihydroxy-4-oxobenzopyran-2-yl) benzodioxin, silybin, and equimolar uncapped poly(D,L-lactide-coglycolide) (PLGA) (Resomer RG504H, Mw 40 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, Milan, Italy). Poloxamers F127 (a = 100 and b = 65) and F68 (a = 76 and b = 29) were obtained from Lutrol (BASF, Ludwigshafen, Germany). Potassium chloride (KCl) from Carlo Erba (Cornaredo, Italy) was used. Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethanol (EtOH), acetone, dibasic sodium phosphate (Na₂HPO₄), and sodium chloride (NaCl) were obtained from J-Baker (Chicago, USA). For cell culture experiments, Roswell Park Memorial Institute (RPMI-1640) medium supplemented with fetal bovine

serum (FBS), 50 UI/ml penicillin, 0.05 mg/ml streptomycin, sodium pyruvate, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Euroclone (Rome, Italy) were used. All chemicals and media were used as received without any further purification. The concentration of the SLB stock solution was 150 mM in DMSO.

3.2.1 Methods

3.2.1.1 Preparation of nanoparticles

NPs were produced by the nanoprecipitation–solvent evaporation technique⁸². In brief, 5 ml of an oil phase composed of a PLGA or PLGA–poloxamer (PLGA:F68:F127, 2:1:1 weight ratio) solution (2% w/v polymers) were combined with 5 or 10 mg of SLB by vortexing for 10 min, as reported in Table 1. The obtained solution was then precipitated through a syringe needle (22 G) in 40 ml of an aqueous phase, containing F127 and F68 as surfactants (1:1 wt ratio; 0.375 mg/ml overall concentration), by a syringe pump (flow rate = 333.3 μ l/min; d = 11.99 mm). After acetone evaporation by overnight stirring at room temperature, the obtained NP suspension was washed three times at 13,000 rpm for 10 min by centrifugation (Hettich Zentri-Fugen, Tuttlingen, Germany). Finally, NPs were stored at 4 °C.

Table 2: Composition and acronyms of the different concentrations of the NP formulations.

Samples	Polymers in the Organic Phase Oil			Polox in the Aqueous Phase	
	% w/v			% w/v	
	PLGA	F68	F127	F68	F127
P	2			0.0175	0.0175
PP	1	0.5	0.5	0.0175	0.0175
PP-SLB5	1	0.5	0.5	0.0175	0.0175
PP-SLB10	1	0.5	0.5	0.0175	0.0175

3.2.1.2 Physicochemical analysis of the NPs

3.2.1.2.1 Particle Size and Surface Charge Analyses

The mean size, size distribution, and zeta potential of the produced NPs were attained by photon correlation spectroscopy (PCS; N5 Submicron Particle Size Analyser,

Beckman Coulter, and ζ -potential (ZP)) by dynamic light scattering (DLS) analyses carried out with a Zetasizer Ultra apparatus (Malvern Instruments, Malvern, UK) at room temperature. To perform the measurements, NPs were suspended in ultrapure water at a concentration of 0.1 mg/ml. Each sample underwent twelve runs at room temperature.

3.2.1.2.2 Stability Study

The stability of the NPs was evaluated by tracking their size over time in an aqueous suspension at 4 °C, as well as in cell culture medium at 37 °C. The time trends of hydrodynamic diameters of unloaded, PP-SLB5, and PP-SLB10 NPs were tracked for up to 30 days in double-distilled water (storage conditions) and in RPMI-1640 medium supplemented with 10% FBS at 37 °C for up to 72 h. Additionally, NP size measurements were conducted on cell culture medium alone to check for any potential self-aggregation. The results presented were averaged from at least five individual measurements.

3.2.1.3 .3 Nanoparticle Morphology

Transmission electron microscopy (TEM) images of NPs were characterized using a TEM, TECNAI-12, FEI, USA. For this analysis, 100 μ l of ultradiluted NP suspensions in water was placed onto a copper TEM grid (300 mesh, 3 mm in diameter).

3.2.1.3.4 Yield, Drug Entrapment Efficiency, and Drug Loading of Nanoparticles

The yield of the NPs and SLB entrapment efficiency (η) and drug loading (λ) were determined from preliminarily freeze-dried NPs (0.01 atm, 24 h; Modulyo, Edwards, UK). Specifically, the NP yield was calculated based on the actual mass of the recovered freeze-dried NPs. For entrapment efficiency and drug loading tests, 100 μ L of PP-SLB5 or PP-SLB10 NPs was mixed with 900 μ l of DMSO and gently stirred for 30 min at room temperature to dissolve the particles and allow total SLB dissolution in the medium. The obtained solution was centrifuged for 15 min at

13,000 rpm, and SLB was quantified by a spectrophotometric assay (UV-1800, Shimadzu Laboratory World, Kyoto, Japan) at $\lambda = 289.0$ nm. The linearity of the response was verified over the concentration range of 0.2–50 $\mu\text{g/ml}$ ($y = 0.0367x - 0.0023$; $R^2 > 0.999$) [10,28]. η and λ were expressed as follows:

$$\eta = 100 \cdot \frac{m(\text{SLB}_{\text{entrapped}})}{m(\text{SLB}_{\text{total}})}$$

$$\lambda = 100 \cdot \frac{[m(\text{SLB})_{\text{total}} - m(\text{SLB})_{\text{entrapped}}]}{[m(\text{NPs}) + m(\text{SLB}_{\text{total}})]}$$

$\pm$ the standard deviation (SD) calculated on three separate batches.

3.2.1.3.2.5 Thermal Characterization via Differential Scanning Calorimetry (DSC)

Thermal analyses were performed by differential scanning calorimetry (DSC) on pure SLB and lyophilized SLB-loaded NPs to investigate drug–polymer and polymer–polymer interactions. Tests were carried out on powders of pure SLB and freeze-dried PP, PP-SLB5, and PP-SLB10 powders (24 h, 0.01 atm, -50 $^{\circ}\text{C}$; Buchi, Flawil, Switzerland). The samples were inserted in aluminum pans and analyzed using a 10–250 $^{\circ}\text{C}$ temperature with a heating rate of 10 $^{\circ}\text{C/min}$. The measurements were conducted in an inert nitrogen atmosphere purged at a 50.0 ml/min flow rate. The heat evolved during the thermic event (W/g) was calculated from the recorded DSC thermograms by integrating the exothermic/endothermic peaks, while the glass transition temperature (T_g) was obtained from the thermogram inflection point.

3.2.1.3 In Vitro Drug Release Studies

The release of SLB from the NPs was assessed by loading 4 ml of a NP suspension (at 14.56 mg/ml for PP-SLB5 and 19.33 mg/mL for PP-SLB10) into a dialysis membrane (Spectra/Por[®] Biotech Cellular ester; molecular cut-off 12 kDa), which was immersed at 80 rpm in an orbital incubator (SI50, Stuart R, UK) containing phosphate buffer solution (PBS) 90% v/v or DMF 10% v/v, with the pH adjusted to 7.4. At predetermined time points, 1 ml aliquots of the release medium were taken

and replaced with an equivalent volume of fresh medium. SLB was quantified by a spectrophotometric assay (UV-1800, Shimadzu, Kyoto, Japan) at a wavelength of 324.5 nm^{79,97}. The instrument response was linear over the 0.1–50 µg/ml concentration range ($y = 0.0358x + 0.0454$; $R^2 > 0.997$). The experiments were run in triplicate. In this study, we compared the results of various models to interpret release data, aiming to select the most suitable one for SLB release from formulated NPs [29]⁹⁸. The models are reported in the following:

The zero-order equation is as follows:

$$F = K_0 t \quad (1)$$

where F is the released drug fraction at time t and k_0 is the zero-order release rate constant⁹⁹.

The first-order equation is as follows:

$$\ln(1 - F) = -k_1 t \quad (2)$$

where k_1 is the first-order release rate constant¹⁰⁰.

Higuchi's equation is as follows:

$$F = k_H t^{0.5} \quad (3)$$

where k_H is the Higuchi release rate constant¹⁰¹.

The Korsmeyer–Peppas semiempirical model is as follows:

$$\ln(F) = \ln(K_{KP}) + n \ln(t) \quad (4)$$

where k_{KP} is the Korsmeyer–Peppas constant, which describes the structural and geometric characteristics of the device, and n is the release exponent, which indicates the main drug release. Each model was fit to average release data.

3.2.1.4 Cell Culture

The human lung cancer cell lines NCI-H1299 (ATCC CRL-5803), NCI-H1975 (ATCC CRL-5908), and NCI-H358 (ATCC CRL-358) were obtained from the ATCC

(Manassas, VA, USA). The cells were routinely propagated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% sodium pyruvate, 50 UI/ml penicillin, and 0.05 mg/mL streptomycin (Euroclone, Milan, Italy). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and were in the logarithmic growth phase at the start of the experiments. Subculturing was performed twice every week, beginning with a low-passage cell stock, for a period of 2–3 months following thawing. The cell lines were routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, Verviers, Belgium).

3.2.1.4.1 In vitro bioactivity of PP-SLB NPs

For the cell proliferation assay and for each cell line, 3×10^4 cells/well in 1 ml of growth medium were seeded in 12-well plates. Sixteen hours after seeding, the cells were treated with different concentrations of SLB alone or encapsulated in PP-SLB5 NPs. Before cell treatment with PP-SLB5 NPs, the formulation was filtered twice through 0.45 µm filters (filter size: 25 mm; AlfaTech, Genova, Italy) for enhanced sterility assurance. Cells treated with vehicle (DMSO) or unloaded NPs were used as controls. After 24 h of treatment, the cells were collected and counted with Trypan blue solution (Sigma-Aldrich, Merck KGaA group, Darmstadt, Germany). Cell viability was assessed by counting live cells using an MTS assay (CellTiter 96; Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. For the MTS assay, the absorbance was measured on a microplate reader at a wavelength of 490 nm (VICTOR Multilabel Plate Reader; PerkinElmer, Inc., Waltham, MA, USA). Cytotoxicity was expressed as a percentage compared to the control cells. The 50% inhibition concentration (IC₅₀) was calculated from the growth curves using GraphPad Prism 10. All experiments were conducted in triplicate, and the results are presented as the mean ± standard deviation (SD).

3.2.1.4.2 Statistical Analysis

The statistical data analysis was conducted using GraphPad Prism 10 software (GraphPad Software, Inc., San Diego, CA, USA). An unpaired t test was utilized to identify significant differences between the two treatment groups. For multiple comparisons, Tukey's multiple comparison tests followed by one-way ANOVA were used. Statistical significance was set at $p < 0.0001$ and $p < 0.05$.

3.3 Results and discussion

The pharmacological potential of SLB is severely restricted by its extensive first-pass metabolism in the liver, thus resulting in an incomplete or irrelevant intestinal absorption, mainly due to its low aqueous solubility¹⁰². To face these solubility and bioavailability issues, we propose loading SLB in PLGA-based nanoparticles (NPs), both with and without poloxamers (PP and P formulations, respectively)⁸² to attain controlled and targeted SLB delivery to the lung. In an earlier study we demonstrated that, after a single intraperitoneal injection, PP NPs were sequestered from the lungs, whereas P NPs were not detected in any organ⁸⁹. We hypothesized that the incorporation of amphiphilic poloxamers into the organic phase used for NP production led to the spontaneous organization of hydrophilic ethylene oxide units toward the NP surface, thus providing the NPs with a hydrophilic surface that may change their pharmacokinetics, thereby promoting passive lung targeting.

3.3.1 Morphology and Characterization of NPs

The results of TEM observations show that NPs are spherical with a regular surface (Figure 2)⁸². The sizes of the unloaded P and PPNPs were around 150 and 90 nm, respectively; the SLB-loaded NPs were around 100 and 124 nm for the PP-SLB5 and PP-SLB10 NPs, respectively (Table 2). Notably, SLB loading in NPs increased their mean diameter compared to unloaded NPs ($p < 0.05$), likely due to physical interactions of SLB with the poloxamers and PLGA, as previously demonstrated⁷⁸. The polydispersity index (PDI) of PP NPs (0.132 ± 0.01) was significantly lower than those of PP-SLB5 and PP-SLB10 NPs (0.212 ± 0.03 and 0.302 ± 0.06 ,

respectively) (Table 2). The inclusion of poloxamers brought about a reduction in the ZP from ~ -20 mV (PP NPs) to ~ -31 mV (P NPs), which is crucial for their dimensional stability in suspension. In contrast, the presence of SLB within the NPs did not affect ZP values (Table 2) ($p < 0.05$)^{85,103}.

Table 3: Size, polydispersity index (PDI), and zeta potential of formulation in distilled water at 4 °C. The entrapment efficiency, drug loading, yield values, and standard deviations were calculated from the last three independent experiments.

Formulation	Size (nm)	PDI	ζ (mV)	η	λ	Yield (%)
P	153 $\pm$ 1.6 *	0.140 $\pm$ 0.01	-31.4 $\pm$ 2.0 *			
PP	89 $\pm$ 0.6 *#\$	0.132 $\pm$ 0.01 \$	-19.2 $\pm$ 2.0 *			57.02 $\pm$ 0.4 #
PP-SLB5	98 $\pm$ 2.2 #&	0.212 $\pm$ 0.03 &	-20.9 $\pm$ 0.4	84.8 $\pm$ 3.0	1.61 $\pm$ 1.8	74.3 $\pm$ 2.5 #
PP-SLB10	124 $\pm$ 2.0 \$&	0.302 $\pm$ 0.06 \$&	-21 $\pm$ 2.0	93.9 $\pm$ 2.1	1.20 $\pm$ 0.1	72.8 $\pm$ 1.8 \$

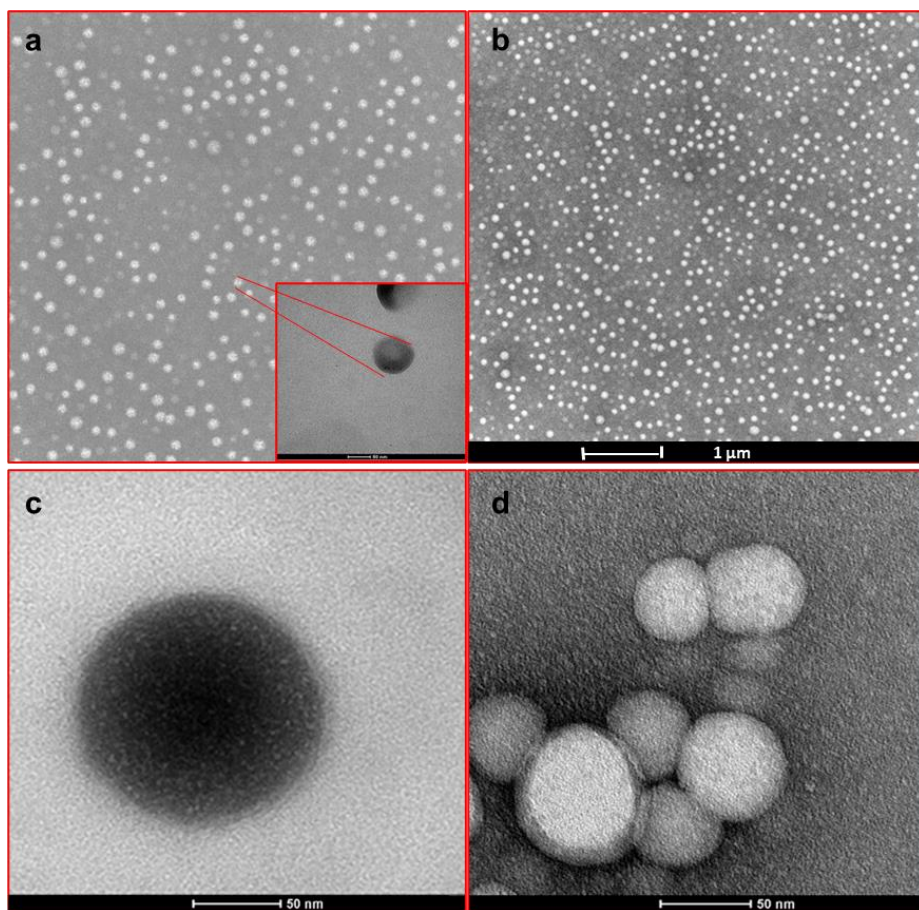


Figure 10: Representative TEM images at different magnifications of SLB-loaded PP NPs (a-d).

The encapsulation efficiencies of the SLBs were 84.8% and 93.9%, with actual loadings of 4.24 mg and 9.39 mg of SLB per 100 mg of polymer for PP-SLB5 and PP-SLB10 NPs, respectively. Furthermore, the drug loading percentages for the SLBs were found to be 1.61% for PP-SLB5 and 1.20% for PP-SLB10. The hydrophobic nature of SLB may be responsible for its high encapsulation efficiency. Interestingly, the increase in entrapment efficiency was not accompanied by significant changes in NP zeta potential, whereas it increased the NP size, polydispersity index, and yield ($p < 0.05$) (Table 2). Studies of stability in double-

distilled water at 4 °C for 30 days and in complete RPMI-1640 medium at 37 °C for 72 h proved that PP-NPs possess a satisfactory dimensional stability (Table 3).

Table 4: Time evolution of mean NP diameters in water at 4 °C and in cell culture medium (RPMI) at 37 °C for different NP formulations loaded with SLB. All the results are expressed as the mean value $\pm$ the standard deviation of three independent runs.

Formulation	NP size in Water 4 °C [nm]				NP in RPMI 37 °C [nm]		
	Day 0	Day 10	Day 20	Day 30	Day 1	Day 2	Day 3
P	153 $\pm$ 1.6	154 $\pm$ 0.2	160 $\pm$ 1.1	182 $\pm$ 5.4	166 $\pm$ 9.4	168 $\pm$ 2.5	191 $\pm$ 1.5
PP	89 $\pm$ 0.6	92 $\pm$ 0.4	89 $\pm$ 0.6	90 $\pm$ 1.0	85 $\pm$ 0.5	84 $\pm$ 0.8	84 $\pm$ 0.3
PP-SLB5	98 $\pm$ 2.2	156 $\pm$ 3.3	153 $\pm$ 2.3	156 $\pm$ 1.9	153 $\pm$ 4.0	160 $\pm$ 2.0	164 $\pm$ 1.2
PP-SLB10	124 $\pm$ 2.9	185 $\pm$ 1.5	163 $\pm$ 1.0	186 $\pm$ 4.7	130 $\pm$ 2.5	197 $\pm$ 1.8	198 $\pm$ 1.5

The outcomes of thermal analyses are detailed in Figure 3 and Table 4. As can be observed from the thermograms depicted in Figure 3, all the NP formulations displayed an endothermic peak associated with the melting of poloxamers at 53.3 °C, while that of SLB presented a melting peak at 171.3 °C. The disappearance of the melting peak of SLB in drug-loaded NPs can be attributed to the loss of the crystalline forms of SLB. Therefore, DSC outcomes indicate that the active molecule is present as a molecular dispersion in the NPs^{89,104}.

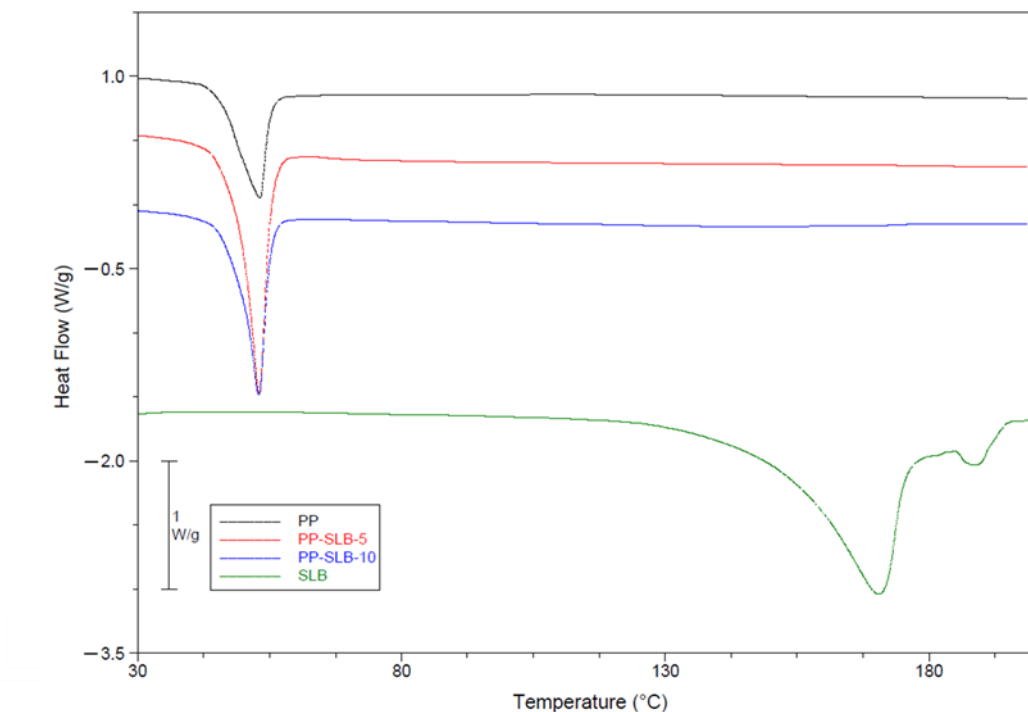


Figure 11: Thermograms of SLB and PLGA–poloxamer nanoparticles (PP NPs), with/without SLB. The exotherm is upwards.

Table 5: Melting temperature (T_m), onset temperature (T_{onset}), and enthalpy change (ΔH°_m) of the unloaded and SLB-loaded PLGA-poloxamer nanoparticles (PP NPs).

Sample	ΔH° (J/g)	T_{onset} (°C)	T_m (°C)
PP	28.6 ± 2.5	47.0 ± 1.9	53.3 ± 0.2
PP-SLB5	52.8 ± 3.7	46.9 ± 2.6	52.3 ± 1.1
PP-SLB10	40.7 ± 1.2	49.4 ± 1.0	53.3 ± 0.5
SLB	172.3 ± 10.4	152.6 ± 2.2	171.3 ± 1.0

3.3.2 In Vitro Drug Release Kinetics

In vitro release curves of SLB from PP-SLB5 and PP-SLB10 NPs are shown in Figure 4. The release profiles were reproducible, thus showing the ability of PP-NPs to control and sustain SLB release. A significant burst effect was detected, in which approximately 97% and 73% of the SLB was released by PP-SLB5 and PP-SLB10

in 24 h, respectively, followed by a slower release phase. The release was completed within approximately 5 days.

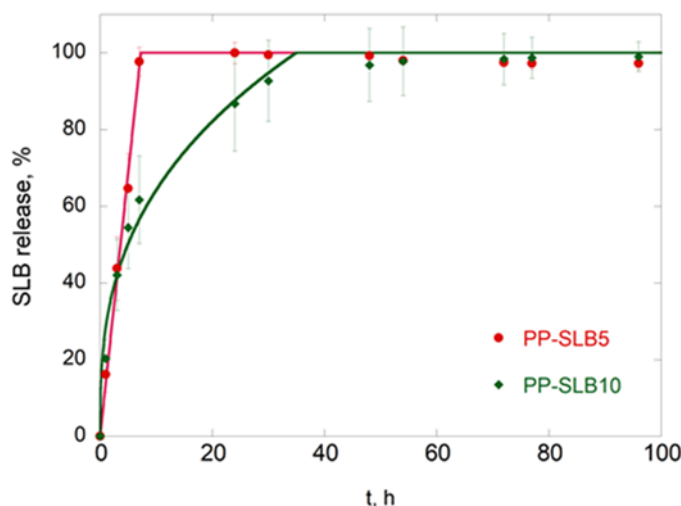


Figure 12: *In vitro* release profiles of SLB from PP-SLB5 and PP-SLB10 of NPs in PBS:DMF (9:1) at 37°C. * $p < 0.05$ of PP-SLB5 and PP-SLB10. Solid lines represent best-fit simulations.

Release data were fitted to Equations (2)– (5) to identify the conventional mathematical model that best describes SLB release and to verify whether different SLB loadings alter the release mechanism. Zero-order kinetics refer to a constant drug release rate over time. In contrast, the first-order model describes the release rate depending on the concentration of the remaining drug. The Higuchi equation models drug release as proportional to the square root of time. Finally, the semiempirical Korsmeyer–Peppas model describes drug release primarily dictated by diffusion, possibly coupled with other mechanisms that facilitate it ¹⁰⁵. Specifically, when applying the Korsmeyer–Peppas model to spherical particles, a value of $n \leq 0.43$ indicates pure Fickian diffusion, while when $0.43 < n < 0.85$, the release mechanism is considered anomalous diffusion, in which Fickian transport is associated with other mechanisms such as swelling and/or degradation ^{106,107}.

The values of the fitting parameters used in the simulations are listed in Table 5.

Table 6: Fitting parameters used in the simulations.

Model	Parameter	SLB 5		SLB 10	
			R ²		R ²
Zero order	k_0 [h ⁻¹]	0.133	0.997	0.082	0.949
First order	k_1 [h ⁻¹]	0.227	0.937	0.084	0.971
Higuchi	k_H [h ^{-0.5}]	0.300	0.942	0.158	0.977
Korsmeyer–Peppas	k_{KP} [h ⁻ⁿ]	0.151	0.935	0.259	0.982
	n	0.927		307	

The observation of R² values indicates that for the PP-SLB 5 NPs, the best-fit release curves were obtained with the zero-order model (R² = 0.997), while the Korsmeyer–Peppas equation better describes the release from the PP-SLB 10 NPs (R² = 0.982). This indicates that at the lowest SLB loading, the driving force of release is constant over time; hence, desorption of the active molecule from the NP surface or nanoporosities prevails. In contrast, for PP-SLB 10 NPs, an n value of 0.307 indicates that diffusion is the primary mechanism governing SLB release from PLGA-based NPs within the experimental time frame, with negligible contributions from other mechanisms such as NP degradation¹⁰⁸. This finding implies that SLB is also located in the inner regions of the NPs and clearly indicates that PLGA degradation occurs over a longer period than SLB release.

In general, drug release from polyester-based biodegradable devices in an aqueous environment is dictated by a complex diffusion–degradation mechanism triggered by water diffusion into the polymeric matrix^{89,106,109}. PLGA degradation consists of the hydrolytic cleavage of ester bonds in the polymer backbone, which generates acidic degradation products. If these products accumulate in the polymeric matrix, they accelerate device degradation, thus establishing first-order autocatalytic degradation^{110–112}. Once solubilized, the encapsulated molecule diffuses outwards

through the interconnected pores of the polymer matrix¹⁰⁶. This phenomenon has been observed in micrometric devices, for which it is necessary to use high concentrations of polymers in the organic phase (10–24% w/v in the organic phase)^{106,109,113}. This favors the trapping of degradation products and therefore autocatalysis. In contrast, at the nanometric scale, polymer concentrations of approximately 2–4% w/v are used, leading to a much higher porosity, with a reduced possibility of degradation product accumulation within the NPs. Therefore, although the very high surface area of NPs rapidly triggers degradation, autocatalysis does not occur to a significant extent. Overall, NPs need a prolonged time for complete degradation, and other formulation strategies would be beneficial for optimizing and further sustaining drug release. In any case, SLB-PP5 formulation was selected to test the effectiveness of nanoencapsulation in promoting the bioactivity of SLB, since in a previous paper we had demonstrated that a mean size ≤ 100 nm strongly promotes NP cell internalization¹¹⁴.

3.3.3 In Vitro Bioactivity of PP-SLB Nanoparticles

The bioactivity of PP-SLB was tested in vitro on three different human lung cancer cell lines that differ in *p53* expression: H1299¹¹⁵ and H358 are *p53*-deficient¹¹⁶, while H1975 expresses wild-type *p53*¹¹⁷. These cells were selected since NPs based on a PLGA–poloxamer blend showed a tropism toward lung tissue⁸⁹. Preliminarily, to study in vitro cytotoxicity, cells were treated with increasing concentrations of SLB for 24 h, after which cell viability was measured. The results of cell cytotoxicity tests are reported in Figure 5. The IC_{50} was 54.70 ± 2 $\mu\text{g/ml}$ for H1299 cells, 55.43 ± 2 $\mu\text{g/ml}$ for H1975 cells, and 11.43 ± 2 $\mu\text{g/ml}$ for H358 cells, showing that this latter cell line is the most sensitive (Figure 5).

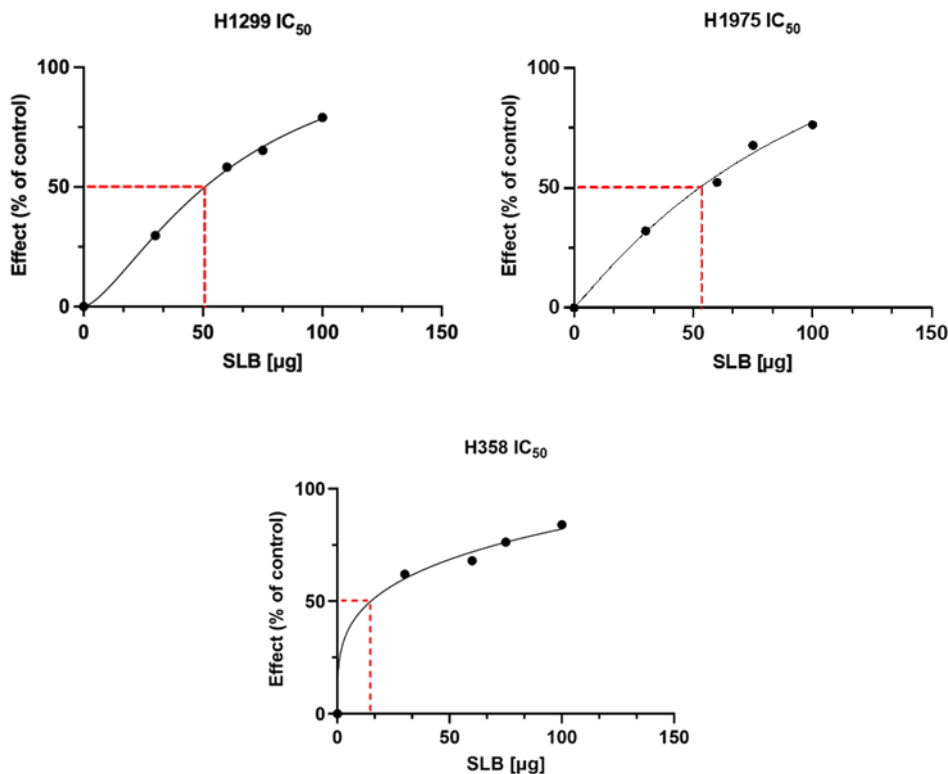


Figure 13: Analysis of the IC₅₀ values. All cell lines were treated with increasing doses (30, 60, 75, 100 μg/ml) of SLB for 24 hours.

Subsequently, the bioactivity of PP-SLB5 NPs was assessed from cytotoxicity tests against the three cell lines. The PP-SLB5 formulation, selected for its smaller particle size and rapid SLB release, was compared to the equivalent concentrations of free SLB. It must be underlined that, following filtration, the actual concentration of SLB in the NP suspension was found to be 2.53 μg /ml. The results of these experiments are shown in Figure 6.

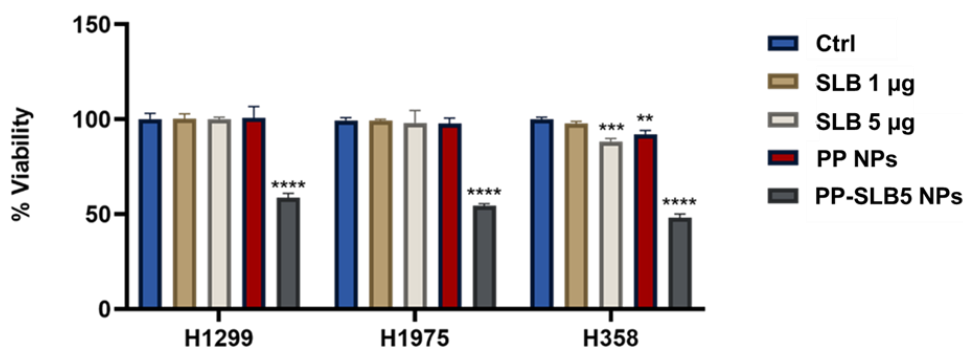


Figure 14: Efficacy of the PP-SLB5 NPs compared to the same amount of free drug. Untreated cells and cells treated with unloaded NPs were used as controls (Ctrl, PP NPs). The data are presented as the mean $\pm$ standard deviation and represents at least three independent experiments ($n = 3$). **** $p < 0.0001$ vs. Control.

As shown in Figure 6, PP-SLB5 NPs displayed a cytotoxic effect around the IC₅₀ of all cell lines tested, while the same concentration of the free drug produced no detectable effect. In more detail, NPs induced cell death around 50% at an equivalent SLB concentration as low as 2.53 µg/ml, while the free drug caused no effect even at higher concentrations (5 µg/mL). Thus, these findings show that the cytotoxicity of SLB against human lung tumor cells was promoted by >10-fold. This result is particularly promising considering that the produced NPs displayed a tropism for the lung in vivo⁸⁹.

These outcomes are in line with the literature findings, which show that a significant amount of research interest has been devoted to testing the synergistic effect of SLB with currently used chemotherapeutics and improve its bioavailability profile^{76,118,119}. In detail, in the field of nanomedicine, SLB has been tested in combination with glycyrrhizic acid and loaded in PEGylated nanoliposomes, which showed a >10-fold increase in SLB bioactivity against human hepatocellular carcinoma cells¹²⁰. In another report, Kuen et al. demonstrated that SLB loaded in chitosan-based NPs displayed enhanced cytotoxicity against the A549 lung tumor cell line¹²¹. To the best of our knowledge, this is the first time that PLGA–poloxamer NPs have been

tested for SLB delivery and bioactivity toward the human lung cancer cells used in this study.

3.4 Conclusions

The formulated NPs proved to be safe both in vitro and in vivo, as they demonstrated preferential accumulation in the lungs⁸⁹. Furthermore, PP NPs can increase the bioactivity of SLBs, as shown by in vitro experiments on lung cancer cells. Despite the promising results obtained in this study, the proposed formulations still present quick release, and it may be desirable to sustain them for longer periods of time in specific therapies. However, the results of the mathematical model comparison demonstrated that by varying the amount of drug loaded into the NPs, it is possible to exploit different release mechanisms. Specifically, in the case of the formulations with the highest SLBs, the release mechanism is diffusive. The diffusion of SLBs is likely to occur through the polymeric matrix and nanoporosities of the device. This suggests that by narrowing the nanoporosities of the NPs through proper formulation changes, it is in principle possible to sustain SLB release. This goal can be achieved by varying the molecular weight and/or concentration of PLGA in the organic phase and/or by further increasing the drug loading. Future studies will therefore focus on optimizing the formulation to prolong SLB release.

Chapter 4

Preliminary Investigation of X-Ray Irradiation on the Cellular Uptake of Hyaluronic Acid-Decorated Nanoparticles in Breast Cancer Cells

4.1 Introduction

Breast cancer (BC) is recognized being the second most common tumor worldwide, accounting for approximately 2.3 million cases annually, and the most prevalent cancer among women. The onset of BC is related to different factors that can be categorized as genetic or non-genetic¹²². Several therapeutic approaches are employed for the treatment based on features of clinicopathologic and tumor grade for patients that include mastectomy, radiotherapy (RT), chemotherapy (CT), hormone therapy (HT), and other novel therapies. The development of new protocols and therapies is strictly linked to the chemoresistance in BC, related to the tumor microenvironment (TME) and the crosstalk between surrounding stromal cell forming the extracellular matrix ¹²³.

The TME provides cancer cells with the support they need to acquire distinctive characteristics, such as uncontrolled proliferation, resistance to programmed cell death (apoptosis), the ability to invade other tissues and form metastases, the promotion of inflammatory processes and the ability to evade the immune system, thus contributing to resistance to treatment^{124,125}.

An increasingly studied aspect in the context of TME is the role of mechanical factors, particularly the stiffness of the extracellular matrix. It has been shown that the stiffness of the tumor microenvironment can modulate the expression of specific cell receptors involved in disease progression, such as the CD44 receptor. This receptor is involved in several key processes of tumorigenesis, including cell adhesion, migration, and resistance to treatment. The dependence of tumor cells on

their microenvironment has opened up new therapeutic perspectives, and the study of the CD44 receptor and its role in tumor resistance could offer new opportunities for the development of innovative therapies capable of specifically targeting the most aggressive tumor cells that are resistant to conventional treatments^{126–128}.

X-ray irradiation, which is widely used in the treatment of solid tumors such as breast cancer, can induce significant changes in the expression of the CD44 receptor, affecting the survival and metastatic behaviour of tumor cells. The ionising radiation causes an increase in the expression of CD44, specifically its CD44v variant, known for its key role in treatment resistance and phenotypic plasticity of cancer cells. Notably, this phenomenon has been observed in breast cancer, where post-irradiation overexpression of CD44 has been associated with an increased ability of cancer cells to adapt to treatment-induced stress, promoting disease progression and increasing the risk of recurrence¹²⁶.

In this context, nanoparticles (NPs) represent one of the most promising platforms for cancer therapy. These are particles measuring between 1 and 1000 nm, which, thanks to their structure and unique physical and chemical properties, such as biocompatibility, biodegradability, the possibility of modifying their surface with specific ligands and their high surface-to-volume ratio, can interact effectively with biological systems^{129,130}.

The aim of this study is to evaluate the effect of X-ray irradiation on the internalisation of polymeric nanoparticles decorated with hyaluronic acid (HA) in tumor cells. Specifically, we intend to explore the potential synergistic effect between radiotherapy-induced overexpression of the CD44 receptor, and the internalisation mechanism based on the interaction between HA and the receptor itself. Its interaction is expected to promote selective accumulation of HA-coated nanoparticles in CD44-overexpressing tumor cells, thereby amplifying the cytotoxic

response, reducing systemic toxicity and, at the same time, offering an innovative approach in the context of targeted and enhanced treatment.

4.2. Materials and methods

4.2.1 Materials

The materials used for these experiments were: 830 kDa hyaluronic acid purchased from Altergon Italia. Rhodamine-labelled PLGA ($M_w = 13\text{--}30$ kDa, (50:50)), poloxamers F127 (Pluronic F127) and F68 (Kolliphor P188), the salts present in phosphate buffered saline (PBS), namely dibasic sodium phosphate (Na_2HPO_4), sodium chloride (NaCl) and potassium chloride (KCl), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (USA). The MDA-MB-231 cells were donated by Francesco Paolo Cammarata, IBFM-CNR, Cefalù, while the MCF10A cells were donated by Stefano Piccolo, AIRC Institute of Molecular Oncology, Milan.

4.2.2 Methods

4.2.2.1 Production of nanoparticles

Two different NP formulations were prepared: one functionalised with hyaluronic acid (NP-PP-HA) and one without hyaluronic acid (NP-PP). Both formulations used rhodamine-labelled PLGA, i.e. a modified version of PLGA in which the polymer was decorated with rhodamine, a fluorescent probe commonly used in biochemistry and cell biology to track and visualise NPs in specific microscopes that exploit fluorescence. The nanoparticles were obtained using the nanoprecipitation technique. The production of NPs involved the preparation of three distinct phases:

- Phase W_0 : 320 μl of ethanol
- Phase Oil: 18.75 mg PLGA-Rod + 9.38 mg di F68 + 9.38 mg di F127
- Phase W_1 : 18.75 ml H_2O DDW + 0.75 ml F68 0.5% p/v + 0.75 ml F127 0.5%p/v. In the case of formulation with HA 15 mg of HA was added.

Initially, phases W₀ and Oil were emulsified by sonication for 5 minutes at 4 W in an ice bath (sonicator, Misonix 3000, USA), using a 419 microtip probe. The W₀/Oil phase thus obtained was then withdrawn with a 5 ml syringe and precipitated using a syringe pump (New Era Pump Systems Inc NE-300 (internal diameter of the 5 ml syringe: 11.99 mm) at controlled flow rate: 333.3 µl/min) in the W₁ phase, which was kept under magnetic stirring at 500 RPM. The resulting formulation is left under a fume hood overnight, under constant stirring, until complete evaporation of the organic solvents.

The formulation was then divided into 2 ml Eppendorf tubes, and three centrifugation cycles (DLAB D1524R, China) were performed at 10,000 RPM for 10 minutes at 4°C. At the end of the first centrifugation, 1 ml of supernatant was removed, the NPs were resuspended, and 1 ml of distilled water was added, while after the second centrifugation, the supernatant was replaced with PBS (pH=7.4). After the last centrifugation, 1.5 ml of supernatant was taken, the NPs were resuspended, and each formulation was combined in a 15 ml Falcon tube. Finally, the formulations were sterilised by 0.2 µm filtration, frozen at -80°C, and lyophilised. The freeze-drying process was carried out for 24 hours (Lio5P, 5Pascal, Italy).

4.2.2.2 Nanoparticles characterisation

To evaluate the average size and zeta potential (ζ), the nanoparticles were analysed by dynamic light scattering (DLS) using a Zetasizer Nano (Malvern Instruments, Malvern, UK). To perform these measurements, the NPs were suspended in ultra-pure water, thus ensuring accurate analysis of their physicochemical characteristics.

4.2.2.3 Cell cultures

The cell cultures used in the experiments are:

- MCF10A cells were cultured in DMEM/F-12 supplemented with 5% horse serum (HS), 1% L-glutamine, 1% penicillin-streptomycin, 0.1% epithelial growth factor

(EGF), 0.1% insulin and 0.1% hydrocortisone. While the final concentration of BPE (bovine pituitary extract) added to the medium was 0.4%, the other growth factors were added at a final concentration of 0.1%.

- MDA-MB-231 cells were cultured in the same base medium used for MCF10A cells, supplemented with 10% foetal bovine serum (FBS, Gibco), 1% L-glutamine (Sigma, St. Louis, MO) and 1% penicillin-streptomycin (Sigma, St. Louis, MO).

4.2.2.4 Cell irradiation

The MCF10A and MDA-MB-231 cell lines were seeded in glass-bottomed Petri dishes, 35 mm in diameter, at a density of approximately 1200 cells/cm². Approximately 24 hours after seeding, they were exposed to X-rays (photons) delivered by the LINAC Synergy Agility (ELEKTA), using a 6 MeV energy beam, at the “PASCALE” National Cancer Institute in Naples. The treatment plans with three-dimensional conformal radiotherapy (3D-CRT) were carried out with Elekta's Monaco v5.11.03 TPS (treatment planning station) to administer doses of 2 and 10 Gy, following the setup below. The cells were placed between two phantom plates in solid water (2 and 3 cm each) and exposed to two opposing fields. The selected dose rate was 200 UM/min. The prescribed doses were administered over a uniform square field of 20 × 20 cm². The 3D-CRT approach was chosen for the simulation, as it is widely used in clinical practice.

4.2.2.5 NPs internalisation multiple particles tracking (MPT)

72 hours after irradiation, both cell lines were incubated for a further 24 hours with the two types of nanoparticles (NPs), with and without HA, at a final concentration of 0.5 mg/ml. At the end of incubation, the cells were thoroughly washed to remove any non-internalised NPs. Images of the internalised NPs were acquired in time-lapse for 50 seconds, with a sampling interval of 0.5 seconds, using a wide-field fluorescence microscope (Olympus Cell-R) and a 60× water immersion objective with NA = 1.35.

MPT analysis was performed using an algorithm described in detail in previous works^{131,132}. After obtaining the trajectories of the nanoparticles, the mean square displacements (MSD) were calculated from the trajectories of the NP centres and correlated with diffusion using the following equation:

$$\langle \Delta r^2(t) \rangle = 4Dt^\alpha$$

where D represents the diffusion coefficient and the exponent α provides an indication of the mode of movement. In the following analyses, only regressions with an R^2 greater than 0.8 were considered, and NPs with an MSD curve following a power law with exponent $\alpha = 1$ and $\alpha < 1$, respectively, were defined as Brownian or subdiffusive motion, while actively transported particles were those whose MSD curve had a scalar exponent between 1 and 2. NPs showing an MSD curve with a time exponent α close to zero were classified as trapped (random caged) and not calculated. The total number of tracked and transported NPs was calculated for both types of NPs and the radiation doses used.

4.3. Results

4.3.1 Nanoparticle characterisation

Technological characterisation studies were carried out on each formulation and the data obtained are summarised in Table 1, which shows information on the average diameter, polydispersity index (PDI) and zeta potential (δ).

Table 1: *Characterization of the two formulations.*

NPs	Size(nm)	PDI	ζ (mV)
PP	157 ± 5	0.09 ± 0.02	-18.6 ± 0.9
PP HA	276 ± 15	0.21 ± 0.06	-25.8 ± 1.7

The data obtained showed that HA-functionalised nanoparticles exhibit larger hydrodynamic diameters compared to their uncoated counterparts, attributable to the presence of HA on the surface of the NPs. Zeta potential analyses further confirm successful surface functionalisation: NP-PP-HA show a more negative zeta potential value than uncoated ones. This result is consistent with the anionic nature of hyaluronic acid, which, upon adsorption, modifies surface charge of NPs, making their more negative.

4.3.2 Analysis of internalisation and multiple particle tracking (MPT)

Internalisation analyses of NP-PP-HA and NP-PP were performed using fluorescence microscopy on two different cell lines, MCF10A and MDA-MB-231. This dual-cell model allows for the comparative assessment of nanoparticle uptake between healthy and tumor cells. MCF10A is an immortalised cell line derived from benign breast tissue and retains characteristics typical of normal epithelial cells ¹³⁰. In contrast, MDA-MB-231 cell line originates from human breast cancer and is widely used in cancer research to study advanced breast tumors. This cell type is characterised by high aggressiveness and invasiveness, attributed to its ability to proteolytically degrade the extracellular matrix (ECM), facilitating tumor migration and dissemination. In addition, the analyses were performed under control conditions and after irradiation with two different doses of X-rays: 2 Gy and 10 Gy. The 2 Gy dose represents the conventional fraction used in standard radiotherapy protocols, while the 10 Gy dose was chosen to simulate the effect of high-intensity treatment. Although rarely used in the breast cancer therapy, high doses can be effective for the treatment of localised lesions by delivering therapeutic doses in fewer sessions. Two different mechanisms of nanoparticle movement were evaluated for both cell lines: diffusive-subdiffusive and transported. Diffusive-subdiffusive movement is characterised by limited diffusion within the cell, often influenced by the viscosity of the intracellular medium and the presence of physical barriers, such as the

cytoskeleton. In contrast, transported movement indicates the active involvement of cellular mechanisms, such as active transport.

Through these analyses, the Mean Squared Displacement (MSD), i.e. the average quadratic displacements of the nanoparticles, was calculated., it As shown in Figure 1, for both cell lines , NP-PP-HA exhibited higher MSD values than their uncoated counterparts across all time points, with a notable difference at 5.5 s (Figure 1 C-F), even after active transport had ceased. This result highlights that, once internalized and likely localized near the perinuclear region, NP-PP-HA retain greater intracellular mobility. This behavior is attributed to CD44-mediated internalization, which appears to facilitate sustained NP dynamics compared to the more heterogeneous uptake mechanisms of bare NPs.

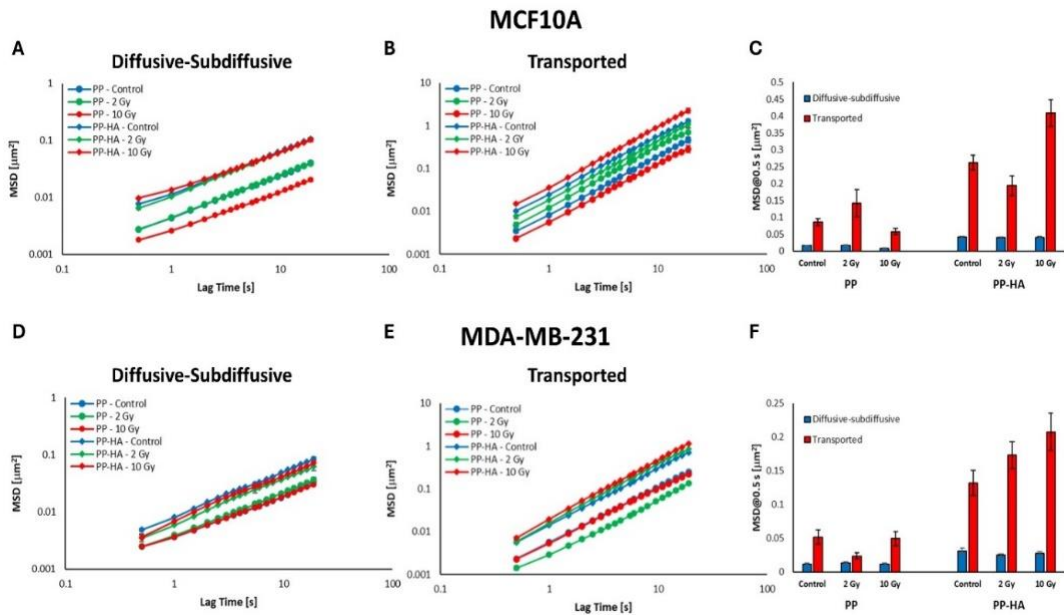


Figure 15: Trend of MSD values for nanoparticles without (●) and with HA (◆) in the two cell lines: MCF10A (A–C) and MDA-MB-231 (D–E).

To evaluate the impact of radiotherapy on CD-44 mediated internalisation, quantitative analyses of the internalised NPs were performed on both cell lines under

three experimental conditions: untreated control, irradiation with 2 Gy, and irradiation with 10 Gy. As shown in Figure 2, the percentage of internalized NPs increased in the tumor cells (MDA-MB-231) compared to the healthy cells (MCF10A), regardless of NP type. On the other hand, in both cell lines, the percentage of internalized nanoparticles is significantly higher for those functionalized with hyaluronic acid (HA) compared to the uncoated nanoparticles, regardless of the conditions. These results confirm that, even after 24 hours from the start of the experiment, the NP-PP-HA are internalized through CD44 receptor-mediated mechanisms, which is specific for HA.

Furthermore, when comparing the percentage of internalized NPs following exposure to 10 Gy irradiation dose, regardless of the cell line, a significant difference emerges between the NP-PP-HA and the NP-PP. This result suggests that exposure to a higher dose of radiation induces an increase in CD44 receptor expression, favouring greater internalisation of the NP-PP-HA through the receptor-mediated mechanism. This hypothesis is confirmed by the lack of significant variations in the percentage of internalised naked (uncoated) NPs following exposure to 10 Gy irradiation dose. The observed trend indicates that the increased radiation dose does not directly affect the internalisation of the naked nanoparticles, reinforcing the conclusion that the increase in the percentage of internalized NP-PP-HA is due exclusively to active targeting *via* CD44.

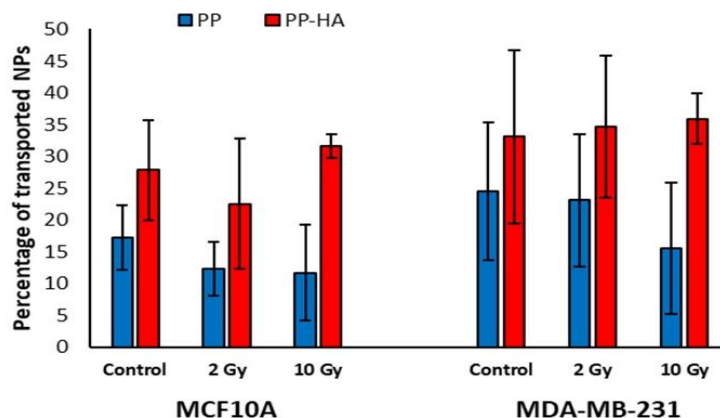


Figure 16: Percentages of internalized nanoparticles in the two cell lines under the three experimental conditions: control, irradiation with 2 Gy, and irradiation with 10 Gy.

4.4. Conclusions

The results of this study demonstrate that coating the NPs with hyaluronic acid (HA) allows for the effective achievement of active targeting. Notably, X-ray irradiation, particularly at higher doses, significantly promotes the internalisation of NP-PP-HA nanoparticles, highlighting the key role of the CD44 receptor in this process. The observed radiation-induced upregulation in CD44 expression supports a synergistic interaction between radiotherapy and HA-mediated active targeting.

This study, therefore, lays the groundwork for the potential application of NP-PP-HA as targeted vectors for the treatment of tumors that overexpress CD44, such as breast carcinoma. Consequently, future studies will focus on the encapsulation of chemotherapeutic agents, including those of natural origin, within the NP-PP-HA and, subsequently, the evaluation of their ability to induce cell death in tumor cells after internalization. Finally, comprehensive toxicity assessments on healthy cells will be essential to establish the safety profile of these nanocarriers and support their translational potential.

General conclusions

The research presented in this doctoral thesis focused on the surface engineering of self-assembling, amphiphilic PLGA-based polymeric nanoparticles (NPs) with the primary objective of overcoming the inherent pharmacokinetic limitations and lack of specificity associated with conventional cancer treatments. In this work it has been shown how the rational surface functionalization of NPs can modulate *in vivo* biodistribution, enhance the therapeutic efficacy of poorly bioavailable compounds, and promote a combined approach relying on active targeting and radiotherapy.

The initial phase provided a critical comparative analysis of the *in vivo* biodistribution profiles between two NP formulations with different surface hydrophilicity. The main outcomes of this research activity indicated that surface modification with Poloxamer impacts not only the colloidal stability of the NPs but, more importantly, the metabolic fate of the nanocarrier *in vivo*.

The tropism for lungs paves the way toward the development of targeted therapies for pulmonary carcinomas. Starting from this knowledge, the study addressed the therapeutic limitations of Silibinin (SLB), a promising flavonolignan compromised by poor solubility and bioavailability. Encapsulation of SLB within the PP NPs proved safe *in vitro* and resulted in a strongly promoted the bioactivity of the compound compared to the free drug, as confirmed by experiments on lung cancer cell lines. Another key finding refers the release mechanism of SLB, which depends on the drug-loading amount and is primarily diffusional in high-SLB-loading formulations. This suggests that the release kinetics of SLB can be modulated by tailoring NP nanoporosities through suitable formulation changes, mainly by varying PLGA concentration.

The final research phase established a proof-of-concept for active targeting combined with X-ray irradiation. NPs were superficially coated hyaluronic acid (HA) to target the CD44 receptor, which is overexpressed in many solid tumors,

including breast carcinoma. HA coating endows NPs with active targeting ability. Furthermore, the study indicated that X-ray irradiation, particularly at higher doses, induces a marked upregulation of CD44 expression. This, in turn, further promotes the internalization of the NP-PP-HA nanocarriers *via* a receptor-mediated mechanism. This finding supports an attractive interaction between radiotherapy and HA-based nanomedicine.

Altogether, the main results of this doctoral thesis indicate the versatility and safety of the designed NPs and can be further applied to a wide array of diseases, indicating a significant translational potential.

Also, the discovery that radiotherapy can be actively leveraged to potentiate the internalization of HA-coated NPs by inducing CD44 receptor upregulation suggests a novel clinical approach where irradiation serves can potentiate the action of NPs possessing active targeting.

Future research will focus on the evolution of the current nanocarrier platform, aiming to address the limitations associated with poloxamers. Indeed, although poloxamers are FDA-approved, in the perspective of an increasingly required “green” approach, the next development phase will involve their replacement with selected phospholipids. These substances offer superior biocompatibility, better mimic natural cell membranes, and are generally associated with reduced immune recognition and toxicity.

A crucial aim of this approach is to make the formulation process safer and more sustainable with no detriment for the finale *in vitro/in vivo* performance of the devices. This also involves the substitution of traditional organic solvents to safer alternatives, such as ethyl acetate. This shift will not only enhance environmental safety but will also mitigate potential residual toxicity in the final product due to the use of chlorine-containing solvents, thereby supporting a smoother passage toward clinical translation (Serri, C.; Frigione, M.; Ruponen, M.; Urtti, A.; Borzacchiello,

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